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Quantitative detection of *Potato virus Y* and *Potato leaf roll virus* by real time PCR – a molecular approach with numerous applications in potato research

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Quantitative detection of *Potato virus Y* and *Potato leaf roll virus* by real time PCR – a molecular approach with numerous applications in potato research

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Abstract

Virus resistance research on potato has a high priority. In African potato growing countries the majority of seed tubers offered on rural markets are infected with potato viruses, such as *Potato virus Y* (PVY) and/or *Potato leaf roll virus* (PLRV) resulting in a catastrophic reduction of yields. By contrast, in industrial nations virus infection of potatoes is virtually irrelevant due to efficient seed certification schemes. Nevertheless, the high yield loss potential has led PVY and PLRV becoming two of the most important pathogens on the potato crop worldwide. Due to their high replication rate viruses evolve quickly and could overcome existing resistances. In the recent history, common PVY strains, were more and more replaced by recombinant strains, such as PVY^{N-Wi}, $PVY^{N:O}$ or PVY^{NTN} that are highly virulent and able to cause the potato tuber necrotic ring spot disease (PTNRD). Taken together, these challenges in potato research and production call for an increased knowledge about the epidemiology of PVY and PLRV strains. Furthermore, breeding programs have to focus on the introduction of new resistance genes and generation of varieties that are adapted to the climatic conditions of their growing regions. These efforts can be supported by reliable and sensitive virus detection methods.

In this thesis the development and applications of reverse transcription real-time PCR (RT-qPCR) detection assays are presented that are able to quantify the worldwide most important potato viruses PVY and PLRV and thus can contribute to several areas of potato research. It is demonstrated that the developed RT-qPCR assay for the quantification of PVY is highly sensitive and can be utilized for the direct testing even of freshly harvested tubers during seed certification. A similar sensitive assay was applied to evaluate *Solanum* species and progenies of somatic hybrids regarding their level of resistance to PVY. The accumulation of PVY differed in progenies of wild potato species that previously were uniformly classified as extreme resistant. Therefore, RT-qPCR may be an interesting tool for a resistance evaluation in breeding programs. Another application of RT-qPCR is the estimation of virulence of different PLRV isolates. A correlation between the PLRV titer in potato plants and virulence could not be assessed. However, a discriminating quantification of different PLRV RNA species allows further epidemiological studies. Finally, RT-qPCR was demonstrated to be a useful tool to evaluate the equivalence of genetically modified potatoes regarding their level of susceptibility to PVY. An increased susceptibility to PVY could be a reliable indicator for possible unintended effects caused by the genetic modification. It is shown that equivalence is difficult to approve if the non-transgenic comparator is non-equivalent and if the classification into equivalent or non-equivalent is dependent on environmental conditions.

Zusammenfassung

Virusresistenzforschung an der Kartoffel hat hohe Priorität. In Kartoffelanbauregionen Afrikas ist die Mehrzahl der auf regionalen Märkten angebotenen Knollen mit Kartoffelviren, wie *Potato virus Y* (PVY) und/oder *Potato leaf roll virus* (PLRV) infiziert, was regelmäßig zu katastrophalen Ernteausfällen führt. Im Gegensatz dazu spielen Viruskrankheiten der Kartoffel aufgrund effizienter Saatgutzertifizierungssysteme in Industrienationen kaum eine Rolle. Nichtsdestotrotz zählen PVY und PLRV aufgrund des hohen potentiellen Ertragsverlustes weltweit zu den bedeutendsten Pathogenen der Kartoffel. Wegen ihrer hohen Replikationsrate entwickeln sich Viren sehr schnell und könnten bestehende Resistenzen überwinden. In jüngster Zeit wurden die gewöhnlichen PVY-Stämme immer mehr durch rekombinante Stämme, wie PVY^{N-Wi} oder PVY$^{N:O}$ und PVY^{NTN} verdrängt. Diese sind höchst virulent und sind Verursacher von Ringnekrosen an Kartoffelknollen (potato tuber necrotic ringspot disease: PTNRD). Diese Herausforderungen in der Kartoffelforschung und im Kartoffelanbau erfordern erweiterte Kenntnisse über die Epidemiologie von PVY- und PLRV-Stämmen. Zudem müssen sich Züchtungsprogramme der Kartoffel auf die Einführung neuer Resistenzgene und der Generierung von Genotypen konzentrieren, die an die klimatischen Bedingungen ihrer Anbauregion angepasst sind. Zuverlässige und sensitive Methoden zur Virusdetektion können bei der Lösung der genannten Herausforderungen helfen.

In dieser Arbeit werden die Entwicklung und Anwendung von *reverse transcription real-time PCR* (RT-qPCR)-Detektionsverfahren vorgestellt, die die weltweit bedeutendsten Kartoffelviren PVY und PLRV quantifizieren und damit einen wichtigen Beitrag auf verschiedenen Gebieten der Kartoffelforschung leisten können. Es wird dargestellt, dass das entwickelte RT-qPCR-Detektionsverfahren für die Quantifizierung von PVY hoch sensitiv ist und für die direkte Testung sogar frisch-geernteter Kartoffelknollen während der Saatgutzertifizierung eingesetzt werden kann. Ein ähnlich sensitives Verfahren wurde verwendet, um *Solanum*-Arten und Nachkommen von somatischen Hybriden auf das Niveau ihrer Resistenz gegen PVY zu bewerten. Die Anreicherung von PVY variierte zwischen den Wildkartoffelarten, die bis dahin einheitlich als extrem resistent eingestuft worden waren. Somit könnte die RT-qPCR ein interessantes Werkzeug für die Resistenz-Evaluierung in Züchtungsprogrammen sein. Eine andere Anwendung der RT-qPCR stellt die Einschätzung der Virulenz verschiedener PLRV-Isolate dar. Ein Zusammenhang zwischen dem PLRV-Titer in Kartoffelpflanzen und der Virulenz der Virusisolate konnte nicht ermittelt werden. Allerdings erlaubt die unterscheidende Quantifizierung verschiedener PLRV RNA-Arten weitere epidemiologische Studien. Abschließend wurde demonstriert, dass die RT-qPCR ein nützliches Werkzeug ist, um die Äquivalenz gentechnisch veränderter Kartoffeln hinsichtlich

ihrer PVY-Anfälligkeit zu untersuchen. Eine erhöhte Anfälligkeit für PVY könnte ein verlässlicher Indikator für mögliche ungewollte Effekte sein, die durch die genetische Modifikation verursacht wurden. Es wird gezeigt, dass der Nachweis von Äquivalenz schwierig ist, wenn der nicht-transgene Komparator selbst nicht äquivalent ist und wenn die Einstufung in äquivalent und nicht-äquivalent abhängig von Umweltbedingungen ist.

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Abbreviations

6K1	first 6 kilodalton protein
6K2	second 6 kilodalton protein
A	adenosine
ABA	abscisic acid
AGO1	Argonaute1
amiRNA	artificial micro RNA
ATP	adenosine triphosphate
bp	base pairs
BWYV	*Beet western yellows virus*
BYDV	*Barley yellow dwarf virus*
CAPS	cleaved amplified polymorphic sequences
CI	cytoplasmic inclusion (protein)
cM	centimorgan
CMV	*Cucumber mosaic virus*
CO_2	Carbon dioxide
CP	coat protein
CPIP	coat protein interacting protein
DNA	deoxyribonucleic acid
DAG	motif: Asp–Ala–Gly
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DEPC	diethylpyrocarbonat
EBN	endosperm balance number
EDTA	ethylenediaminetetraacetic acid
eEF1A	eukaryotic elongation factor 1A
eIF4E	eukaryotic initiation factor 4 E
eIFiso4E	isoform of eIF4E
ER	endoplasmic reticulum
EU	European Union
Fig	figure
FRNK	motif: Phe-Arg-Asn-Lys
FW	fresh weight
GMO	genetically modified organism
ha	hectare
HC-Pro	helper component protease
HDA	helicase-dependent amplification
HSP70	70 kilodalton heat shock protein
IRES	internal ribosome entry site
kb	kilo base
kDa	kilodalton
KITC	motif: Lys–Ile/Leu–Thr/Ser–Cys [K(I/L)(T/S)C]
LNA	locked nucleic acid
LOD	limit of detection
LOQ	limit of quantification
mRNA	messenger RNA
miRNA	micro RNA
MP	movement protein
NIa	first nuclear inclusion protein
NIb	second nuclear inclusion protein
NLS	nuclear localization signal

NPBT	novel plant breeding techniques
nt	nucleotide
NTC	non-template control
ORF	open reading frame
PABP	polyadenosine (polyA)-binding protein
PBS	phosphate-buffered saline
PD	plasmodesmata
PIPO	pretty interesting *Potyviridae* ORF
PLRV	*Potato leaf roll virus*
ppm	parts per million
PPV	*Plum pox virus*
PStV	*Peanut stripe virus*
PTK	motif: Pro–Thr–Lys
PTNRD	potato tuber necrotic ringspot disease
PVA	*Potato virus A*
PVP	polyvinylpyrrolidone
PVX	*Potato virus X*
PVY	*Potato virus Y*
RAPD	random amplified polymorphic DNA
RAT	replication associated translation
RdRp	RNA-dependent RNA polymerases
RISC	RNA-induced silencing complex
RJ	recombinant junction
RNA	ribonucleic acid
RTD	read-through domain
S	Svedberg
SCAR	sequence-characterized amplified region
scFv	single-chain variable fragment
SEL	size exclusion limit
SMV	*Soybean mosaic virus*
siRNA	small interfering RNA
SSR	simple sequence repeats
STS	sequence-tagged sites
TaMV	*Tamarillo mosaic virus*
TEV	*Tobacco etch virus*
TGA	total glycoalkaloid
TMV	*Tobacco mosaic virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
UTR	untranslated region
VPg	viral genome-linked protein
ZYMK	*Zucchini yellow mosaic virus*

List of figures and tables

1 An introduction to the potato, its viral diseases and breeding techniques

Unlike the botanical term suggests, the potato (*Solanum tuberosum* L.) was firstly described and named by the Swiss naturalist Gaspard Bauhin in 1596 (Bauhin 1596) after it was brought by Spanish colonialists from South America (Andean region) to the European continent a few decades before. From the Canary Islands and Spain the potato was introduced to Central and Northern Europe until the end of the 16th century (Hawkes 1990), and primarily it was a botanical novelty. The analysis of DNA from herbarium specimens has confirmed that from the early 18th century primarily potatoes of the Andean-type were introduced to Europe. This changed in the early years of the 19th century when nearly all analyzed potatoes were of the Chilean-type since they were better adapted to the long summer days and the climatic conditions in Northern Europe (Ames and Spooner 2008). In the 17th and 18th centuries many European empires were hit by several devastating famines. The introduction of the potato into the daily diet of the population finished almost all famines in Europe and the population size of the continent enormously increased, especially after guano was discovered to be an effective plant fertilizer. The guano trade led to large-scale traffic between Peru and Northern Europe and it is believed that the agent of the late blight disease (*Phytophthora infestans*) was carried by the ships from South America to Central Europe in the middle of the 19th century (Andrivon 1996; Ristaino 2002). The late blight epidemic became known as one of the most tragic events in history of plant pathology. Ireland was the most affected country where about one million people starved to death in the years after the outbreak of a late blight epidemic in 1845. As a result of the epidemic millions of people emigrated from Ireland and other European countries in the decade after the famine. Nevertheless, until the end of the 19th century the potato was considered as a major food crop (Burton 1989) and colonists and missionaries took with them the potato to the rest of the world.

Today, potatoes are grown on six continents and the expansion to China and India led these countries becoming the first and second most important producers of potatoes worldwide with an annual production of 96 million and 45 million tones, respectively, in the year 2013 (FAOSTAT 2015). Altogether, potato is the world's fourth most important food crop behind maize, wheat and rice with nearly 376 million tons produced in 2013 (FAOSTAT 2015). The nutritional value of baked, boiled, steamed or microwave cooked potatoes especially in mixed meals is very useful and beneficial to the human diet. Potatoes are rich in dietary fiber and antioxidants comprising polyphenols, vitamin C, carotenoids, and tocopherols (Lachman and Hamouz 2005). Furthermore, potatoes are a good source of vitamin B6, B1, potassium, iron, magnesium and folate (Nutrition Data 2015).

However, like in all nightshades, glycoalkaloids occur in nearly all potato tissues, of which α-solanine and α-chaconine generally contribute about 90% to 95% of total glycoalkaloids (TGAs) (Maga 1980). In recent cultivars the levels of TGAs range from 2 to 10 mg/100 g FW and 30% to 80% of TGAs are restricted to the potato skin. The generally accepted safe level of TGAs in potato is 20 mg/100 g FW (Bömer and Mattis 1924). However, an adult human weighing 70 kg would have to eat 1050 g potatoes with skin, that have an average TGA content of 20 mg/100 FW to obtain a TGA uptake of 3 mg/kg body weight, which is in the range of a toxic dose between 1 and 5 mg/kg body weight (Kuiper-Goodman and Nawrot).

The TGA levels in some wild species, such as *S. stoloniferum* and *S. cardiophyllum*, can be many times higher than in cultivated *S. tuberosum* varieties (Sotelo et al. 1998; Ruiz de Galarreta et al. 2015), which can be noticed by a very bitter taste. Nevertheless, a high TGA content in potato upper and lower plant organs ensures a good protection against pests such as the Colorado potato beetle (*Leptinotarsa decemlineata*), potato feeding aphids (Tingey 1984; Guntner et al. 1997) and wireworm larvae of *Agriotes obscures* (Jonasson and Olsson 1994). Furthermore, many wild potato species are a valuable source of resistance genes against viral, fungal and bacterial pathogens making them essential in potato breeding. It is on the breeders to integrate the desired traits while eliminating the adverse ones.

1.2   Potato diseases

Due to their vegetative propagation, potatoes are particularly susceptible to the accumulation of diseases. Several hundred pests and pathogens are known and Oerke and Dehne (2004) calculated that "the loss estimates for pathogens, viruses, animal pests and weeds in 1996–1998 totaled 22%, 8%, 18% and 23%, respectively, worldwide". The number of potato diseases of higher significance increases due to the global trade of potato tubers and also due to the climate change. Diseases not common in northern areas in the past have been introduced and adapted to new geographic regions, such as the potato wart disease (*Synchytrium endobioticum*) in Canada, an aggressive type of *P. infestans* in Great Britain or the zebra chip disease (*Candidatus Liberibacter solanacearum*) in the USA (Hampson 1993; Jones and Baker 2007; Secor et al. 2009).

Potato late blight disease caused by *P. infestans* is the most important potato disease based on the numbers of records listed in literature databases (Allefs et al. 2005). It causes annual yield losses of $6.7 billion in potato (Chowdappa et al. 2015) and outbreaks can have global impacts as, for example, seen during the Irish potato famine in 1845-1846. The oommycete is very adaptable and has the ability to evolve quite rapidly. In many potato growing regions worldwide new lineages were observed (e. g. A2 mating types) that are more aggressive and

overcome fungicide treatments with metalaxyl (Lee and Fry 1997) and previously strong sources of resistances (Oyarzun et al. 1997; Cooke et al. 2012; Fry et al. 2013; Chowdappa et al. 2015). Other nematodal, fungal and bacterial diseases such as potato cyst nematodes (*Globodera rostochiensis*, *G. pallida*), the wart disease (*Synchitrium endobioticum*), silver scurf (*Helminthosporium solani*), black scurf (*Rhizoctonia solani*), ring rot (*Clavibacter michiganensis* subsp. *sepedonicus*) or brown rot (*Ralstonia solanacearum*) have also a high damaging potential to the crop because of their considerable risk of contagion. Some of them are listed as quarantine pests by the European and Mediterranean Plant Protection Organization (EPPO 2014). In addition, viroids, such as the Potato Spindle Tuber Viroid (PSTVd) and phytoplasmas, such as the Potato purple-top wilt phytoplasma, which is also an EPPO quarantine organism, have a high damaging potential to the potato crop.

Viral diseases of potato are of major concern in Europe since a severe condition termed "curl" occurred in the middle of the 18th century in England and Ireland. The crop was so severely attacked that growers abandoned cultivation. Later on, the degeneration was described as a mixture of diseases, one of which leaf roll was a major agent. The yield losses caused by the leaf roll disease were not as weighty as during the late blight famine in Ireland. However, the cause and effect of the disease were heavily discussed on the political and scientific level. This becomes obvious considering that already in 1911 more than 600 articles were available that addressed the disease (Appel and Schlumberger 1911). The eminent phytopathologist Julius Kühn attributed the "Kräuselkrankheit" to plants that apparently have an excessive growth. He noted "Solche in ihrer Entwicklung übermäßig gesteigerte Pflanzen müssen allen ungünstig auf das Pflanzenleben wirkenden Einflüssen leichter unterliegen, als normal entwickelte […]" (Kühn 1859 p. 202).

Since these first reports of a disease, which is now known to be caused by at least one potato virus, today approximately 40 different viruses are known to infect the potato crop, naturally (Valkonen 2007). Potato viruses with the most damaging potential on a worldwide scale are *Potato virus Y* (PVY), *Potato leaf roll virus* (PLRV), *Potato virus A* (PVA) and *Potato virus X* (PVX) (Salazar 2003). However, viruses that were not of major concern until now, currently are getting more attention, since global trade flows and the climate change convey these viruses around the world as stowaways whereupon they adapt to new geographic regions. On the one hand, movement of virus-infected plant material, especially from wild potatoes in the Andes, constitutes a risk for the distribution of yet unknown viruses to other parts of the world. On the other hand, in warm climates, where potato growing is currently expanding, viruses transmitted by whiteflies (e. g. members of the genus *Begomovirus*) and thrips (e. g. *Tomato spotted wilt virus*) have become more and more prevalent. The reasons for this are not fully understood (Valkonen 2007). Nevertheless, PVY and PLRV are

considered to be the most important potato viruses on a worldwide scale. Therefore, these two shall be described in detail in the next chapter.

1.2.1 PVY

PVY is the type species of the *Potyvirus* genus in the family *Potyviridae* (Shukla et al. 1994). It was first isolated from potato by means of aphids by Smith (1931). However, the same author detected cytoplasmic inclusions in chlorotic mosaics of potato leaves, which he called "amoeba-like bodies", already seven years before (Smith 1924). Today PVY is considered to be the most harmful virus in cultivated potatoes and is present wherever potatoes are grown. In the leaves PVY causes variable symptoms depending on the strain, the potato cultivar, climatic conditions and on the time of infection. Primarily infected plants develop mild to severe mottle of young leaves. Older leaves often exhibit yellowing and necrosis. In secondary infections, when the mother tuber was infected, the diseased plants are dwarfed and brittle with puckered or crinkled leaves. The tubers of infected plants are reduced in number and size (Kerlan 2006). The percentage yield loss can be calculated by:

$$Yield\ loss = \ 0{,}5\ \times (\beta - 10)$$

where β is the percentage of PVY-infected plants in the crop (Valkonen 2007). In the United Kingdom alone the estimated annual yield loss due to PVY infections totals £30-40 million (Valkonen 2007). Furthermore, not only absolute yield losses represent the damage to the growers. The quality of tubers can also be affected by some PVY strains inducing the potato tuber necrotic ringspot disease (PTNRD) (Boonham et al. 2002) or cracked tubers (Inglis and Gundersen 2015). Since potatoes are also widely grown in home gardens, the disease is hard to eradicate. In addition, PVY has a rather large natural host range comprising many solanaceous crops, such as tomato, tobacco or pepper, ornamentals, such as *Dahlia* and *Petunia* spp., but also many weed species, such as *Datura* spp., *Physalis* spp., *S. dulcamara* and *S. nigrum* (Edwardson 1974). These species my act as reservoirs for PVY, from where the virus is transmitted into the potato fields. However, infected seed tubers display the most important initial PVY source for infections within the crop. Therefore, seed potatoes require costly certification to be marketed in the EU and many other countries (European Community 2002; UNECE 2006). Recently these directives and standards, respectively, were amended to the effect that field inspection no longer differentiates between mild and severe mosaic virus symptoms (European Union 2013; UNECE 2014), because in recent studies no correlation was found between the amount of viral RNA from PVY and the severity of symptoms (Hühnlein et al. 2013; Lindner et al. 2015). To control the disease, the elimination of aphid vectors is usually ineffective because of the non-persistent mode of transmission. PVY virions

bind to the food canal and foregut of aphids during sap ingestion and are inoculated into new plants by salivary secretion (Revers and García 2015). This occurs often during the first minutes after landing when aphids make the first probes. Before an insecticide is effective against the aphids, many new infections will have been accomplished, already. Moreover, some insecticide can lead to prolonged probes, which might increase the risk of PVY transmission (Thieme et al. 2009). Therefore, certified seeds and PVY resistant cultivars should be grown such as 'Arosa', 'Fioretta' and 'Bettina' (table varieties) or 'Django', 'Toccata' and 'Maxi' (processing varieties) (Federal Plant Variety Office of Germany 2014). However, the use of cultivars by growers is primarily based on market needs, such as good processing or cooking properties. In industrial nations the production efficiency is of secondary importance.

1.2.1.1 Particle morphology and genome structure and function

As it is shown in Fig. 1 the non-enveloped PVY virions are 730-740 nm in length and 12 nm in width. They have a filamentous and flexuous shape with a helical symmetry. In infected parenchyma cells PVY particles occur scattered or bundled throughout the cytoplasm (Weintraub et al. 1974; Edwardson and Christie 1991). Furthermore, PVY induces the formation of cylindrical (e. g. pinwheels), amorphous and/or crystalline inclusions within the cytoplasm (Christie and Edwardson 1977; Edwardson and Christie 1991).
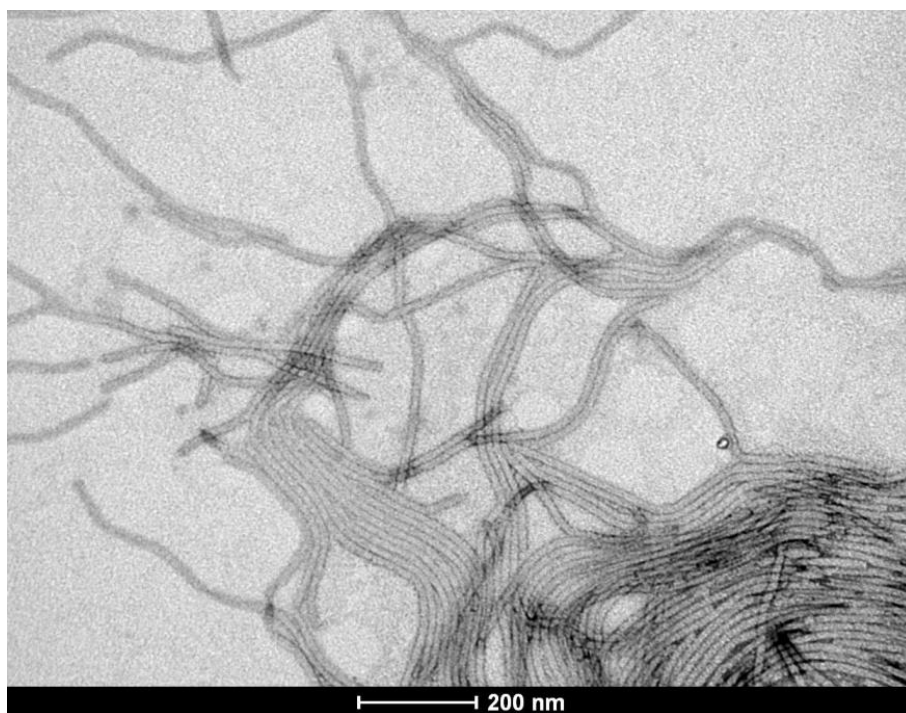
Fig. 1 PVY virions (isolate Gr99) purified from *Nicotiana tabacum* 'Samsun NN'

The genome of PVY consists of a single-stranded sense mRNA that has a length of approximately 9.7 kb. A schematic illustration of the genome organization of PVY is presented in Fig. 2A. The 5' end of the genome is not capped but a viral protein (VPg) is covalently linked by a phosphodiester bond and thus blocks the 5'-terminus of the viral RNA (Mandahar 2006). The 185 nt long 5' UTR of PVY facilitates cap-independent translation. However, it is not clear, whether the translation relies on an internal ribosome entry site within the 5' UTR (Gallie and Walbot 1992; Levis and Astiermanifacier 1993) or another (currently unknown) plant viral translation enhancer (Zhang et al. 2015). Like the mRNA in eukaryotes the RNA of PVY contains a polyadenosine (A)-tail at the 3' end of the genome, which protects the RNA from degradation and plays together with the 3' untranslated region an important role in RNA replication and translation (Mandahar 2006). The length of the poly (A)-tail of potyviruses varies considerably between species but also between isolates within a potyvirus species. It ranges between less than 15 to more than 500 residues (Hari 1981; Lain et al. 1988; Rosner and Raccah 1988), whereby the minimum number of A residues required for maintaining high infectivity is between 5 and 10 (Tacahashi and Uyeda 1999). Between the non-coding regions at the 5' and 3' end a single open reading frame codes for a polyprotein of 3061 or 3063 amino acid residues (depending on the PVY strain), which is cleaved into ten multifunctional proteins by P1 serine, HC-Pro cysteine and NIa-Pro cysteine proteinases (Shukla et al. 1994). An eleventh protein PIPO (Pretty Interesting *Potyviridae* ORF) is translated in the +2 reading-frame of the P3 cistron, as a P3-PIPO fusion product (Chung et al. 2008).

Most of the PVY proteins are multifunctional (Revers and García 2015). The protein 1 (P1) is the most variable protein among all of the potyviruses (Shukla et al. 1994 p. 103). It functions as a proteinase by cleaving itself from the adjacent helper component protease (HC-Pro) (Verchot et al. 1991). In addition, for *Potato virus A* (PVA), *Tobacco vein mottling virus* (TVMV) and *Turnip mosaic virus* (TuMV) it has been shown to bind single- and double-stranded RNA (Brantley and Hunt 1993; Soumounou and Laliberte 1994; Merits et al. 1998). P1 enhances viral suppression of RNA silencing mediated by HC-Pro (Tena Fernandez et al. 2013; Pasin et al. 2014) and is important in defining virus host range (Salvador et al. 2008). Recently, it was determined that P1 stimulates the translation of viral proteins during infection (Martinez and Daros 2014). However, P1 remains the most mysterious protein among all the potyvirus proteins, since it seems not to be essential. A virus deletion mutant completely lacking the P1 cistron is still viable (Verchot and Carrington 1995).

Next to its autoproteolytic activity, the helper component protease (HC-Pro) is involved in aphid transmission by acting as a "bridge" between virion particles and the aphid stylet (Blanc et al. 1998). Within the HC-Pro two conserved motifs can be found for aphid transmission:

the N-terminal KITC, which is involved in binding to the aphid stylet, and the PTK within the central region of HC-Pro, which can bind to and stabilizes the coat protein (CP) (Atreya and Pirone 1993; Peng et al. 1998; Valli et al. 2014). For aphid transmission a physical interaction between the CP and HC-Pro is important (Seo et al. 2010). In higher plants RNA silencing is based on the processing of double stranded (ds) RNAs that occur in plants amongst others during viral replication. The dsRNAs are fragmented into 21-24 nt short interfering RNA (siRNA) and microRNA (miRNA) duplexes by the RNase III-type DICER enzymes (Lakatos et al. 2006). The small RNAs are then incorporated into the RNA induced silencing complex (RISC), which induces the sequence-specific cleavage of the viral target RNA (Pham et al. 2004). The highly conserved FRNK box within the central region of HC-Pro is involved in suppression of RNA silencing by interacting physically with siRNA and miRNA duplexes (Lakatos et al. 2006; Shiboleth et al. 2007; Varrelmann et al. 2007). Moreover, Lakatos et al. (2006) demonstrated that siRNA sequestration leads to an inhibition of RISC assembly. In contrast to potyviruses with a high RNA silencing suppression activity of HC-Pro, such as *Tobacco etch virus* (TEV) (Anandalakshmi et al. 2000; Llave et al. 2000) or *Zucchini yellow mosaic virus* (ZYMV) (Shiboleth et al. 2007), the HC-Pro of PVY has a weak RNA silencing suppression activity and needs stabilization by the neighboring P1 to function as potent RNA silencing suppressor (Tena Fernandez et al. 2013). Earlier studies on further functions of HC-Pro were often seen individually, such as the involvement in symptom expression and synergy with co-infecting viruses (Vance et al. 1995; Pruss et al. 1997; Shi et al. 1997) or its role in virus multiplication and systemic movement (Kasschau and Carrington 2001). However, these functions are associated with and, what is more, result from the silencing suppressor activity of HC-Pro (Quenouille et al. 2013). HC-Pro has been reported to interact with other PVY proteins, such as the cytoplasmic inclusion (CI) protein (Guo et al. 2001) or the P1 protein (Merits et al. 1999) and many different host factors. For example, the N-terminal region of HC-Pro was suggested to interact with three *Arabidopsis* 20S proteasome subunits, which are related to the antiviral defence in plants (Jin et al. 2007). Recently, a completely new role of HC-Pro was demonstrated. It reduces the photosynthetic rate of PVY-infected plants by decreasing the amount of the chloroplast ATP synthase complex (Tu et al. 2015) and indirectly leads to an increase in abscisic acid (ABA) content (Jameson and Clarke 2002; Li et al. 2015). Furthermore, an interaction was confirmed between HC-Pro of different potyviruses and the eukaryotic initiation factor 4 E (eIF4E) as well as its isoform eIFiso4E, which also interact with the VPg. This interaction may demonstrate a probable role of HC-Pro in the translation initiation complex (Ala-Poikela et al. 2011). Finally, the C-terminal region of HC-Pro is known to elicit the host hypersensitivity genes Nc_{tbr}, Nc_{spl} and Ny_{tbr} (Moury et al. 2011) and the veinal necrosis phenotype in tobacco (Hu et al. 2009c; Tian and Valkonen 2013). Although it was estimated as unlikely in former studies (Glais et al. 2002; Schubert et al.

2007), HC-Pro was recently shown to be involved in the ability to induce necrotic symptoms on potato tubers (Valkonen 2007 p. 631; Glais et al. 2015).

The protein 3 (P3) is suggested to be involved in virus replication, virus movement, systemic infection, pathogenicity and symptom development (Merits et al. 1999; Jenner et al. 2002; Jenner et al. 2003; Choi et al. 2005; Cui et al. 2010; Lin et al. 2011; Vijayapalani et al. 2012; Lu et al. 2015; Wang et al. 2015c). As is the case for 6K2, P3 is a membrane protein containing two hydrophobic domains at the N- and C-terminal ends, respectively (Eiamtanasate et al. 2007). Within the P3 coding region the overlapping ORF PIPO is expressed as a translational fusion with the N-terminus of P3 named P3N-PIPO. P3N-PIPO was shown to be located to the plasmodesmata and plays an important role in cell-to-cell movement by binding to the host plasma membrane protein PCaP1 (Wei et al. 2010b; Vijayapalani et al. 2012).

The functions of the first 6 kDa protein (6K1) remain unclear. It is suggested that is has a relevant role in the potyviral infection cycle bcause it was found as a mature protein in *Nicotiana benthamiana* plants infected with *Plum pox virus* (PPV) (Waltermann and Maiss 2006). For *Soybean mosaic virus* (SMV) it was found to localize to the cell periphery suggesting that the protein might be involved in cell-to-cell movement (Hong et al. 2007). However 6K1 lacks transmembrane domains and the way it may act in cell-to-cell movement has to be different from currently recognized patterns of viral movement proteins (Hong et al. 2007). 6K1 might have an impact on the proteolytic processing of P3 and CI from the polyprotein. Mutant clones of PVA lacking coding sequences for 6K1 were not able to separate P3 from the cytoplasmic inclusion protein (CI), proteolytically (Merits et al. 2002). Furthermore, a deletion of the 6K1 protein-encoding region rendered PVA non-infectious, which may indicate the importance of 6K1 for the replication of PVA (Merits et al. 2002).

The formation of pinwheel-shaped inclusion bodies is a distinctive feature of potyviral infections. The cytoplasmic inclusion (CI) protein forms these inclusion bodies that are involved in cell-to-cell movement of the virus by collaboration with P3N-PIPO (Carrington et al. 1998; Roberts et al. 1998; de Cedron et al. 2006; Wei et al. 2010b). Recently, it was shown that the CI protein interacts with the coat protein (CP), and the N-terminal part of the CI protein modulates cell-to-cell movement by making this protein able to target plasmodesmata (Deng et al. 2015). Because of its RNA binding and RNA duplex unwinding activity, the CI protein was found to be an RNA helicase in *Tamarillo mosaic virus* (TaMV) (Eagles et al. 1994) and in PPV (Lain et al. 1990; Fernandez et al. 1995), and plays an important role in virus replication (Fernandez et al. 1997; Carrington et al. 1998; Deng et al. 2015). Furthermore, the CI protein may be involved in translation initiation, since the C-terminal part

of this protein has been shown to interact with the eIF4E in plants (Abdul-Razzak et al. 2009; Tavert-Roudet et al. 2012).

The second 6 kDa protein (6K2) is membrane bound (Restrepo-Hartwig and Carrington 1994; Merits et al. 2002) and induces the formation of 6K2-containing membranous vesicles at endoplasmic reticulum (ER) exit sites, responsible for potyvirus replication (Kopek et al. 2007; Wei and Wang 2008; Wei et al. 2010a). The 6K2 protein was found to anchor the replication complex to membranous sites, especially the endoplasmic reticulum (Schaad et al. 1997) making it indispensible for virus replication. Furthermore, 6K2 was shown to be involved in inter- and intra-cellular movement of potyviruses as well as in host-specific symptom induction (Rajamäki and Valkonen 1999; Spetz and Valkonen 2004).

The first nuclear inclusion protein (NIa) possesses two domains: an N-terminal VPg and a C-terminal protease domain, which cleaves most proteins of the precursor polyprotein (Carrington and Dougherty 1987; Garcia et al. 1990). The majority of unprocessed NIa protein molecules accumulates within the nucleus of infected cells. A partial quantity of unprocessed NIa protein and the VPg alone are covalently attached to the 5' end of viral RNA (Carrington et al. 1991; Carrington et al. 1993). As the name suggests, in the late stage of infection the NIa protein forms nuclear inclusions in the nucleus of infected cells (Knuhtsen et al. 1974; Edwardson and Christie 1991). However, this is not the case in all potyviruses. No inclusions are formed e. g. in cells infected with the *Peanut stripe virus* (PStV) (Hajimorad et al. 1996). The C-terminal domain of the NIa protein has also a DNAse activity and may degrade host DNA within the nucleus (Anindya and Savithri 2004). In addition, the NIa protease elicits the dominant gene for extreme resistance *Ry* in host plants (Mestre et al. 2000; Song et al. 2005). The NIa protease and - due to its structural flexibility (Rantalainen et al. 2011) - especially the VPg, have many cellular and viral binding partners. These comprise plant translation factors, such as eIF4E (Leonard et al. 2004), polyadenosine (polyA)-binding proteins (PABP) (Beauchemin and Laliberte 2007) and the 70 kDa heat shock protein (HSP70) (Dufresne et al. 2008; Thivierge et al. 2008), the eukaryotic elongation factor 1A (eEF1A) (Thivierge et al. 2008), the nucleolar protein fibrillarin (Rajamäki and Valkonen 2009), viral RNA (Merits et al. 1998) and probably all potyviral proteins, verifiably P1 (Merits et al. 1999), HC-Pro (Roudet-Tavert et al. 2007; Ala-Poikela et al. 2011), P3 (Merits et al. 1999), CI (Tavert-Roudet et al. 2012), NIb (Li et al. 1997; Daros et al. 1999) and the CP (Shen et al. 2010; Zilian and Maiss 2011). Therefore, the VPg is a hub protein that controls many processes resulting in virus production and spread (Jiang and Laliberté 2011).

The second nuclear inclusion protein (NIb) is the RNA-dependent RNA polymerase involved in the replication of viral RNA (Domier et al. 1987; Hong and Hunt 1996). As is the case for NIa, the vast majority of NIb molecules predominantly accumulates in the nucleus of infected

cells and is able to form amorphous or crystalline nuclear inclusions in infected cells (Knuhtsen et al. 1974; Baunoch et al. 1988; Restrepo et al. 1990; Edwardson and Christie 1991). Since NI proteins and their inclusions accumulate in high levels in the nucleus especially towards the later stages of infection, Ivanov et al. (2014) postulated, that the nucleus may serve as a sequestration site for the "overproduced" NI proteins. The NIb interacts with other potyviral proteins, such as P1, P3 and NIa (Fellers et al. 1998; Daros et al. 1999; Merits et al. 1999). Furthermore, NIa and NIb interact with translation factors, such as PABP and HSP70 (Dufresne et al. 2008). NIb was found to uridylylate the VPg and to use the resulting product to prime viral RNA synthesis (Puustinen and Makinen 2004; Anindya et al. 2005).

The coat protein (CP) encapsidates the viral genome and protects it from degradation. However, the CP is a multifunctional protein that is involved in almost every stage of the viral infection cycle. As it was outlined above, the CP plays an important role in aphid transmission by interacting of the N-terminal DAG motif (Lopez-Moya et al. 1999) with the PTK motif of the HC-Pro. In turn, the HC-Pro binds to the aphid stylet resulting in sticking the virions to the aphid stylet (Ng and Falk 2006). The CP is also involved in systemic and cell-to-cell movement of the virus (Dolja et al. 1995; Rojas et al. 1997; Andersen and Johansen 1998). The CP of PVA was found to be phosphorylated, when it was not incorporated into virions (Ivanov et al. 2001). The phosphorylation inhibits the RNA-binding activity of the CP indicating a regulation of the virion assembly (Ivanov et al. 2001). Furthermore, Hafren et al. (2010) and Besong-Ndika et al. (2015) demonstrated that the CP of PVA can inhibit replication-associated translation (RAT) by switching from primary translation to primary virus assembly in later stages of infection. The authors of this study explained it as follows: The HSP70 together with its cochaperone (CPIP) regulate viral infection. At an early stage of infection, when CPIP is more abundant in the cell, the CPIP binds to the CP expressed in *trans* and delivers it to HSP70, which promotes modification of the CP by ubiquitin, leading to CP degradation. At this early stage of infection the RAT can proceed. In later stages of infection, the CPIP is depleted and the CP, which is needed for viral encapsidation, becomes abundant. Then the CP expressed in *trans* forms a complex with the *cis*-expressed CP. This complex binds to the CP cistron in the viral RNA and may initiate virus assembly.

1.2.1.2 Strains

In the last ten years the nomenclature of PVY strains became the topic of particularly intense discussions (Singh et al. 2008; Kehoe and Jones 2011; Karasev and Gray 2013b; Jones 2014; Kehoe and Jones 2015). The actual strain nomenclature (Fig. 2B) contains a mixture of

differences identified by whole genome sequencing, geographical designations and single biological and serological properties.

The strains PVY^C, PVY^O and PVY^Z induce mosaic and vein clearing in tobacco and are defined by phenotypes induced by inoculation to potato cultivar differentials with the hypersensitivity genes *Nc*, *Ny* and *Nz*, respectively (Jones 1990; Le Romancer et al. 1994). PVY^N elicits necrosis and stunting in tobacco and overcoms all three hypersensitivity genes (Cockerham 1970; De Bokx and Huttinga 1981). In the 1990s the strain group PVY^E was recognized (Kerlan et al. 1999; Singh et al. 2008). As PVY^N it overcomes all three hypersensitivity genes, but does not induce necrosis in tobacco (Jones 1990; Kerlan et al. 1999; Singh et al. 2008). However, the differentiation of strains on the basis of biological properties is insufficient because in the past literature some so-called PVY^O isolates are PVY isolates that do not produce any necrosis on tobacco but an inoculation to potato cultivar differentials with *Nc*, *Ny* or *Nz* genes was never used to distinguish them (Nie et al. 2004; Hu et al. 2009b; Hu et al. 2009c). A further classification of PVY strains on the basis of biological properties was done with PVY^N strains that cause the PTNRD and vein necrosis on tobacco. These strains were named PVY^{NTN} (Le Romancer et al. 1994). In addition, PVY^{NTN} isolates were differentiated into PVY$^{(EU-)NTN}$, comprising isolates with a RJ in their CP, and PVY^{NA-NTN}, comprising isolates without a RJ in their CP and primarily were found in North America (Nie and Singh 2002a; Nie and Singh 2002b).

The distinction between PVY$^{O:N}$ and PVY^{N-Wi} is based on the number of recombinant junctions (RJ). Whereas PVY$^{O:N}$ has only one RJ, PVY^{N-Wi} displays two or more RJs (Singh et al. 2008; Karasev and Gray 2013b). Other authors do not discriminate between PVY$^{O:N}$ and PVY^{N-Wi}. They classified all PVY$^{O/N}$ recombinants with one or two RJ as PVY^{N-Wi} (Hu et al. 2009b). The differentiation between the recombinants PVY^{N-Wi} and PVY^{NTN} was done on the basis of the position of the RJ in the ORFs coding for the NIb protein and the CP, respectively (Schubert et al. 2007). Whereas isolates that display a continuous PVY^N-type specific sequence at least until nt ~9190 are classified as PVY^{NTN}, isolates that have a RJ upstream or in the region of the interface between NIb and CP are classified as PVY^{N-Wi}. However, recent phylogenetic studies found latter isolates clustering in a new strain group called PVY^{SYR-II} or PVY^{NTN-NW} (Chikh Ali et al. 2007; Chikh Ali et al. 2010; Kehoe and Jones 2015).

Based on sequence analysis, other types of recombinants are also being found, such as isolate NE-11, which is a recombinant between PVY^N and an unknown PVY variant (Lorenzen et al. 2008), isolate PVYnnp, which is a PVY^N/PVY^C/PVY^O recombinant (Schubert et al. 2007) or isolate PVY-MON displaying PVY^N-type, PVY^O-type and unknown PVY sequences. On the basis of its biological properties the latter isolate was classed into the strain group PVY^E (Galvino-Costa et al. 2012).
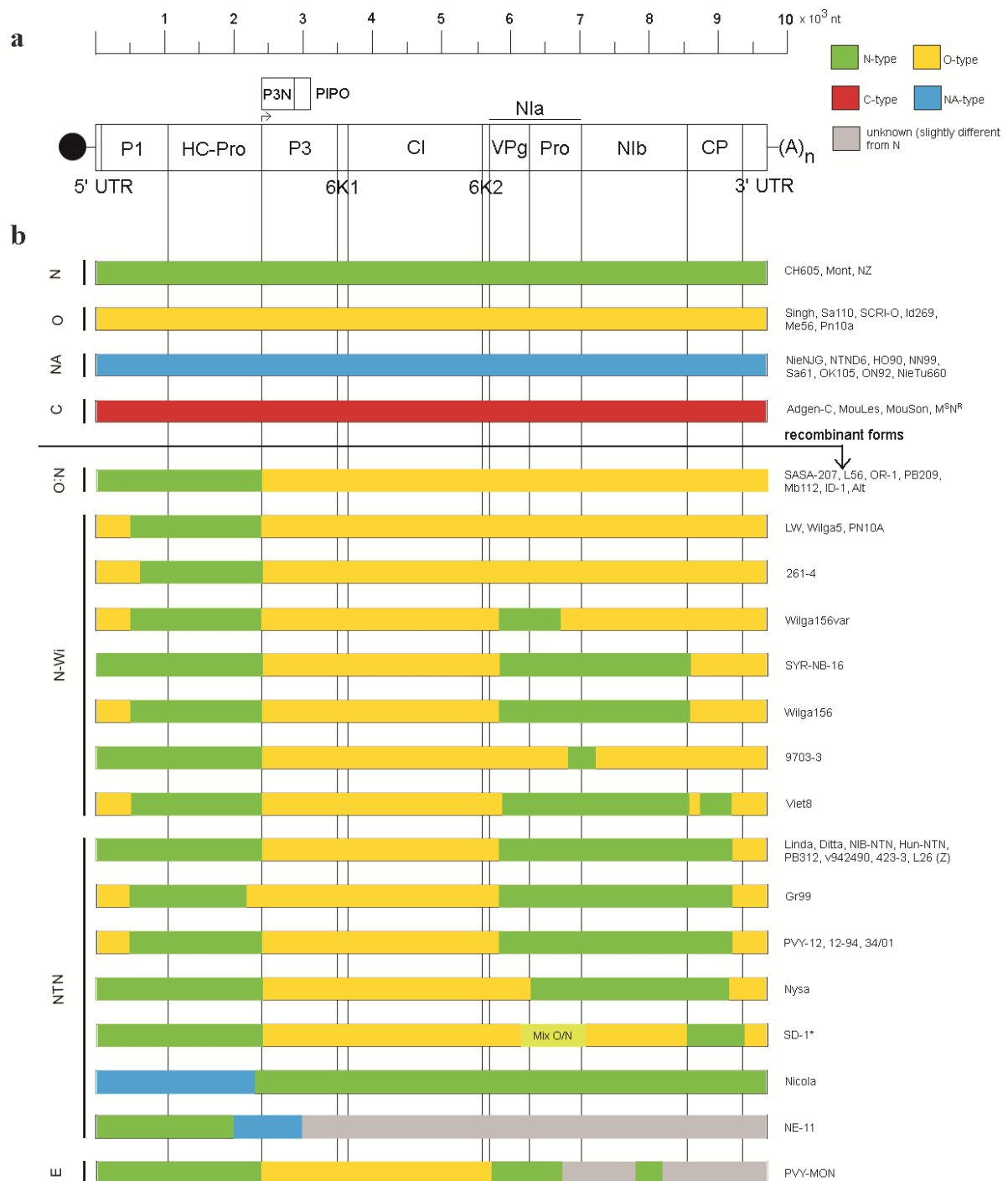
Fig. 2 The genome of PVY. **a** From 5' to 3': black circle = viral genome-linked protein (VPg); UTR = untranslated region; P1= protein 1; HC-Pro = helper component protease; P3 = protein 3; P3N-PIPO = fusion of the amino (N)-terminal part of P3 with PIPO (Pretty Interesting *Potyviridae* ORF), protein is produced after +2 frame shifting (arrow) from an overlapping ORF; 6K1 = first protein with a length of 6 kDa; CI = cytoplasmic inclusion protein; 6K2 = second protein with a length of 6 kDa; VPg and Pro (protease) are two possessed domains of the NIa = first nuclear inclusion protein; Nib = second nuclear inclusion protein; CP = coat protein; $(A)_n$ = polyadenosine (poly-A) tail. **b** Genome and recombination structure of different isolates assigned to known strains and variants of PVY. Strains are indicated on the left, names of isolates on the right.

In the last years a reclassification of the strain PVY^Z was proposed, because the single PVY^Z isolate that was sequenced (L26) is molecularly identical to standard PVY^{NTN} isolates, such as Linda or Ditta (Glais et al. 2002; Lorenzen et al. 2006; Schubert et al. 2007) and causes the PTNRD (Hu et al. 2009c). Furthermore, like L26, all PVY^{NTN} isolates seem to elicit a HR in potato indicator cultivars carrying the *Nz* gene (Kerlan et al. 2011). PVY^Z and PVY^{NTN} isolates only differ in their symptoms in tobacco: PVY^Z induces mosaic and vein clearing, while PVY^{NTN} induces vein necrosis (Kerlan et al. 2011; Karasev and Gray 2013b). Therefore, Kerlan et al. (2011) suggested to rename PVY^Z into PVY^Z-NTN. Furthermore, Kehoe and Jones (2015) sequenced and analyzed four historical PVY^Z isolates that were biologically characterized by Jones in 1990. Surprisingly, in a phylogenetic analysis, they clustered with PVY^O isolates, although they did not elicit HR in potato indicator cultivars carrying the *Ny* gene. This supports the finding that some PVY^Z isolates also induce the "leaf-drop streak" symptom (Kerlan et al. 2011), described as a PVY^O strain-specific trait (De Bokx and Huttinga 1981). In addition, a PVY^Z isolate found in France was recognized by anti-PVY^{OC} antibodies but not by anti-PVY^N antibodies, which isolate L26 reacts with (Kerlan et al. 1999).

Initially, the classification of isolates into the strain group PVY^{NTN} was done on their biological property to cause the PTNRD. However, until now many more isolates were found causing the PTNRD that molecularly do not fit into the pattern of the PVY^{NTN} strain, such as Tu660 (PVY^{NA}) (Nie and Singh 2002a), ID-1 ($PVY^{O:N}$) (Piche et al. 2004), SYR-NB-16 (PVY^{N-Wi}) (Chikh Ali et al. 2007) or PVY-MON (PVY^E) (Galvino-Costa et al. 2012). Conversely, infected indicator potato cultivars sometimes lacked the PTNRD in some or all tubers (Beczner et al. 1984; Xu et al. 2005; Singh et al. 2008).

These findings reveal a disagreement between the biological, serological and phylogenetic nomenclature systems of PVY strains and some authors call for a replacement of the actual strain nomenclature (Fig. 2b) with a system that uses Latinized numerals for phylogenetic groups (I-XIII) while retaining the naming of strains on the basis of biological properties (Jones 2014; Kehoe and Jones 2015).

Within the last three decades recombinant strains, especially PVY^{NTN}, $PVY^{O:N}$ and PVY^{N-Wi} have become the most prevalent PVY strains worldwide (Blanco-Urgoiti et al. 1998; Salazar et al. 2000; Lindner and Billenkamp 2005; Baldauf et al. 2006; Crosslin et al. 2006; Lorenzen et al. 2006; Varveri 2006; Chikh Ali et al. 2007; Boukhris-Bouhachem et al. 2010; Galvino-Costa et al. 2012; Nanayakkara et al. 2012; Ogawa et al. 2012; Visser et al. 2012; Gao et al. 2014; Benedict et al. 2015; Lindner et al. 2015) and are believed to possess a fitness advantage over non-recombinant strains (Kerlan 2004; Karasev and Gray 2013a). Verbeek et al. (2010) and Srinivasan and Alvarez (2007) found that *M. persicae* transmits PVY^{N-Wi} and

PVY^{NTN}, respectively, more efficiently than other PVY strains. Furthermore, recombinant strains tend to be less symptomatic on many cultivars (Chrzanowska 1991; McDonald and Singh 1996a, b; Nie et al. 2012), which often might lead to visual field inspections that fail to recognize PVY infections resulting from recombinant strains (Gray et al. 2010). However, a recent study showed that potato plants of different cultivars infected with PVY^{N-Wi} or PVY^{NTN} caused mild and severe symptoms in nearly equal numbers of infected samples, independently from the virus titer within the plants (Lindner et al. 2015). Moreover, PVY^{N-Wi} and PVY^{NTN} isolates were found in Vietnam that caused very severe symptoms on locally grown potato plants and different potato cultivars tested under experimental conditions (Schubert et al. 2015).

1.2.2 PLRV

Potato leaf roll virus (PLRV) is a Polerovirus in the family *Luteoviridae*. Symptoms of a primary PLRV infection consist of chlorosis, upward rolling of young leaves at the base of the stem and stunting of the whole plant. Plants grown from infected tubers develop severe stunting and upward rolling of basal leaves. Necrosis may develop in the phloem tissue in the upper parts of plants and in tubers (net necrosis) especially in cultivars, such as 'Russet Burbank' or 'Green Mountain'. Due to the impaired phloem transport of carbohydrates (Herbers et al. 1997; Loebenstein 2001), the tubers of PLRV infected plants are considerably reduced in size, and yields may be diminished by 33-50% (Loebenstein 2001). In comparison to PVY, PLRV has a narrower host range that is mainly restricted to species of the Solanacae, such as *Datura stramonium* (diagnostic species), *D. tatula*, *Physalis floridana* (diagnostic species), *S. nigrum*, *S. villosum* or *S. paniculatum* (Thomas 1987; Loebenstein 2001). However, beyond *S. tuberosum,* several other crop plants can also be infected, such as *Lycopersicon esculentum*, *Nicotiana tabacum*, *Medicago sativa*, *Allium cepa*, *Malus domestica*, *Fragaria* x *ananassa* and *Prunus persica*.

Once, systemic insecticides were not as common in potato production as today, PLRV was classified as the most prevalent potato virus reducing both, yield and tuber quality (Douglas and Pavek 1972; Killick 1979). Over the last 30 years, the significance of PLRV decreased remarkably due to the implementation of statutory schemes for the production of healthy seed potatoes in many countries (European Union 2002; UNECE 2006). Furthermore, the control of aphid vectors became very successful due to the application of systemic insecticides. The group of neonicotinoids, developed in the late 1980s, today belongs to the most widely used systemic insecticides worldwide and has taken a share of 27 % of the total insecticide market of 6.330 billion Euros in 2010 (Jeschke et al. 2011; Simon-Delso et al. 2015). PLRV is a phloem-limited virus (Harrison 1999; Peter et al. 2008a; Peter et al. 2009) and is transmitted

by aphids in a persistent manner. The aphids die after the ingestion of systemic insecticides before the virus would reach the salivary glands from where the virus would have been transported to the salivary canal and thus to phloem cells. Therefore, systemic insecticides have a large share on the decrease of the significance of PLRV within the last 30 years. However, in the past it was repeatedly reported that very mild strains of PLRV exist that do not cause typical symptoms in some cultivars, especially if the plants are infected late in the season (Wright and Cole 1966; Wright et al. 1967; Bradley 1978; Barker and Woodford 1987). This could lead to undetected PLRV infections with a risk to the seed potato production, when infected plant material is not recognized and serves as source of infection (Hühnlein et al. 2016b). Furthermore, PLRV is rated among the plant viruses that benefit from climate change (Boland et al. 2004; van der Waals et al. 2013). Increasing mean winter temperatures facilitate the winter survival of aphid vectors and the second growth of potato plants that serve as a source of inoculums (Boland et al. 2004). For Germany, meteorologists register and predict an increase of mean winter temperatures and years with early summer drought as a result of the climate change (Adelphi et al. 2015). Therefore, it can be assumed that the number of PLRV infections may increase within future decades since a clear positive correlation could be shown between temperature and the size of aphid population, which in turn correlates with the frequency of PLRV infections (Ioannou 1989; Basky 2002; Khan 2002). Additionally, in 2013 the European Commission (EC) compiled a proposal about the restriction of the use of three neonicotinoids (clothianidin, imidacloprid and thiametoxam) for seed treatment, soil application (granules) and foliar treatment on bee attractive plants and cereals (European Commission 2013). However, the EU Member States did not reach a majority either in favour or against the proposal. In 2015 the European Food Safety Authority (EFSA) confirmed that the above mentioned neonicotinoids are a risk to bees when used as foliar sprays (EFSA 2015). Until January 2017 the EFSA will update its assessments of the risks to bees posed by these pesticides (EFSA 2016). The updated assessment then will serve as a basis of the decision of the EC on the adoption of the proposed restrictions. If the EC will decide to restrict the use of neonicotinoids, many of the systemic insecticides will be dropped from the list of permitted insecticides that can be used in potato growing (Peter Steinbach, personal communication, 2016). An insufficient control of aphid vectors may lead to an increase of PLRV infections in potato fields.

1.2.2.1 Particle morphology and genome structure

The positive-sense single-stranded RNA of PLRV is approximately 5.8 kb in length (Loebenstein 2001) and is packed into non-enveloped icosahedral particles with a diameter of 24-25 nm (see Fig. 3). The capsid comprises 180 CP subunits with a percentage containing a

read-through (RT) extension located on the particle's surface (Bahner et al. 1990; Peter et al. 2008b; Chavez et al. 2012). However, the number of subunits of the CP replaced by the RT protein is still unknown.
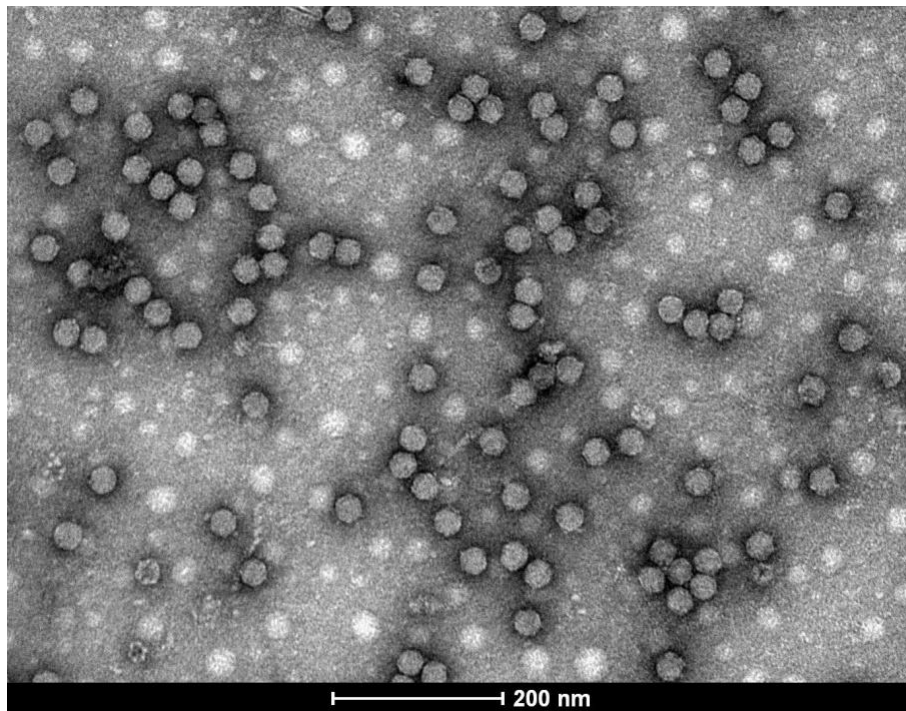
Fig. 3 Isometric virions of PLRV isolate JokerMV10 (accession number JQ346191) purified from potato cultivar 'Joker'

Sequences of PLRV isolates originating from different geographical regions were sequenced and analyzed (Mayo et al. 1989; van der Wilk et al. 1989; Keese et al. 1990; Pałucha et al. 1994; Guyader and Ducray 2002; Hühnlein et al. 2016b). Sequence homologies were found reaching from 95% to 97%. Phylogenetic analyses reveal no differentiation of strains on the basis of sequence patterns or geographical origins (Guyader and Ducray 2002; Djilani-Khouadja et al. 2005; Zarghani et al. 2012; Hühnlein et al. 2016b).
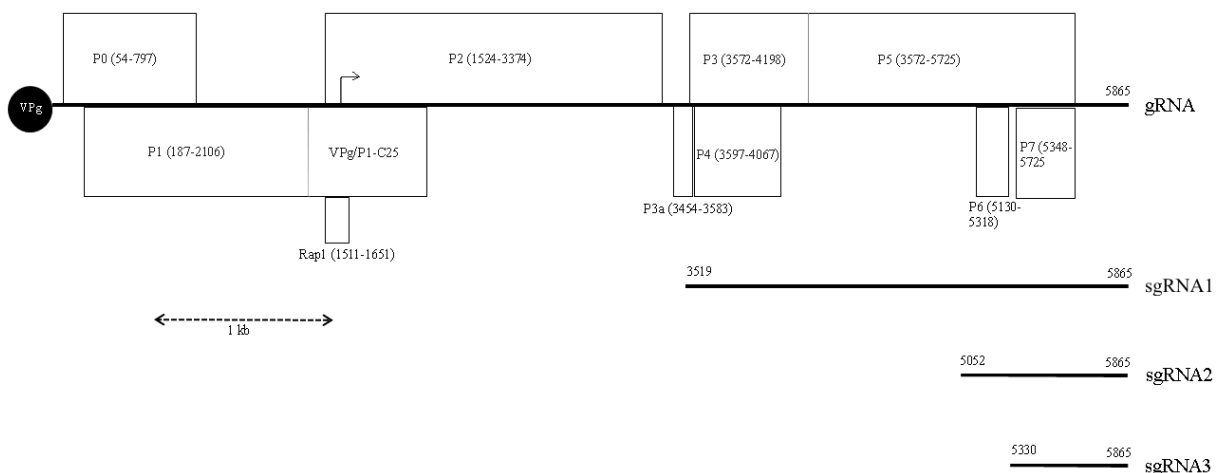
Fig. 4 Schematic diagram of the genome structure of PLRV, isolate JokerMV10 (accession number JQ346191). The position and length of ORFs and the proteins, which are translated from them, are displayed as oblongs. The lengths of genomic and sub-genomic RNAs (gRNA and sgRNA) are illustrated as black lines. The viral genome-linked protein (VPg) is indicated by a black circle at the 5' end. The arrowhead in the lower left corner of oblong P2 indicates the approximate position of a programmed ribosomal frame shift.

The genome of PLRV consists of ten ORFs, which encode eleven proteins (see Fig. 4). Five proteins are translated from the genomic RNA (gRNA): P0, P1/VPg-C-25, P2 and the replication associated protein (Rap1). The proteins P3, P3a, P4 and P5 are translated from the first sub-genomic RNA (sgRNA1) (Smith and Harris 1990; Miller and Mayo 1991; Miller et al. 1995). sgRNA2 encodes for the proteins P6 and P7 (Ashoub et al. 1998) and a recently found sgRNA3 encodes only for P7 (Hwang et al. 2013). The two protein clusters translated either from g or sgRNA1 are separated by a 94 nt long intergenic region containing the leader sequence (LS), which is thought to maintain a 1:1 ratio in the synthesis of P3 (coat protein) and P4 (movement protein) (Juszczuk et al. 2000).

1.2.2.2 Functions of ORFs and translated proteins

The expression of ORF0 was former correlated with the induction of PLRV symptoms (van der Wilk et al. 1997a). However, the development of symptoms after the expression of ORF0 not merely is a direct result, but rather a consequence of the meanwhile known role of P0 in the suppression of the plant`s RNA silencing mechanism (Baumberger et al. 2007; Derrien et al. 2012; Almasi et al. 2015; Cascardo et al. 2015; Wang et al. 2015a). Argonaute1 (AGO1) is an endonuclease that binds siRNAs generated by Dicer-like enzymatic degradation of viral double-stranded RNAs and is the key component of the RNA-induced silencing complex (RISC) in plants (Baumberger et al. 2007; Bortolamiol et al. 2007). Recent experiments with transformed *Arabidopsis* lines expressing the P0 of *Beet western yellows virus* (BWYV) upon β-estradiol treatment reveal that P0 targets the autophagic degradation of AGO1 by hijacking an ubiquitin E3 ligase to ubiquitylate AGO1. The ubiquitylated AGO1 is then targeted for degradation by selective autophagy before the RISC assembles (Derrien et al. 2012). However, Zhuo et al. (2014) reveal that the P0 protein of a Mongolian PLRV isolate (P0[PL-IM]) does not interact with the ubiquitin E3 ligase and sugggest that P0[PL-IM] may suppress RNA silencing by using an alternative pathway to target AGO1 for degradation. The P0 protein contains an F-Box-like domain (Pazhouhandeh et al. 2006; Zhuo et al. 2014), which has, together with the C-terminal conserved P0 sequence, an important role in the suppression of RNA silencing (Han et al. 2010). However, the F-Box-like domain of P0[PL-IM] differed from F-Box-like motifs of other Poleroviruses and was declared as "unusual" by Zhuo et al. (2014). It

has a Trp/Gly (W/G) sequence instead of Leu/Pro (L/P), requirements for the suppressor activity. Furthermore, it has an additional GW/WG-like motif that is lacking in P0 proteins of other Poleroviruses. Nevertheless, the silencing suppressor activity of P0^{PL-IM} was shown to be strong, which is not the case for all Polerovirus P0 proteins (Kozlowska-Makulska et al. 2010; Delfosse et al. 2014).

The P1 (and P2) protein is required for virus replication and thus multiplication (Sadowy et al. 2001a; Nickel et al. 2008). The 70 kDa protein P1 comprises multiple domains. The central domain contains a serine protease motif (Miller et al. 1995) and the C-terminal domain codes for the VPg (van der Wilk et al. 1997b). During the virus life cycle P1 undergoes autoproteolytic processing (Sadowy et al. 2001a; Sadowy et al. 2001b; Li et al. 2007). The hydrophobic N-terminal end of P1 targets the protein to cellular membranes while the hydrophilic C-terminal end of P1 binds to the 5' end of the viral RNA. Afterwards, a 25 kDa P1-C25 protein is proteolytically processed from the C-terminal end, which contains either the VPg or is located to it (Prüfer et al. 1999). Herby, the VPg and the 5' end of PLRV RNA are juxtaposed, which is essential for virus multiplication. Prüfer et al. (1999) assume that P1-C25 may protect the VPg from cellular proteases through protein folding. Additionally, a small ORF within the P1 region was found, the replication-associated protein 1 (*Rap1*). It is translated through an unusual internal ribosome entry site (IRES) and is essential for viral multiplication (Jaag et al. 2003).

P2 is translated by ribosomal frame shift from ORF 1 and carries the conserved extended GDD motif typical of RNA-dependent RNA polymerases (RdRp) (Kamer and Argos 1984; Koonin 1991; Prufer et al. 1992).

The 23,2 kDa coat protein (CP) of PLRV (Smith and Harris 1990) is translated from ORF 3 and has an N-terminal arginine-rich domain, which is found in the inner part of the capsid interacting with the viral RNA and a shell domain, which forms the core of the capsid (Rossmann and Johnson 1989; Terradot et al. 2001). The CP gene is highly conserved among PLRV isolates and also between the members of the *Luteoviridae* family (Mukherjee et al. 2003). Although a nuclear localization of PLRV has not been demonstrated, a conserved nuclear localization signal (NLS) motif was found in an Indian isolate that may be responsible for the transport of virus particles through nuclear pores. However, the NLS motif of the Indian PLRV CP was located on the inside of particles, which makes an involvement in nuclear pore translocation improbable (Garcia-Bustos et al. 1991; Mukherjee et al. 2003). As it was mentioned above, a small portion of the CP is enlarged by its read-through domain (RTD) forming the read-through protein (RTP). The RTD is fused to the CP and is translated by a suppression of the CP amber stop codon (Bahner et al. 1990). It could be shown that the incorporated and non-incorporated form of the RTP is required for systemic movement in

plants (Peter et al. 2008b). Particularly, the RTD is involved in efficient virus transmission by aphids (Brault et al. 1995; Brault et al. 2000; Brault et al. 2003) by regulating the efficiency of virus movement across the gut and salivary tissues (Reinbold et al. 2001). In addition, virus retention in aphids is regulated by the interaction of the RTD with the protein GroEL (also known as symbionin) produced by *Buchnera* spp., the primary endosymbiotic bacterium of aphids (van den Heuvel et al. 1999). The amino-terminal region of the RTD mediates the binding to the N- and C-terminal regions of the so-called equatorial domain of GroEL (Hogenhout et al. 1998). This interaction might protect the virion from proteolytic breakdown during trafficking within the aphid vector (Young and Filichkin 1999). Finally, it could be demonstrated that both 5' and 3' UTR and the LS have an influence on the protein expression of gRNA and sgRNA (Juszczuk et al. 1997; Juszczuk et al. 2000). Within the coding sequence of the CP and its RTD an additional translation regulatory element was detected that may modulate the protein synthesis of sgRNA1 (Loniewska-Lwowska et al. 2009).

P3a was recently identified by Smirnova et al. (2015). This protein is translated from sgRNA1 at a non-AUG codon initiation site. P3a is involved in long-distance movement and like P4 (movement protein) it is also targeted to the Golgi apparatus and plasmodesmata (PD), indicating also a role in the cell-to-cell spread of the virus.

P4 or the viral movement protein (MP) mediates cell-to-cell spread of viral particles through PD (Carrington et al. 1996). It is translated from sgRNA1 by internal translation initiation. The C-terminal half of the MP binds to single-stranded nucleic acids in a sequence-unspecific manner (Tacke et al. 1991). P4 is transported to the PD via the ER-Golgi network (Hofius et al. 2001; Vogel et al. 2007) where the serines S71 and/or S79 in its nucleic acid binding domain are phosphorylated (Lucas and Lee 2004; Link et al. 2011). Phosphorylation is essential for targeting host PD (Schmitz et al. 1997; Link et al. 2011) but it does not inhibit the nucleic acid-binding activity (Tacke et al. 1993). At the PD site P4 is able to increase the size exclusion limit (SEL) (Lucas and Lee 2004). As a plant defense response to a PLRV infection, large amounts of callose is deposited in the sieve tubes and at the extracellular region adjacent to the plasma membrane domains at both sites of the PD channel, which decreases the SEL. With the aid of the MP PLRV is able to take advantage of the host β-1.3-glucanases to reduce PD callose accumulation (Iglesias and Meins 2000; Bucher et al. 2001). De Storme and Geelen (2014) assume that the MP may target host β-1.3-glucanases to the PD or that it inhibits the stress induced production of PD callose.

P6 is translated from sgRNA2 and its role is still unknown. It is speculated that it has a minor supporting role in replication (Mohan et al. 1995). A point mutation and deletion analysis, accomplished by these authors, revealed that knocking-out of ORF6 prevents the accumulation of sgRNA2. Young et al. (1991) showed that a +1 frame shift mutation in the

ORF6 of *Barley yellow dwarf virus* (BYDV-PAV) results in an interruption in the corresponding amino acid sequence and the loss of infectivity. Recent *in vitro* studies using BYDV-GAV demonstrated a RNA-silencing suppressor activity of P6 (Liu et al. 2012).

P7 is translated from sgRNA2 and sgRNA3 and is known to have nucleic acid binding properties (Rohde et al. 1994; Ashoub et al. 1998; Taliansky et al. 2003).

1.3 Potato breeding for resistance to viruses

Of the genus *Solanum* approximately 200 tuber-bearing potato species are classified originating from the Central and South American continent. The cultivated species *S. tuberosum* can be classified into nine informal groups: Tuberosum, Andigenum, Chaucha, Phureja, Stenotomum, Curtilobum, Ajanhuiri and Juzepczukii (Dodds and Paxman 1962; Huaman and Spooner 2002). Instead of groups it is also common to use cultigenic species such as *S. phureja* or *S. andigena*. Within the cultivated species The World Catalogue of Potato Varieties (Pieterse and Judd 2014) lists more than 4800 cultivars grown around the world considerably differing in shape, taste, processing properties, adaptation to the length of growing season or the resistance to pests and pathogens. For reasons of environmental protection the application of very toxic pesticides today is prohibited in many countries worldwide. Instead, plant protection measures have to comply with integrated pest management approaches, which include the cultivation of resistant varieties if possible. Therefore, the resistance to pests and pathogens of (potato) cultivars is an important value-determining trait within the licensing procedure of new varieties.

1.3.1 Conventional breeding methods

Most of the common cultivated potato species are autotetraploid ($2n = 4x = 48$). However, the majority of the approximately 200 wild potato species is diploid. Nevertheless, they are potentially rich sources of genes that can be conductive for potato breeding improving the resistance of cultivated potatoes against biotic and abiotic stress factors. The endosperm balance number (EBN) is a genetic model for predicting the interspecific crossing success of potato species (Johnston and Hanneman 1980). In fact, varieties with the same ploidy level do not necessarily hybridize if they have different EBNs. For example, the tetraploid *S. acaule* (2EBN) is not crossable with the tertraploid *S. tuberosum* (4EBN). In turn, *S. acaule* hybridizes with diploid species that have an EBN of 2 (Carputo et al. 2003). Crossability between diploid wild species and tetraploid varieties can be achieved by manipulating the EBN. Increasing the EBN can be achieved by using gametes from diploid species with the somatic chromosome number ($2n$ gametes) (Watanabe and Peloquin 1989; Carputo et al.

2003). Decreasing the EBN is possibly by using (di-)haploids from tetraploid varieties as bridging genotypes to transfer useful traits from diploids to the tetraploid cultivated gene pool (Watanabe et al. 1995). Distantly related diploid wild potato species with an EBN of 1 are an important genetic pool for many resistances to pathogens (Spooner and Bamberg 1994). However, direct crossing with cultivated potato lines result in odd ploidies, which hamper further breeding (Watanabe 2015). If crossing barriers exist that make hybridizing of varieties impossible, then mentor/double pollination and/or embryo rescue may lead to success. The first strategy involves the application of pollen from the incompatible species followed by that of the compatible variety (mentor), one or two days later (Brown and Adiwilaga 1991). The mentor pollen stimulates the development of fruit and the pollen from the incompatible variety may fertilize some ovules. Marker assisted selection helps later to eliminate the offspring of the compatible hybridization (Jansky 2006). Embryo rescue can be applied in cases the endosperm is not expected to develop normally resulting in abortion of the seeds. Shortly before the embryo becomes aborted, it is removed from the ovule. Subsequently, the embryo is placed on nutrient medium in tissue culture allowing it to grow to maturity. A combination of both techniques, mentor pollination and embryo rescue, may yield in successful generation of hybrids differing in their ENB (Singsit and Hanneman 1991).

A process circumventing sexual reproduction is somatic fusion, where protoplasts are fused either by chemical or electro-fusion. Somatic fusion was applied especially to fuse 2ENB and 1EBN genotypes, such as *S. bulbocastanum* (Helgeson et al. 1988), *S. brevidens* (Fish et al. 1988) or *S. pinnatisectum* (Menke et al. 1996). Such hybrids from somatic fusion can be used in crosses with cultivated potato to produce backcross generations. In every backcross generation it has to be proven if the new desired traits are still present. Recently, somatic hybridization was done with *S. bulbocastanum* (1EBN) and *S. tuberosum* (4EBN) in order to shorten the breeding process since classical breeding by bridge species is very time consuming taking up to 50 years of continuous crossings (Rakosy-Tican et al. 2015). However, the results of this study suggest that *S. bulbocastanum* and cultivated potatoes are somatically incompatible producing many asymmetric somatic hybrids lacking several genes or even whole chromosomes especially from the wild species (Rakosy-Tican et al. 2015). Such incompatibilities can be overcome by using molecular methods (see chapter 1.3.2). In addition, plants derived from somatic fusion that cannot be obtained by conventional breeding are regulated by the European legislation for genetically modified organisms (GMOs) (European Union 2001). This constrains the application of somatic fusion in European potato breeding.

Virus resistance genes were found in cultivated and wild potato species. Most of the breeding programs have concentrated on resistances mediating hypersensistive resistance (HR) or

extreme resistance (ER), because the inheritance of HR and ER to viruses is monogenic and dominant and therefore effective and durable (Solomon-Blackburn and Barker 2001a). Extreme resistant potatoes are often resistant to several strains of a virus or even to several viruses, but they are less common among potato cultivars. A plant extremely resistant to a virus prevents the virus from multiplication and does not show any symptoms (Solomon-Blackburn and Barker 2001b). The HR response of potatoes to virus inoculation is more strain specific but it is more widely available in cultivars than ER. A plant with HR to a virus prevents virus spreading by programmed cell death leading to local lesions or even systemic necrosis. However, HR can be affected by the physiology of the host or by environmental conditions (Solomon-Blackburn and Barker 2001b; Hühnlein et al. 2016a). An example is the temperature-dependent expression of necrotic reactions of cultivars 'Rywal', 'Albatros' and 'Sekwana' (Szajko et al. 2008; Szajko et al. 2014). HR genes in potato cultivars have been known for a long time and are used as sources for resistance against PVA (Na), PVX (Nx), PVY^C (Nc), PVY^O (Ny) or PVY^Z (Nz) (Cockerham 1943; Jones 1990). Many of them have their origin in *S. tuberosum*. However, HR genes from wild species are also known, such as Ny_{adg} from the cultigen *S. andigena*, Ny_{chc} from *S. chacoense* or Ny_{dms} from *S. demissum* (Solomon-Blackburn and Barker 2001b). In cultivated potatoes many ER genes were introduced from *S. stoloniferum* (Ry_{sto}), *S. hougasii* (Ry_{hou}) or from the cultigen *S. andigena* (Ry_{adg}). They are resistant against PVY and/or PVA. Potato plants harboring the Ry_{adg} or Ry_{sto} gene are resistant to all known PVY strains (Machida-Hirano 2015).

PLRV resistance in commercial potato cultivars seems to have arisen from germplasm of *S. demissum*, which originally was used as a source for late blight resistance (Brown and Corsini 2001). However, this kind of resistance is of a polygenic nature, which has several disadvantages. Individual potato plants can be infected with a high number of PLRV particles and often remain symptomless (tolerant). Furthermore, the number of infected plants is dependent on the virus pressure and environmental conditions resulting in high PLRV infection rates in some years with favorable conditions for aphid flights and virus accumulation (Brown et al. 1997; Ummad-ud-Din et al. 2011). Additionally, the inheritance of polygenic resistances is complicated because of the tetraploid potato genome. Extreme resistance to PLRV was only found in diploid *Solanum* species, such as *S. brevidens*, *S. etuberosum*, *S. acaule* (Swiezynski et al. 1989) or *S. chacoense* (Brown and Thomas 1993).

Conventional breeding is associated with permanent selection of lines harboring the desired traits. Tuber characteristics or resistances to pathogens are traits whose selection is costly and time-consuming. Tuber traits can be determined not before harvest and the tests are often destructive limiting the amount of vegetative propagation material. The screening for pathogen resistance needs expensive inoculation and detection methods and some potato

breeders do not have the equipment to accomplish all the laboratory work. Diagnostic DNA-based markers can enhance the efficiency and precision in potato breeding reducing the time and costs for extensive field trials (Tiwari et al. 2012; Gebhardt 2013). Potato plants, which harbor the Ry_{adg} gene, are resistant to all known PVY strains. This gene was localized on chromosome number eleven (Hämäläinen et al. 1998) and can be detected by DNA-based makers, such as sequence-characterized amplified regions (SCARs) (Kasai et al. 2000) or cleaved amplified polymorphic sequences (CAPS) (Flis et al. 2005a). The gene for ER Ry_{sto} was localized on chromosome number twelve (Flis et al. 2005a; Song et al. 2005) and several markers were developed detecting the gene in potato plants, such as simple sequence repeats (SSR) (Milbourne et al. 1998), sequence-tagged sites (STS) (Song and Schwarzfischer 2008) or CAPS (Witek et al. 2006). Using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers, the Ry_{chc} gene of *S. chacoense* was mapped to the most distal end of the chromosome number nine (Sato et al. 2006). Recently, a microsatellite marker (SSR) was developed detecting the ER in *S. phureja*, whose Ry_{phu} gene was also localized on chromosome number nine (Torrance et al. 2009; Al-Abedy et al. 2012).

Rlr_{adg} is a resistance gene from *S. andigena* making potato plants extremely resistant to PLRV. It can be detected by SCAR and CAPS markers (Mihovilovich et al. 2014) and is located on the upper arm of chromosome number five (Velasquez et al. 2007). The gene for ER against PLRV in *S. etuberosum* (Rlr_{etb}) was mapped to chromosome number 4. CAPS markers were developed flanking the Rlr_{etb} gene at 2.1 cM (Kuhl et al. 2016). The resistance genes responsible for the relative resistance in some potato cultivars were analyzed by quantitative trait loci (QTL), namely *PLRV1* on potato chromosome number 1, *PLRV2* on chromosome number 6, *PLRV3* on chromosome number 5 (Marczewski et al. 2001) and *PLRV4* on chromosome number eleven (Marczewski et al. 2004).

However, if a marker is not located directly within the resistance gene, a biological evaluation additionally is needed to determine the presence of the desired trait. Furthermore, not for all virus resistance traits DNA-based markers will have an impact on potato breeding. Some traits may be tightly linked to undesired traits, such as fertility reduction, low yield or non-marketable tuber traits (Ross 1986). Molecular methods, such as genetic engineering or genome editing may therefore be more precise and efficient and allow direct and intergeneric modifications of the potato genome, which was recently sequenced, completely (PGSC 2011).

1.3.2 Breeding using molecular methods

With conventional breeding many important tuber and resistance traits have been successfully introgressed into the cultivated potato. Therefore, in potato breeding the use of molecular

methods is not as common as in other crops, such as rape or maize. Nevertheless, the advantage of genetic engineering or genome editing is the introgression, repair, change or knock-out of specific genes in plants without the need of backcrossing. The desired trait(s) can be added to a selected cultivar, which considerably fastens the breeding process (De Koeyer et al. 2011). Furthermore, the barrier of incompatibility can be overcome, since no crossing is needed to introduce a desired trait.

First approaches of genetic engineering to achieve resistance to virus infection used the expression of viral coat proteins (CP) in plants (CP-mediated resistance). Already in 1986 Abel et al. reported that the expression of a *Tobacco mosaic virus* (TMV) CP gene in transgenic tobacco plants confers protection against an infection by TMV. Three years later, CP-mediated resistance was also reported for transgenic potatoes: In 1989 the potato cultivars 'Bintje' and 'Escort' were transformed expressing the CP of PVX (Hoekema et al.). Kaniewski et al. (1990) demonstrated that transgenic 'Russet Burbank' potato plants expressing CP genes of PVX and PVY were resistant to simultaneous infection by these viruses. However, the mechanisms that govern CP-mediated resistance are not fully understood and they are different in different viruses (Bendahmane et al. 2007). As it was mentioned in chapter 1.2.1.1, the CP of potyviruses is also involved in the inhibition of viral RNA translation in a dose-dependent manner (Besong-Ndika et al. 2015). In transgenic plants expressing high amounts of CP, invading viral RNAs may be encapsidated and ribosomes in the process of translation may be removed from the viral RNA by the CPs. Disassembled virions may subsequently be reassembled and no further translation takes place, which possibly leads to resistance of the transgenic plant. However, reports exist that the resistance in CP-expressing transgenic lines may not be directly related to the CP expression level but rather to a RNA-mediated mechanism leading to RNA silencing (Galvez et al. 2014). Currently, CP-mediated resistance is used commercially for PVY resistance in the so-called Hi-Lite or Shepody New Leaf™ Y potato from the Monsanto company authorized as food and/or feed in the United Stated of America, Australia, Canada, Japan, Mexico, New Zealand and South Korea (ISAAA 2015). van der Wilk et al. (1991) and Kawchuk et al. (1991) regenerated PLRV resistant 'Desiree' and 'Russet Burbank' potato plants, respectively, containing the CP gene of PLRV. The transformed plants were resistant although no accumulation of CP could be detected in the transgenic tissue. Furthermore, transgenic plants whose construct was inserted in the reverse orientation (anti-sense) showed a similar level of resistance to PLRV. Therefore, these transgenes confer RNA-mediated protection (Prins et al. 2008). The RNA-mediated resistance leads to viral RNA silencing. Sequence identity between the transgenic and the viral RNA leads to the formation of dsRNA, which is a substrate for the RNaseIII-like enzyme Dicer. The Dicer cleaves the long dsRNA into siRNAs, which are

incorporated into the RISC complex and finally lead to viral RNA silencing (Novina and Sharp 2004). However, simple sense or anti-sense viral transgenes usually are unstable and sometimes yield in partial resistance (Maki-Valkama et al. 2000; Arif et al. 2012).

Transgenic plants producing dsRNAs prior to the infection are transformed with inverted-repeat (IR) constructs designed to express hairpin RNAs (Waterhouse et al. 1998; Prins et al. 2008). These plants have active RISCs with sequence-specific antiviral recognition prior to a virus infection. Upon inoculation with the respective virus, the virus RNA can be rapidly targeted and degraded, even before virus-encoded RNA silencing suppressor proteins are produced. This approach is very efficient with up to 90 % of transgenic plants being resistant to the homologous virus (Waterhouse and Helliwell 2003). An example for the construction of IR transgenesis is the study of Missiou et al. (2004), who generated transgenic potato plants that were highly resistant to PVY through RNA silencing. The potato plants were not only resistant to the homologous PVY isolate but also to several other isolates with sequence similarities between 93% and 98%. Chen et al. (2010) generated transgenic tobacco plants by introducing hairpin RNAs consisting of fragments from the 3' ends either of the P1, HC-Pro, P3, CI, NIa-VPg, NIa-Pro, NIb or CP gene of PVY[N]. It seemed as if the different sequences of the PVY genome had different abilities to protect tobacco from viral infection. Plants transformed with vectors containing the 3' ends of the CP and the VPg gene, respectively, showed the highest ratio of resistance of above 60%. The resistance ratio was reduced (only 40%) when vectors were used containing the 3' ends of the HC-Pro and NIb genes, respectively (Chen et al. 2010). Schubert et al. (2005), Chen et al. (2010) and Jiang et al. (2011) found a linear relationship between the local free energy in the target region and the efficiency of siRNAs to cause virus resistance. The higher the local free energy was, the more stable was the secondary structure of the hairpin RNA. Ai et al. (2011) transformed tobacco plants with artificial micro RNAs (amiRNAs) targeting the p25 of PVX and the HC-Pro of PVY. They observed a similar relation between the local free energy and the ratio of virus resistance. They concluded that a high stem-loop stability would make the pre-amiRNA transcript fold more rapidly into its secondary structure, resulting in faster formation of the RISC and more potent gene silencing (Ai et al. 2011). Song et al. (2014) created amiRNAs targeting different PVY genes. In contrast to the authors mentioned before, they found only a weak correlation between the local free energy of amiRNAs and the level of virus resistance. More likely, they ascertained that the presence of the 30 terminal free nucleotides within the amiRNA structures may influence RNA silencing efficiency. However, they observed that amiRNA that targeted the NIb and CP genes displayed a higher silencing efficiency than did the amiRNAs that targeted the NIa or CI gene. Naveed et al. (2014) did deep sequencing of PVY-derived siRNAs. They found that siRNAs derived from every position in the PVY

genome, but with a distinct accumulation of unique siRNAs in different PVY strains. In plants infected either with PVY^O or PVY^{NTN}, the highest number of siRNAs derived from the CI gene. However in plants infected with PVY^N the highest number of siRNAs originated from the NIb gene. Moreover, they observed that uracil and adenine were the most frequent 5' nucleotide in all PVY-derived siRNAs, which probably stabilizes the siRNAs through an association with AGO1, the main AGO protein that forms the RISC (Mi et al. 2008). To sum up, the silencing efficiency of siRNAs and amiRNAs is different dependent on their sequence and priority to be incorporated into the RISC. Thereby, PVY genes are primarily concerned that have the most essential roles during virus replication, such as the RdRp (NIb) or the CP.

The mechanism of replicase-mediated resistance is still unknown and contradictory results were obtained by searching for the mediator (protein or RNA) of this kind of resistance (Galvez et al. 2014). Tobacco plants transformed with the RdRp gene of PVX were resistant to PVX, regardless whether the construct was modified or unmodified (Mueller et al. 1995). PVX resistance of these plants was absolutely dependent on low-level accumulation of the transgenic RdRp RNA. Therefore, in that case replicase-mediated resistance seems to be based on a RNA-mediated mechanism (Mueller et al. 1995). However, replicase-mediated resistance was demonstrated to be protein-mediated in tobacco plants that were transformed with the NIb gene of PVY^O, although it was not described to which extent the NIb gene was expressed (Audy et al. 1994; Prins et al. 2008). Deletions in the conserved GDD motif of the NIb gene lead to susceptibility. This resistance was very specific and limited to homologous PVY^O strains (Audy et al. 1994). Replicase-mediated resistance was also demonstrated on 'Russet Burbank' potatoes transformed with either a construct containing a full-length unmodified replicase gene of PLRV, an antisense-orientation of the full-length cDNA or a truncated replicase gene coding only for the 3' portion (Lawson et al. 2001). All transgenic lines showed a variable degree of resistance to PLRV, with the full-length sense RdRp gene construct being the most effective gene for conferring a high level of resistance to a PLRV infection. The replicase protein itself was not detected in the transgenic plants (Lawson et al. 2001) indicating a RNA-mediated resistance. Transgenic potatoes with PLRV replicase-mediated resistance (New Leaf™ Plus Russet Burbank potato from the Monsanto company) are commercially available as food and/or feed in the United Stated of America, Australia, Canada, Japan, Mexico, New Zealand and South Korea (ISAAA 2015).

The production of virus resistant transgenic plants based on pathogen-derived gene expression of the RdRp, the CP or the MP gene is under discussion. In laboratory experiments and under high selective pressure it was demonstrated that recombination events may occur between the resistance gene and an innocuous virus leading to the increase of its virulence (Schoelz and Wintermantel 1993; Greene and Allison 1994; Aaziz and Tepfer 1999; Tepfer et al. 2015). It

was shown that recombination does occur in transgenic plants expressing *Cucumber mosaic virus* (CMV) CP genes on the infection with a divergent strain of CMV (Turturo et al. 2008). However, by pyrosequencing Morroni et al. (2013) ascertained that all recombinants observed in transgenic plants were also observed in non-transgenic plants, infected with both strains of CMV. Additionally, Flatken (2006) and Dietrich et al. (2007) monitored recombination events of several potato viruses in transgenic and non-transgenic potato plants. No recombinant potyviral sequence was identified from those plants. Therefore, the likelihood of the emergence of novel viruses as a potential harm, is low (Dietrich et al. 2007; Tepfer et al. 2015). Nevertheless, it is possible to avoid any kind of recombination by using short viral sequences of siRNAs (IR technology, hairpin RNA) because recombination primarily occurred when longer viral sequences are expressed, as it is the case in transgenic plants expressing pathogen-derived genes (Tenllado et al. 2004). In addition, recombination can also be prevented when non-viral sequences are expressed in transgenic plants. One such approach is the expression of plantibodies either in full-size or as fragments. They can bind to the functional domain of viral proteins, inactivate them and thus prevent the disease (Safarnejad et al. 2011). The success of early approaches was limited by the instability of many single-chain variable fragment (scFv) antibodies in plant cells and the antibodies usually only slowed the progress of infection (Ziegler and Torrance 2002). Furthermore, the incomplete resistance in plants expressing recombinant antibodies targeting viral CPs was probably the result of the large amount of CPs accumulating in infected plant cells, which could not be completely neutralized by the lower amounts of scFvs produced in the transgenic plants (Gargouri-Bouzid et al. 2006). A very high degree of resistance to PVY was observed in transgenic potato plants that expressed scFv antibodies targeting the PVY NIa (Gargouri-Bouzid et al. 2006; Ayadi et al. 2012). In these plants the precursor protein of PVY could not be cleaved and virus accumulation was prevented, successfully. Non-structural proteins, such as NIa of PVY or P1 of PLRV, do not accumulate in such large amounts like structural proteins, and antibodies expressed at very low levels may achieve complete neutralization (Gargouri-Bouzid et al. 2006; Nickel et al. 2008; Safarnejad et al. 2011; Ayadi et al. 2012).

However, in the European Union plants or products declared as or containing GMOs are currently not accepted by the consumers and they are regulated by the Regulation (EC) No 1829/2003 on GM food and feed (European Union 2003) and by the Directive 2001/18/EC (European Union 2001). For every single trait the absence of harm and the substantial equivalence of the GM line to commercial varieties have to be proven (Wendt and Mullins 2011). To examine whether a GM line is substantially equivalent, is hardly to be answered, in particular with regard to safety evaluations. This is mainly because the safety of the traditional varieties often has not been sufficiently clarified and the reliability of conclusions of

toxicological and immunological safety on the basis of partial compositional analysis is at least doubtful (Spök et al. 2002). Therefore, in Europe the technology of genetic engineering is a victim of ambiguity and misinformation leading to non-marketability of every GM plant or its product in the EU for a long time or probably forever.

However, research proceeds in the area of genetic modification because the technologies have irrefutable advantages over conventional breeding methodologies (see chapter 1.3.1 and this chapter). In the last decade several techniques were developed, which are in the discussion of whether they should be exempted from the EU Directive 2001/18/EC or not. In cisgenic-derived crops only plant own genes are inserted, called cisgenes, allowing variety improvement with only natural alleles from the breeders gene pool (Schouten et al. 2006). That is the reason why several researches recommend exemption of this technology from the GMO regulation process considering cisgenesis as a new sub-invention in the traditional breeding field (Jacobsen and Schouten 2009; Wendt and Mullins 2011). However, the argument that a cisgenic plant may also be obtained by conventional breeding methods is not correct because the introgression of the target gene(s) takes place at indeterminable locations within the plant's genome and the introgression in some locations would not have been achieved by traditional breeding techniques (European Food Safety Authority 2012a). Therefore, jurisdiction currently demands cisgenic crops to be regulated as GMOs.

In recent years, novel plant breeding techniques (NPBT) were developed, whose resulting modifications in the plant's genome are indistinguishable from natural mutations in the conventional counterparts. In contrast to the specific undirected genome alterations achieved by genomic modification, genome alterations generated by NPBT are specific and directed (Hartung and Schiemann 2014; Small and Puchta 2014). Examples for NPBT are (i) accelerated breeding following early flowering, (ii) reverse breeding, (iii) grafting onto GM rootstocks, (iv) oligonucleotide-directed mutagenesis (ODM), (v) zink finger nucleases (ZFN), (vi) meganucleases, (vii) transcription activator-like effector nucleases (TALENs) and (viii) clustered regularly interspaced short palindromic repeats /associated protein nucleases (CRISPR/Cas). Since NPBT are quite new technologies, currently only few examples of applications on potato exist. Nicolia et al. (2015) and Butler et al. (2015) used TALENs and CRISPR/Cas, respectively, to target the acetolactate synthase gene in potato. Yasumoto et al. (2014) lowered the level of steroidal glycol alkaloids in potatoes by using TALENs. By using the same NPBT Clasen et al. (2015) obtained potato tubers that had improved cold storage and processing traits. The CRISPR/Cas9 system was used to introduce gene knock-outs in potato (Wang et al. 2015b) leading to a knock-out of the auxin/indole-3-acetic acid gene of *S. tuberosum* (*StIAA2*) that is involved in petiole hyponasty and shoot morphogenesis (Kloosterman et al. 2006). Additionally, New Generation Sequencing (NGS) techniques, such

as Targeting Induced Local Lesions in Genomes (TILLING), allow the discovery of false insertions or even single nucleotide mutations resulting from somaclonal variation, and thus provide further options to control the success and accuracy of genome editing techniques.

Whether the NPBT should fall under the scope of the European GMO legislation or not, is not yet decided by the European Commission (European Commission 2015). The EFSA has issued an opinion on side directed nucleases and recommended that NPBT should be regulated by the European GMO legislation but with the need of "lesser amounts of event specific data" (European Food Safety Authority 2012b).

2 An introduction to real-time polymerase chain reaction

Since its development in 1983 by Mullis et al. (1987) conventional PCR has revolutionized all areas of the life science disciplines, because of its efficiency, specificity and fidelity. However, the quantification of nucleic acids by PCR is problematic, because there are numerous experimental variables that become exaggerated by the exponential amplification (Bustin 2004a). Therefore, it is difficult to draw conclusions from the intensity of end-point signal to the absolute amount of starting and amplified product. Results are often inconsistent with a very variable reproducibility (Bustin 2004a). Therefore, a technique is needed, that measures the amount of amplified product very early in the reaction, when the chemicals are not yet depleted and the impact of experimental variables is still low. Real-time polymerase chain reaction (qPCR) fulfills this reqirement.

2.1 Technical considerations

qPCR measures the amplification of a targeted DNA molecule continuously during the PCR - in real-time - and not at the end, as it is the case in conventional PCR (Higuchi et al. 1993; Heid et al. 1996). In a reverse transcription (RT) qPCR the amplified target is a complementary (c) DNA derived from the RT of RNA or mRNA molecules (Gibson et al. 1996). The detection of the amplified product is based on the measurement of fluorescence emitted either from dsDNA intercalating dyes, such as SYBR® Green I and EvaGreen, or from fluorophore-labeled probes (e. g. TaqMan®, molecular beacon or scorpion®) (Navarro et al. 2015). As long as all reaction components in the qPCR are abounding, the amount of PCR product approximately doubles in each cycle. During this exponential phase (Fig. 5) the initial amount of template present in the reaction can be calculated, because there is an inverse relationship between the amount of target DNA or cDNA present at the start of a PCR and the amount of amplified product measured during the exponential phase (Bustin 2004a). However, the quantification starts not before the qPCR instrument first detects fluorescence above background noise. This point is defined as threshold (C_t) or quantification cycle (C_q) (Fig 5). The higher the initial amount of target in the qPCR is, the sooner the fluorescence crosses the threshold and the lower is the C_q value. If the difference between two C_q values is 1, then the amount of the amplified product has doubled, theoretically. After 30 cycles 2^{30} molecules should be amplified from one target DNA or cDNA molecule.
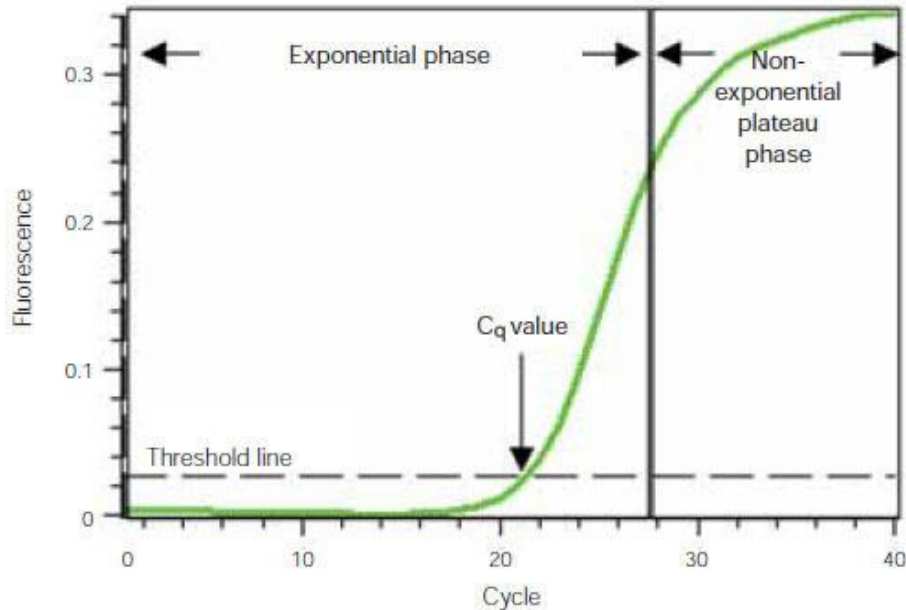
Fig. 5 Amplification plot of a qPCR run. The baseline-subtracted fluorescence is shown on the y-axis. The number of PCR cycles is depicted on the x-axis. During the exponential phase the amount of amplified product is approximately doubled in each cycle. Once the fluorescence exceeds the background noise, the C_q value can be determined. Later in the reaction - during the plateau phase - enzymes, primers, probes and dNTPs get depleted and no further amplification occurs.
The graph was copied with kind permission from the website of the Bio-Rad Laboratories, Inc. (Bio-Rad 2016).

However, in the real reaction the efficiency is not 100%, since not all target molecules are replicated. The overall efficiency of a RT-qPCR is dependent on many factors, such as the template secondary structure, the efficiency of the RT, the efficiencies of primers and probes, the initial copy number of target DNA or cDNA, an accurate pre-qPCR processing, the efficiency of the qPCR instrument, the presence of PCR inhibitors, the efficiency of enzymes, the efficiency of the thermal profile or the concentration of qPCR chemicals (Bustin 2004b; Kontanis and Reed 2006; Poma et al. 2012; Rogers‑Broadway and Karteris 2015). Furthermore, sample storage and preparation has a crucial impact on the reliability and reproducibility of the quantification of target nucleic acids. In order to determine the efficiency of a qPCR or RT-qPCR and to achieve an accurate quantification of the target nucleic acid, a standard curve has to be constructed. A standard curve consists of a dilution series with a known concentration of target (e. g. number of RNA copies). The C_q values for each dilution is then plotted against the log of the starting concentration. From the linear regression line the correlation coefficient "R^2", the slope and the Y-intercept are calculated. From the slope the efficiency can be calculated:

$$theoretical\ slope = \frac{ln(x)}{ln2}$$

and

$$E = 10^{-1/slope} - 1$$

where x is the dilution factor and E is the efficiency. An efficiency of 1 or 100% means that the amount of amplicon has doubled in each cycle: 2^n (n = number of cycles). If the efficiency is lowered to 99% by one of the above mentioned factors, then only 90% of the theoretically expected amplified product can be found after 10 cycles (1.98^{10}). After 30 cycles even only 74% of the expected amplified product can be found (1.98^{30}). Therefore, the uncertainty increases with the increase of the C_q value and thus with the decrease of the initial target copy number (Bustin and Nolan 2004). Therefore, it is essential to determine the limit of quantification (LOQ), especially when doing absolute quantification. The LOQ is the highest dilution of template that is still linear within the plot of C_q values and the log of the starting concentration (Linnet and Kondratovich 2004). In contrast, the limit of detection (LOD) is the lowest quantity of a template that can be distinguished from the non-template control (NTC) in 95 % of the cases (Waiblinger et al. 2011). During the optimization of a qPCR assay both, the LOD and the LOQ should be determined for the certainty of results. Since the results of a qPCR experiment may vary from laboratory to laboratory or even between two qPCR runs from the same sample, it is essential that sufficient experimental details are recorded and published with the results making them reliable and reproducible (Bustin et al. 2009). These requirements were specified in the guidelines for Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) by Bustin et al. (2009).

qPCR assays are divided into relative and absolute quantification. In relative quantification, changes in the gene expression relative to an untreated control or to a co-amplified reference gene can be analyzed. A reference gene is a constitutively expressed gene that ideally is found in constant copy numbers under all tested conditions (Pfaffl 2004). Commonly used reference genes, also called endogenous controls, are albumin, actins, 18S rRNA or 28S rRNA. However, the expression level of some of the so-called "housekeeping" genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-tubulin, can alter dramatically with different experimental conditions (Greer et al. 2010) and should not be used as reference genes. Prior to the relative quantification of a sample it has to be validated that the amplification efficiencies of the assay for the gene of interest and the reference are approximately equal. However, no standards with known concentrations are needed. The calculation of the relative changes in the gene expression is based on the comparison of the C_q values of target and reference gene resulting in an out-put that is expressed as a fold-difference (e. g. 10-fold) of expression levels.

In the absolute quantification a single target sequence (e. g. within a viral genome) is quantified based on the comparison of the C_q values of a standard with known concentrations. If the copy number of a RNA sequence should be determined the standard should also undergo the RT step because the efficiency of the RT may vary, considerably (Pfaffl 2004). However, the production of a RNA standard is costly in time and resources. Therefore, plasmid DNA can be used with a sufficient reliability to quantify RNA when the copy number of template DNA for the standard exceeds 100 DNA copies (Bowers and Dhar 2011; Ziegler et al. 2014). However, if it is expected that the template RNA copy number in the samples may be less than 100 RNA copies, then a RNA standard is essential. A RNA standard can consist of *in vitro* transcribed plasmid DNA, into which a RT-PCR fragment of the target sequence was cloned. The concentration of the recombinant RNA standard has to be determined, exactly. From that concentration the copy number of RNA transcripts can be determined:

1   Determination of the molecular weight of the transcribed insert

2   Equation with the Avogadro constant

Here is a sample calculation:

Length of the insert:	1,275 nt
Molecular weight of RNA:	$340 \frac{g}{mol}$
Avogadro constant:	$6.022 \times 10^{23}\ mol^{-1}$
Molecular weight of the insert:	$1{,}275\ bp \times 340 \frac{g}{mol} = 4.335 \times 10^5\ \frac{g}{mol}$
Equation with the Avogadro constant:	$4.335 \times 10^5\ \frac{g}{mol} = 6.022 \times 10^{23}\ mol^{-1}$
	$4.335 \times 10^{14}\ ng = 6.022 \times 10^{23}$
	$4.335\ ng = 6.022 \times 10^9$
	$1\ ng = 1.389 \times 10^9$ copies of RNA transcript

The transcribed RNA should be treated with a DNase and stored in a non-diluted form, since diluted RNA undergoes degradation when it is stored over a long period. For every new qPCR the RNA standard has to be diluted and a serial of six to eight 10-fold dilutions has to be produced. If all components of a qPCR, except the samples, remain constant, then an interplate calibrator (IC) can be used for normalization and variation compensation of all plates. The IC can consist of diluted PCR product of the target DNA or cDNA sequence. By using an IC, the standard dilution series has to be added only to the first plate. All other plates are normalized by the aid of the IC. Next to the reduced costs, the use of an IC is much more

accurate than running a separate standard curve on all plates, since the random noise in the separate standard curves introduces systematic run-to-run variation (Svec et al. 2015).

2.2   New developments in quantitative PCR

In recent years new developments in the qPCR technology can be recorded (Gadkar and Filion 2014). Helicase-dependent amplification (HDA) represents an isothermal detection method without the need of thermal cyclers (Goldmeyer et al. 2007; Gadkar and Filion 2014). With this method the dsDNA is unwinded by a helicase enzyme prior to primer (and probe) annealing and elongation. However, only short fragments with a maximum length of approximately 200 bp can be amplified, since the unwinding speed of available helicases is limited (Vincent et al. 2004; An et al. 2005; Goldmeyer et al. 2007). In recent studies a bifunctional protein was engineered that physically links the helicase to the DNA polymerase resulting in an increase of the length of fragments to approximately 2.3 kb that can be amplified with this method (Motré et al. 2008). A further isothermal detection method is the loop-mediated isothermal amplification of DNA (LAMP), which uses a DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers producing a stem-loop DNA structure. This very sensitive method can also be used for quantification purposes and produces high amounts of amplified DNA, which makes it possible to detect the target in the form of turbidity with the naked eye (Notomi et al. 2000; Mori et al. 2004). However, the design of primers is complicated and needs the application of special software. Furthermore, based on their special requirements to the sequence, primers often cannot be placed to desired locations within the target sequence, making it difficult to use this method e. g. for the discrimination of virus strains based on few nucleotide polymorphisms.

The use of DNA analogs, such as locked nucleic acids (LNAs), is a further novelty in qPCR. The incorporation of LNAs into oligonucleotides results in a significant increase in the thermal stability of duplexes with complementary DNA. Probes should have a higher melting temperature than that of primers to ensure strong binding of the probes during the annealing step. However, as a consequence probes usually have a length of about 25 to 30 nt making their design sometimes difficult. The incorporation of LNAs into probe sequences makes it possible to create shorter probes with a sufficient high melting temperature (Koshkin et al. 1998). Furthermore, the level of fluorescence intensity can be increased because the interference during the amplification process is lowered (Costa et al. 2004).

Digital PCR (dPCR) or, more recently, droplet digital PCR (ddPCR) is a conventional end-point PCR-based method, combined with fluorescence-based detection that enables absolute

quantification without the need of any standard curve. In dPCR a reaction mix (typically 20 µl) containing a sample is partitioned into hundreds, thousands or even millions of minute sub-reactions (Bizouarn 2014). The partitioned sub-reactions are separated either on a chip or, in the case of ddPCR, by carefully titrated emulsion of oil, water and stabilizing chemicals that are transferred into plates for thermo cycling (Manoj 2016). After the PCR amplification, a reader determines, which droplets or reaction chambers contain a target (positive) and which do not (negative). The relative ratio of positives (p) to the total is used to determine the average number of targets per sub-reaction (λ) using the Poisson formula (see below). The result is then multiplied by the number of sub-reactions per µl to get the copy number per µl.

$$\lambda = -ln(1-p)$$

Since no standard curve is needed for absolute quantification, dPCR displays an alternative approach to real-time PCR-based quantification. It showed better reproducibility at low target concentrations and a greater tolerance to inhibitors (Whale et al. 2012). However, to apply the Poisson formula, at least one sub-reaction must remain negative. If all sub-reactions are positive, it is impossible to calculate the target copy number. Therefore, the more sub-reactions are present within a dPCR device, the wider the dynamic range is. However, the number of target molecules analyzed are allowed to exceed the number of partitions, because of the nature of the Poisson distribution, which can be calculated as long at least one sub-reaction is negative. For instance, the practical dynamic range of 50.000 partitions is 1 to approximately 320.000 target copies (Bizouarn 2014). Currently, dPCR and ddPCR, respectively, are used in particular for the quantification of low abundance targets in human virology, such as the measurement of residual HIV infections (Alidjinou et al. 2015) or the difficultly detectable CMV herpes virus (Parry et al. 2016). However, ddPCR was also used to quantify fire blight and potato brown rot (Dreo et al. 2014). They found that ddPCR improved the detection of low concentrations of bacterial pathogens in potato tubers over that of a qPCR by an increase of the analytical sensitivity. Indeed, further applications in plant pathology are certainly dependent on the development of costs for dPCR instruments and consumables, which are between $3 and $30 per sample (Baker 2012).

3 Aims of this work

During this work quantitative detection assays were developed for RT-qPCR, with which a great variety of PVY strains and PLRV isolates can be detected. Furthermore, external standards were designed that allow absolute quantification of PVY and PLRV RNA copies. The determination of PVY and PLRV RNA copy numbers in different organs of the potato plant offers the ability to address several challenges in the scope of potato cultivation and research:

1. Development of a more efficient method to detect PVY in seed potatoes

2. Development of a method to complementarily evaluate *Solanum* species and their progenies regarding their resistance type to PVY for breeding purposes

3. Development of a method to examine the virulence of PVY and PLRV strains

4. Development of a method to complementarily evaluate the equivalence of GM potatoes regarding their susceptibility to PVY

For these purposes PVY and PLRV were intensively studied at a visual and molecular level comprising virus purification, evaluation of symptoms on test plants, sequencing of whole PVY and PLRV genomes and discrimination of strains and RNA species by using different primers or probes. RT-qPCR assays were developed and optimized for the absolute quantification of the two viruses. Afterwards, the assays were applied in different experiments and evaluated regarding their suitability to address the above mentioned challenges.

4 Optimizing the method – successes and setbacks

A part of the laboratory work and greenhouse experiments were done between 2009 and 2011 within the framework of the research project "Development and validation of an analysis method for the detection of altered resistance in transgenic plants to herbivore-pathogen-complexes" at the Julius Kühn-Institut (JKI, Institute for the Biosafety in Plant Biotechnology) in Quedlinburg. This project was part of a funding program of the German Federal Ministry of Education and Research (BMBF) and had the grant number 03WKBN05A. As an entomology specialist the BTL Bio-Test Labor GmbH Sagerheide was the partner of this project. In addition to the work on the BMBF project, which addressed only one of the objectives mentioned in the last chapter, further work was done to obtain results regarding the other three scopes. This chapter describes how PVY and PLRV were intensively studied at a visual and molecular level and which efforts were made to optimize the detection and absolute quantification of the two viruses with regard to sensitivity and efficiency.

4.1 Virus purification

This complex and time-consuming work was done to obtain the purified virus, in order to

1. use it as an internal positive control during the RNA extraction step, since the efficiency of RNA extraction with Concert™ Plant RNA Reagent (Life Technologies™, according to the manufacturer's protocol) was not known,

2. use it as a standard for immuno-capture (IC) RT-qPCR as a possible method to improve the efficiency of qPCR assays,

3. produce IgG antibodies that could be used for the IC RT-qPCR and the low-cost detection of PVY and PLRV by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

4.1.1 PVY

200 g symptomatic leaves of tobacco and potato, respectively, infected with the tuber-necrotic PVY^{NTN} strain (isolate Gr99, accession number AJ890343) were filled into a high speed blender and triturated with additional two volumes (400 ml) of 0.1 M, pH 7.0 potassium citrate buffer containing 20 mM EDTA and 500 µl proteinase inhibitor PMFS (and 5g PVPP in the case of potato for the purpose of binding polyphenols). Afterwards, the homogenized fluid was pressed through a gauze cloth into a funnel filled with glass wool. The extract was then evenly distributed into four centrifuge tubes and centrifugated for 15 min at 5,000 rpm in

a Heraeus™ Biofuge™ Stratos™ centrifuge (Thermo Fisher Scientific, Inc.). The supernatant was filled into a big beaker with 3% (v/v) of the surfactant Triton X-100 and stirred by the aid of a magnetic stirrer for 30 min at 4 °C. Each of six 65 ml-tubes were filled with 50 ml of the cooled mixture. With the aid of a syringe a sucrose solution (40% sucrose in 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA) was injected to the bottom of the centrifuge tubes to form a lower layer. The completely filled tubes were placed into a 45Ti rotor (Beckmann Coulter, Inc, Brea, USA) and centrifugated at 35.000 rpm at 10 °C for 2 h.

Afterwards, each formed pellet was resuspended in 3 ml of 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA by the aid of a glass homogenizer. The suspension was then distributed to two 10.5 ml tubes, filled-up with 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA, placed into a 90Ti rotor (Beckmann Coulter, Inc) and centrifugated at 15,000 rpm at 4 °C for 10 min in a Heraeus™ Biofuge™ Stratos™ centrifuge (Thermo Fisher Scientific, Inc.). The supernatant was mixed with one volume of a caesium chloride solution (CsCl + 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA).

The mixture was then centrifugated in a 90Ti rotor at 45,000 rpm at 10 °C for 16 h without decelerating at the end. After this centrifugation step one opalescent band was visible under a light bulb (Fig. 6).
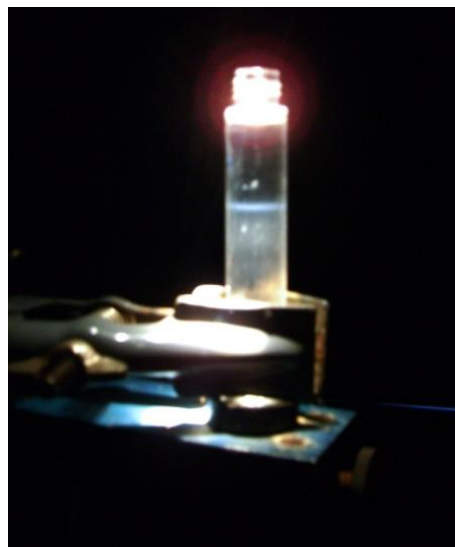
Fig. 6 Opalescent band formed in CsCl by PVY particles after the isopycnic ultracentrifugation step.

The opalescent band was aspirated by the aid of a syringe and mixed with three volumes (v/v) of 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA. The mixture was then distributed to three 1.5 ml centrifuge tubes, placed into a TL-100 rotor and centrifugated in a Beckman Optima TL-100 Benchtop ultracentrifuge at 62,000 rpm at 10 °C for 2 h.

Afterwards, each pellet was resuspended in 170 µl of 0.1 M, pH 7.0 potassium citrate buffer + 20 mM EDTA. The protein concentration was measured by the aid of a spectrophotometer

(NanoDrop 8000, Thermo Fisher Scientific, Inc.). The optical density at 260 nm was 2.2. According to Stace-Smith and Tremaine (1970), who calculated a value of 2.86 at 260 nm for 1 mg/ml of PVY, the amount of the author's purified PVY was 0.77 mg/ml. The purity of the virus was determined by electron microscopy (Fig. 7).
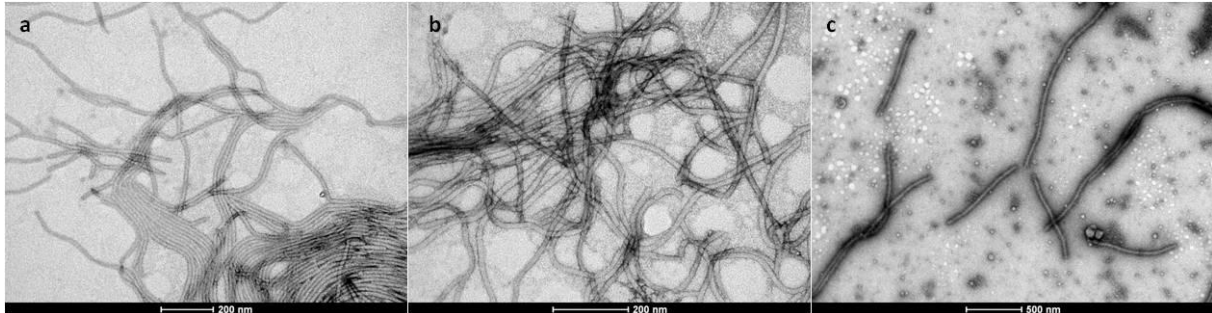
Fig. 7 Electron micrographs of PVY virions purified from *N. tabacum* 'Samsun NN' (**a**) or potato (**b**,**c**). The virions of PVY in picture **c** were decorated with PVY IgG antibodies.

Since the purity of PVY was excellent, a subset of the purified virus was transferred to the production of polyclonal IgG antibodies. The antibodies were prepared by the group of Dr. Frank Rabenstein, Institute for Epidemiology and Pathogen Diagnostics (JKI).

4.1.2 PLRV

Among several tested protocols a successful PLRV purification was obtained by the protocol according to Thomas et al. (1997). 170 g potato leaves infected with PLRV were filled into a high speed blender and triturated with three volumes (500 ml) of 0.1 M, pH 6.0 trisodium citrate buffer + 0.5% (v/v) 2-mercaptoethanol + 2% (v/v) either Rohament® CL (AB Enzymes GmbH, Darmstadt, Germany) or Depol™ 793L (Biocatalysts Limited, Wales, UK) for digestion of cell walls. The mixture was then stirred at room temperature for 2.5 h. Subsequently, 1% (v/v) Triton X-100 was added and the slurry stirred for further 30 min.

Afterwards, the mixture was emulsified with 20% (v/v) of a 1:1 mixture of n-butanol:chloroform. Crude components were then separated by low-speed centrifugation. The aqueous phase was subjected to PEG precipitation by adding 8% (w/v) PEG 8000 (Sigma-Aldrich, St. Louis, USA) and 1% (w/v) NaCl. The mixture was stirred at room temperature until the solids were dissolved. After a further step of low-speed centrifugation (5,000 rpm for 15 min) in a Heraeus™ Biofuge™ Stratos™ centrifuge (Thermo Fisher Scientific, Inc.) the pellet was resuspended over night in 42.5 ml of 0.1 M, pH 6.4 trisodium citrate buffer + 0.01 M EDTA. After a further step of low-speed centrifugation the supernatant was centrifuged in a 45Ti rotor (Beckmann Coulter, Inc.) for 2 h at 30,000 rpm to pellet the virus.

Afterwards, the pellet was resuspended in 1.7 ml of 0.05 M, pH 6.4 trisodium citrate buffer + 5 mM EDTA. Subsequent to low-speed centrifugation, the supernatant was centrifugated in a 45Ti rotor (Beckmann Coulter, Inc) for 2 h at 30,000 rpm. Afterwards, the supernatant was layered on an over-night performed sucrose gradient (40%, 30%, 20% and 10% sucrose in 0.1 M, pH 6.4 trisodium citrate buffer + 0.01 M EDTA). The sucrose density gradient centrifugation was realized in a SW28 rotor (Beckmann Coulter, Inc.) at 25,000 rpm for 2 h.

After this centrifugation step a very weak opalescent band was visible under a light bulb. The band was aspirated by the aid of a syringe and mixed with three volumes of 0.1 M, pH 6.4 trisodium citrate buffer + 0.01 M EDTA. A centrifugation step followed at 64,000 rpm at 10 °C for 2 h in a TL100 rotor (Beckmann Coulter, Inc.). The jelly-like pellet was resuspended in 170 µl of 0.05 M, pH 6.4 trisodium citrate buffer + 5 mM EDTA.

The protein concentration was measured by the aid of a spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific, Inc.). The amount of purified PLRV was 1.3 mg/ml (Rohament® CL, A260/A280: 1.84) and 0.7 mg/ml (Depol™ 793L, A260/A280: 1.83). The purity of the virus preparation was analyzed by electron microscopy (Fig. 8).
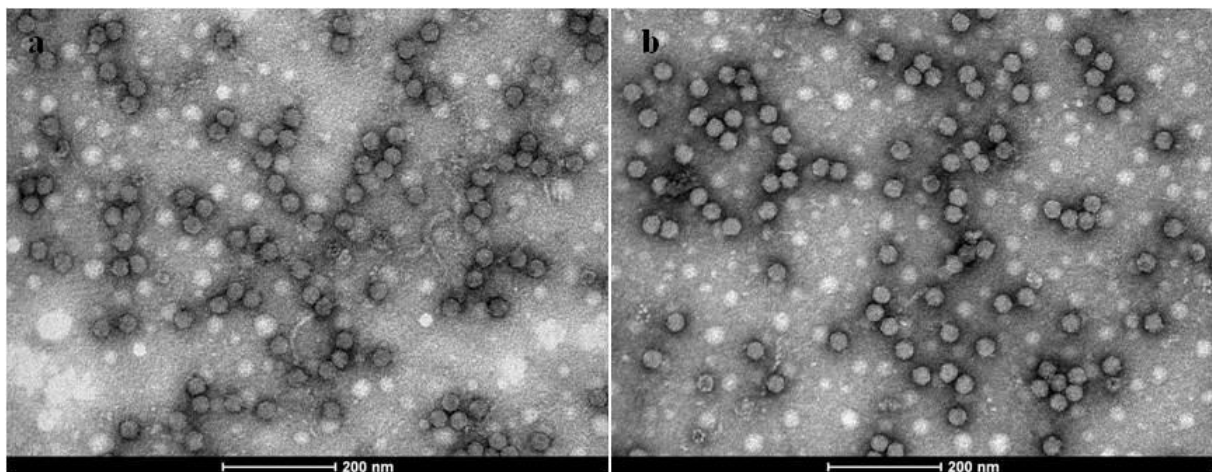
Fig. 8 Electron micrographs of PLRV virions purified from potato. Prior to purification the leaf tissue was digested either with Rohament® CL (**a**) or Depol™ 793L (**b**).

Although the yield was twice as high with the Rohament® CL digestion, no visible differences, such as more or less disabled or conglomerated virions, were observed by the aid of electron microscopy (Fig. 8). The purified virus was transferred to the production of polyclonal IgG antibodies, which were prepared by the group of Dr. Frank Rabenstein, Institute for Epidemiology and Pathogen Diagnostics (JKI). The antibodies were later used for immuno-capture (IC) PCR.

4.2   Evaluation of symptoms on potato plants

The evaluation of plant resistance to a virus (chapter 5.2) or the determination of the virulence of a virus isolate (chapter 5.3) is accompanied by an observation and assessment of disease symptoms. To evaluate the symptoms of PVY and PLRV on their host potato, several plants of a diverse range of cultivars were infected with different isolates of PVY and PLRV, respectively, and maintained either in the greenhouse, *in vitro*, a climate chamber or in the field. The development of symptoms was observed at different developmental stages and documented by photography. In addition, tubers were sporadically harvested from field plants and examined for necrotic rings (PTNRD) and net necrosis indicating an infection with PVY and PLRV, respectively.

4.2.1   PVY

Necrotic rings were rarely documented (Fig. 9). This typical symptom of PTNRD-inducing isolates of PVY was often hidden by other tuber diseases or physiological disorders, such as black dots, silver scurf or cracking.

Fig. 9 Tubers of potato plants infected with a PVY isolate causing the PTNRD.

Susceptible potato cultivars showed typical PVY symptoms on their leaves, such as mottling or leaf chlorosis and necrosis on older leaves. Plants grown from tubers of infected mother plants developed the most severe symptoms (Fig. 10a).
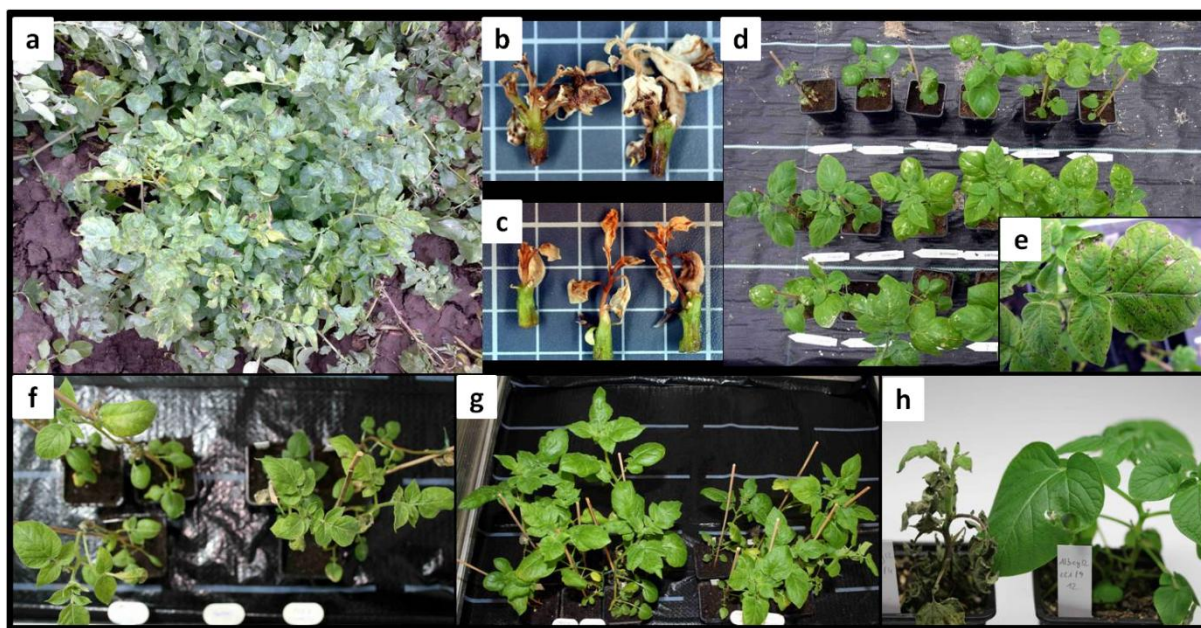
Fig. 10 Symptoms of potato plants infected with different strains of PVY and maintained either in the field (**a**), *in vitro* (**b,c**), in the greenhouse (**d**) or in the climate chamber (**f,g,h**).

Experiments regarding the evaluation of equivalence of transgenic to non-transgenic potatoes regarding their PVY susceptibility should be performed by using PVY-infected *in vitro* plants to minimize the experimental error, which can be substantial if the plants are inoculated mechanically. In order to establish an assortment of different PVY infected *in vitro* cultivars, greenhouse plants infected either with PVY^{NTN} or PVY^{N-Wi} were transferred *in vitro* and maintained at constant 20 °C and 10.000 lx. However, the shoots sprouting from stem segments of PVY^{N-Wi} infected cultivars, such as 'Albatros' (Fig. 10b) and 'Desiree' (Fig. 10c) became necrotic after eight weeks of *in vitro* cultivation, indicating a systemic HR. Therefore, experiments could not be conducted with already infected potato plants originating from *in vitro* cultivation. Hence, all experiments in the greenhouse (Fig. 10d) or in climate chambers (Fig. 10f-h) were performed with healthy *in vitro* plants that were mechanically inoculated with either one of the before mentioned PVY strains (Fig. 11). Nevertheless, the experimental error was minimized by a special handling of the inoculum source. Only the second emerging leaf after mechanical inoculation of PVY-infected *N. tabacum* 'Samsun NN' was used, since it is known that PVY is not evenly distributed in *N. tabacum* due to recovery (Nie and Molen 2015).

In the greenhouse and especially during climate chamber experiments some cultivars showed different responses to a PVY infection dependent on the temperature. At 24 °C cultivar 'Hermes' developed necrosis on older leaves and leaf drop (Fig. 10f), whereas at 20 °C this cultivar primarily showed mottling and crinkling of young leaves (Fig. 10g). At 20 °C cultivar 'Albatros' developed a typical HR with local necrotic lesions on the leaves (Fig. 10e) when infected with PVY^{N-Wi}. However, at 12 °C only few local lesions were visible (Fig. 10h right),

most of them could only be detected by microscopy. At 24 °C the HR in 'Albatros' became systemic, leading to complete necrotization (Fig. 10h left).
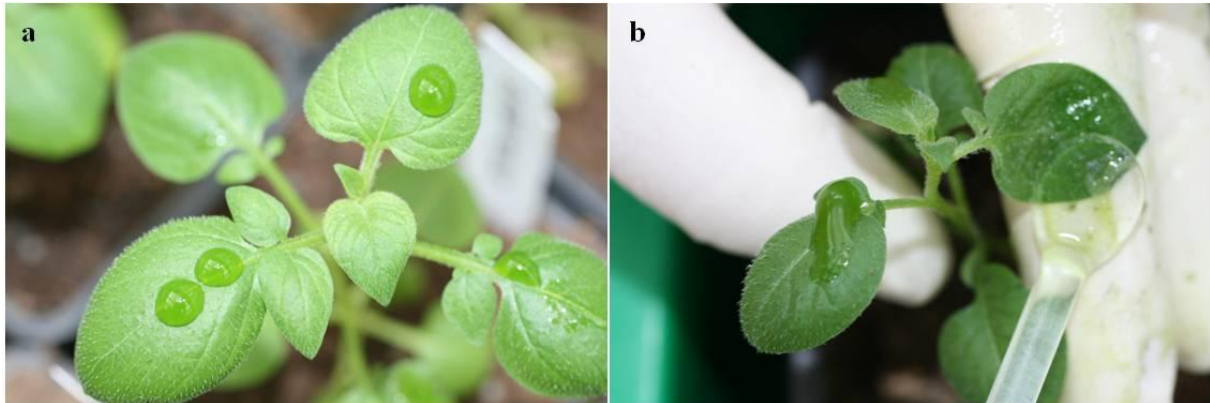
Fig. 11 Mechanical inoculation of potato test plants. 2 g of PVY infected leaves of *N. tabacum* 'Samsun NN' were ground in 10 ml of phosphate buffer, pH 8. Afterwards, 100 µl of the tobacco buffer mixture (**a**) were rubbed onto silicon carbide powdered plantlets (**b**).

4.2.2 PLRV

Symptoms on potato tubers were not observed. The leaves of infected plants showed well known PLRV symptoms, such as leaf roll, chlorosis and necrosis of older leaves, dependent on the light intensity. Whereas in the field and under high light conditions potato plants developed severe symptoms (Fig. 12a, d-f), no symptoms were visible on plants maintained *in vitro* or in the greenhouse without additional UV-irradiation (Fig. 12b, c). Possible reasons for this observation are discussed by Hühnlein et al. (2016b) in chapter 5.3. The influence of the cultivar or the isolate of PLRV was rather low. In an experiment, which is assessed in the aforementioned publication, plants of three different potato cultivars differing in their susceptibility rating according to the Federal Plant Variety Office of Germany were infected with one of three different PLRV isolates and maintained under constant high light conditions. At the end of the experiment no visible differences of symptoms could be observed on the test plants (Fig. 12d-f).
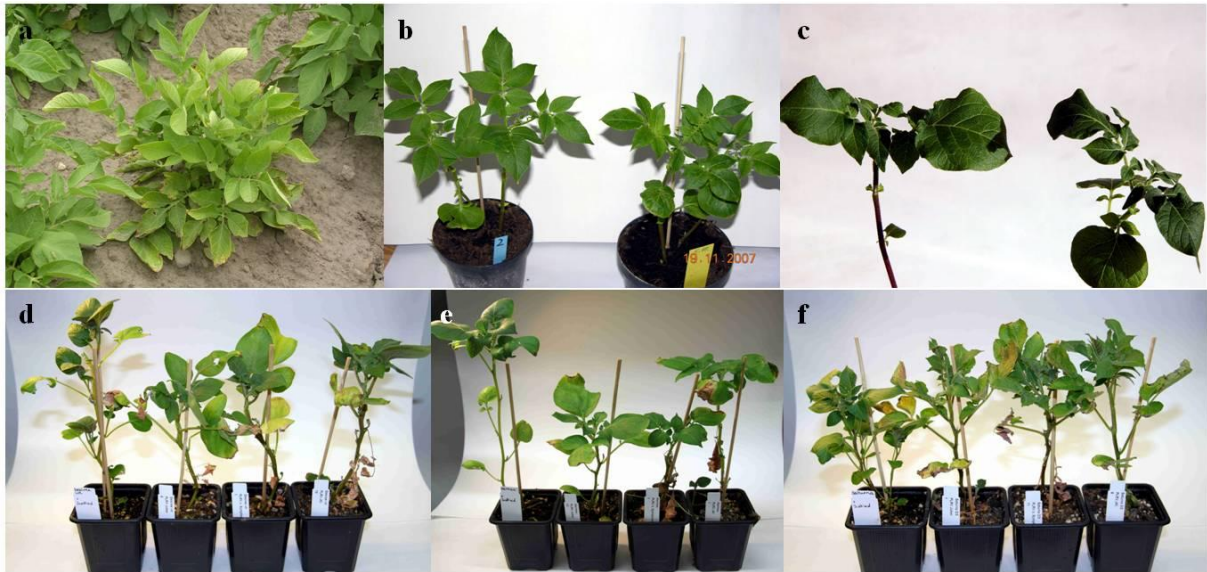
Fig. 12 Symptoms of potato plants infected with several strains of PLRV. Typical PLRV symptoms were observed in the field on cultivar 'Princess' (**a**). No symptoms were visible on cultivars 'Kuras' (**b**) and 'Agria' (**c**) maintained in a greenhouse (left plant: healthy, right plant: infected with PLRV). **d-f**: potato plants evaluated during an experiment assessed by Hühnlein et al. (2016b). Plants of the cultivars 'Desiree' (**d**), 'Hermes' (**e**) and 'Saturna' (**f**) were either mock-inoculated (left plant) or inoculated with PLRV isolates JokerMV10 (accession number JQ346191, second from left), SymlessLS10 (accession number JQ346189, second from right) or ASL2000 (accession number JQ346190, right plant). The pictures and final publication are available at Springer via http://dx.doi.org/ 10.1007/s10658-016-0872-3.

4.3   Sequencing of PVY and PLRV genomes

Two PVY and three PLRV isolates were sequenced in order to compare them with available sequences from the GenBank (National Center for Biotechnology Information: NCBI) and to search for motifs possibly involved in symptom development on potato plants. This was done in addition to address the third above mentioned challenge in the scope of potato cultivation and research: evaluation of the virulence of PVY and PLRV isolates. In order to sequence the full genomes of PVY and PLRV isolates, the following procedure was applied.

First, the RNA of PVY and PLRV-infected plants was isolated by using Concert® Plant RNA Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The isolated total RNA was reverse transcribed and amplified by using RT-PCR and PVY and PLRV-specific primers, respectively, to produce overlapping fragments of approximately 1,500 nucleotides encompassing the whole viral genome. However, the fragment at the very 5' end of the PLRV genome lacked the first 121 nucleotides, since this part was yet sequenced only by Mayo et al. (1989). All the other PLRV sequences availably on the GenBank start with the 122nd nucleotide leading to a sufficient comparison of these sequences with the sequences from this study. The primers to generate the fragments were designed according to available sequence information from the GenBank and published in Hühnlein et al. (2013) for PVY and in Hühnlein et al. (2016b) for PLRV.

The RT was performed by using different 3' primers dependent on the 3' primer that should be used for the PCR. For example, fragments, which were produced near the 5' end of the virus genome, were amplified from cDNA that was reverse transcribed either by using the same 3' primer as for the PCR or by using a 3' primer with a position no more than 1,325 nucleotides downstream of the 3' PCR primer. Thereby, the yield of amplified fragments near the 5' end could be increased in comparison to fragments that were produced from cDNA with a 3' end thousands of nucleotides downstream of the amplified fragment. By using gel electrophoresis, the success of the PCR was confirmed and fragments of the desired length were excised and cleaned by centrifugation at 5,000 rpm for 10 min in centrifugal filter columns (Ultrafree-DA Gel Extraction Kit, Millipore Cooperation, Merck KGaA, Darmstadt, Germany). The purified DNA was then ligated into pGEM®-T Easy vectors (Promega Cooperation, Madison, USA) by mixing 7.5 µl of DNA solution, 0.5 µl of vector solution, 1 µl of T4-ligase buffer and 1 µl of T4 ligase (Promega Cooperation). The mixture was incubated over night with 8 cycles of four 30-min steps (24°C, 20°C, 18 °C and 14°C).

The recombinant DNA was then transformed into NEB® Turbo Competent *Escherichia coli* cells (New England BioLabs, Ipswich, USA) by applying the following steps: 100 µl of competent cells were mixed with 1 µl of 1:10 diluted 2-mercaptoethanol and incubated for 10 min on ice. Subsequently, 2 µl of recombinant DNA solution were added and the cells were incubated for 40 min on ice. A heat shock was performed for 1 min at 42 °C. Finally, the cells were left 2 min on ice. Immediately, 200 µl of super optimal broth with catabolite repression (SOC) was added and the mixture was incubated at 37 °C for 1 h.

Afterwards, 100 µl of the solution with transformed cells was spread onto selection plates containing lysogeny broth (LB) medium, 80 µg/ml of X-gal, 0.3 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 µg/ml of ampicillin. The plates were incubated over night at 37 °C. Afterwards, white colonies were singularized and spread onto selection plates (see above for their composition). After six hours, a colony PCR was performed by using M13 forward and reverse sequencing primers. Clones with inserts of the correct length were inoculated into liquid LB medium containing 0.01 % (v/v) of ampicillin and shaked over night at 37 °C. The plasmid DNA was then prepared by using the NucleoSpin® plasmid preparation kit according to the manufacturer's protocol (Machery-Nagel GmbH & Co. KG, Düren, Germany).

The DNA sequencing reaction was done by using the GenomeLab DTCS Quick Start Kit (Beckmann Coulter, Inc) according to the manufacturer's protocol. 300 ng plasmid DNA were sequenced using M13 reverse and universal primers. The sequencing reaction mixture was purified by ethanol precipitation. The pellet was then resuspended in sample loading solution (provided with the kit) and stored at -20 °C until loading into the sequencing system

(GenomeLab GeXP Genetic Analysis System, Beckman Coulter, Inc.). In order to validate the sequences at least two clones of a fragment were sequenced in parallel. The obtained sequences were published in the GenBank (Tab. 1).

Tab. 1 Isolates of PVY and PLRV that were sequenced during this work

Virus	Strain/isolate	Origin	Acession number
PVY	PVY^{N-Wi}/MV99	Collected 2009 in Mecklenburg-Western Pomerania, Germany	HE608963
	PVY^{N-Wi}/MV175	Collected 2009 in Mecklenburg-Western Pomerania, Germany	HE608964
PLRV	SymlessLS10	Collected 2010 in Lower Saxony, Germany	JQ346189
	ASL2000	Collected 2000 in Saxony-Anhalt, Germany	JQ346190
	JokerMV10	Collected 2009 in Mecklenburg-Western Pomerania, Germany	JQ346191

In order to compare the obtained sequences with available accessions in the GenBank a primary alignment was done using the MUSCLE program (Edgar 2004) provided by the MEGA software version 6 with default parameters (Tamura et al. 2013). Furthermore, by utilizing the CLC Sequence Viewer 7.6 (CLC Bio, Aarhus, Denmark), phylogenetic trees were calculated and drawn using the Neighbour-Joining method and a 1,000 replicate bootstrap analysis. The results obtained by alignments and phylogenetic analyses are presented in Hühnlein et al. (2013, chapter 5.1) for PVY and in Hühnlein et al. (2016b, chapter 5.3) for PLRV.

4.4   Design of primers and standards for RT-qPCR

This chapter describes the primers that were used for the detection and absolute quantification of PVY and PLRV. A software was used supporting the design of primers for RT-qPCR (Beacon Designer™, Premier Biosoft, Palo Alto, USA). However, the software was not able to design PVY strain specific primers. For that application we designed primers with a melting temperature of approximately 64 °C according the rule A/T=2°C, G/C=4°C. Many primer combinations were tested and optimized until a few primer pairs remained that were sufficient in their sensitivity and efficiency. Furthermore, the quantification of virus RNA is only possible by means of a standard consisting of a known quantity of virus RNA. The preparation of a standard dilution series is described at the end of each sub chapter.

4.4.1 PVY

For the detection of PVY in seed potatoes, an assay is needed that detects as many strains of a virus as possible with one pair of primers. Plant protection services take samples from different seed lots, where several strains and isolates, respectively, are present. They need the certainty not to miss virus infections due to failed primer-isolate's RNA homologies. Furthermore, the seed potatoes are tested for many potato viruses. Therefore, the assay for their detection should be as simple and cost-extensive as possible.

Although highly conserved, the CP sequences of all to date published accessions of PVY differ clearly especially in the region of recombination breakpoints at approximately 8550 nt and 9170 nt, respectively. Even single nucleotide mismatches between primer and isolate sequences can lead to a variable efficiency of the amplification (Hühnlein et al. 2013, Tab. 2, chapter 5.1).

Tab. 2 Position of PVY primers with mismatches in the sequences of some PVY isolates

Strain, isolate	MMPVYall5-9194* (9194-9211 nt)	MMPVYall3-9420* (9384-9419 nt)
PVY^{NTN}, Gr99	AGGTCACATCACGAACAC	GTGA-TGTAGTGTCTCTCCG
PVY^{N-Wi}, 261-4	AGGTCACATCACGAACAC	GTGA-TGTAGTGTCTCTCCG
PVY^{O}, SCRI-O	AGGTCACATCACGAACAC	GTGA-TGTAGTGTCTCTCCG
PVY^{N}, Mont	AAGTTACATCACGGACAC	GTGATTGTAGTGTCTTTCCG
PVY$^{NA/NTN}$, Nicola	AAGTTACATCACGGACAC	GTGATTGTAGTGTCTTTCCG
PVY^{C}, M^{S}N^{R}	AAGTCACATCACGAACAC	GTGATTGTGCTGCCTCTCCG

*primer names contain the elongation direction (5=forward, 3=reverse) followed by the numerical position within the PVY RNA genome.
Letters shaded in gray highlight mismatches in the sequences of PVY isolates with the primers.

In contrast, the efficiency of primers is equal if they match with all nucleotides of the sequences. Such a primer pair was designed having its position within the PVY genome at position 8912-8931 nt (CTGTGATGAATGGGCTTATG) for the forward primer (PVYall5-8911) and at position 9072-9090 nt (CTCAGATGTTGCAGAAGCG) for the reverse primer (PVYall3-9090). These primers match perfectly with more than 70 recently published accessions of PVY in the GenBank, even with unusual recombinant isolates, such as NE-11, PVY-MON, SD-1, or L26 (Tab. 3).

Tab. 3 List of PVY isolates matching with the designed primers (PVYall5-8911 and PVYall3-9090)

Strain	Isolate	Accession Number	Reference
N	-	NC_001616	Robaglia et al. (1989)
N	-	D00441	Robaglia et al. (1989)
N	New Zealand (NZ)	AM268435	Schubert et al. (2007)
N	Mont	AY884983	Lorenzen et al. (2006)
N	SCRI-N	AJ585197	Barker and McGeachy (unpublished)
N	SASA 207	AJ584851	Barker and McGeachy (unpublished)
N	N-Egypt	AF522296	El-Mohsen et al. (2003)
N	N-Jg	AY166867	Nie and Singh (2003b)
N	PVYPOLYP	M95491	Thole et al. (1993)
N	T13	AB714135	Hataya et al. (1994)
O	British	Z29526	Welnicki et al. (unpublished)
O	PVY-Oz	EF026074	Baldauf et al. (unpublished)
O	SCRI-O	AJ585196	Barker and McGeachy (unpublished)
O	SASA-110	AJ585195	Barker and McGeachy (unpublished)
O	PO7	U09509	Singh and Singh (1996)
O	CO2140	HQ912914	Karasev et al. (2011)
NA/NTN	ON92.	AB331519	Ogawa et al. (2008)
NA/NTN	NN99	AB331518	Ogawa et al. (2008)
NA/NTN	HO90	AB331517	Ogawa et al. (2008)
NA/NTN	OK105	AB331516	Ogawa et al. (2008)
NA/NTN	D6	AB331515	Ogawa et al. (2008)
NA/NTN	Nicola	AJ890346	Schubert et al. (2007)
NA/NTN	RRA-1	AY884984	Lorenzen et al. (2006)
NA/NTN	SASA-61	AJ585198	Barker and McGeachy (unpublished)
C	Adgen	AJ890348	Schubert et al. (2007)
C	NC57	DQ309028	Hari and Kelly (unpublished)
C	MSNR	AF463399	Fellers et al. (2002)
O:N	PB209	EF026076	Baldauf et al. (unpublished)
O:N	Alt	AY884985	Lorenzen et al. (2006)
O:N	OR-1	DQ157179	Piche et al. (unpublished)
O:N	ID-1	DQ157178	Piche et al. (unpublished)
O:N	L56	AY745492	Nie et al. (2004)
O:N	Mb112	AY745491	Nie et al. (2004)
N-Wi	Syr NB-16	AB270705	Chikh Ali et al. (2007)
N-Wi	Wilga	EF558545	Kosakowski et al. (unpublished)
N-Wi	261-4	AM113988	Schubert et al. (2007)
N-Wi	MV99	HE608963	Hühnlein et al. (2013)
N-Wi	MV175	HE608964	Hühnlein et al. (2013)
N-Wi	iso5	AJ890350	Schubert et al. (2007)
N-Wi	LW	AJ890349	Schubert et al. (2007)
N-Wi	156var	AJ889868	Schubert et al. (2007)
N-Wi	156	AJ889867	Schubert et al. (2007)
N-Wi	PN10A	DQ008213	Lorenzen et al. (unpublished)

Strain	Isolate	Accession Number	Reference
N-Wi	GBVC_PVY_34 N_Wi	JQ969041	Kamangar et al. (2014)
NTN	L26 (Z)	FJ204165	Hu et al. (2009c)
NTN	N4	FJ204164	Hu et al. (2009c)
NTN	PVY-12	AB185833	Chikh Ali et al. (2007)
NTN	N Nysa	FJ666337	Golnik et al. (unpublished)
NTN	SD1	EU182576	Chen et al. (2010)
NTN	Satina	AJ890347	Schubert et al. (2007)
NTN	Linda	AJ890345	Schubert et al. (2007)
NTN	Ditta	AJ890344	Schubert et al. (2007)
NTN	Gr99	AJ890343	Schubert et al. (2007)
NTN	34/01	AJ890342	Schubert et al. (2007)
NTN	12-94	AJ889866	Schubert et al. (2007)
NTN	NE-11	DQ157180	Piche et al. (unpublished)
NTN	v942490	EF016294	Gow et al. (unpublished)
NTN	PB312	EF026075	Baldauf et al. (unpublished)
NTN	423-3	AY884982	Lorenzen et al. (2006)
NTN	P1	AY840083	Sawazaki et al. (unpublished)
NTN	IAC	AY840082	Sawazaki et al. (unpublished)
NTN	NIB-NTN	AJ585342	Barker et al. (unpublished)
NTN	PVY-AST	JF928460	Galvino-Costa et al. (2012)
NTN	HR1	FJ204166	Hu et al. (2009c)
NTN	Tu 660	AY166866	Nie and Singh (2003a)
NTN	11289-1	KC614702	Souza-Richards et al. (unpublished)
E	PVY-AGA	JF928459	Galvino-Costa et al. (2012)
E	PVY-MON	JF928458	Galvino-Costa et al. (2012)
?	Chile3	FJ214726	Moury (2010)
?	SON41	AJ439544	Moury et al. (2002)
?	LYE84.2	AJ439545	Moury et al. (2002)
nnp	pepper	AF237963	Fanigliulo et al. (2005)

Although the efficiency of primers was equal between the mentioned isolates, it ranged only between 95% and 97%. A higher efficiency between 98% and 100% was obtained by using primers with isolate dependent efficiency and when only matching sequences of known isolates (e.g Gr99 and 201-4) were amplified. Such isolates were employed to estimate potato progenies regarding their (PVY) resistance type and to evaluate the equivalence of transgenic to non-transgenic potatoes regarding their amount of detectable PVY RNA copies. Fluorescence during RT-qPCR was emitted from SYBR® Green. Indeed, fluorophore-labeled TaqMan® probes were also designed, but their efficiency was always too low to obtain reliable results for qPCRs (see setbacks, chapter 4.6).

For the estimation whether new, probably more virulent, strains of PVY might be present within positively tested samples (using the PVYall5-8911 and PVYall3-9090 primers), strain

specific primers were used for RT-PCR. If a positively tested sample could not be amplified by these primers, then it might be a new strain. Two of such samples were found and sequenced (see chapter 4.3).

For the strain specific detection of PVY^O, PVY^N, and PVY^{NA-NTN}, primers published by Schubert et al. (2007, Tab. 4, Fig. 14b, c, d) were used. However, the primers for the specific detection of PVY^{NTN} and PVY^{N-Wi}, published by these authors, amplify a very long product of 3867 bp and 5052 bp, respectively. Such long products can be amplified only by the application of specific and expensive Taq polymerases, such as DreamTaq™ DNA polymerase (Thermo Fisher Scientific, Inc.).

Therefore, new primers were designed (Tab. 4). The recombinant junction (RJ) at approximately 9190 nt is typical for all PVY^{NTN} isolates, except for isolates of the recombinant PVY^{NA-NTN}. By designing primers that amplify a product of 890 bp spanning this RJ (Fig. 13), the specific detection of PVY^{NTN} was possible. However, amplifying high virus titers resulted in unspecific binding of the PVY^{NTN} primers with PVY^{N-Wi}, PVY^N and PVY^O isolates, recognizable by weak bands in Fig. 14e. However, by a simultaneous amplification with PVY^{N-Wi}, PVY^N and PVY^O specific primers, the presence of a mixed infection could be examined.

Tab. 4 List of strain specific primers

Name of primers	Sequence of primers	Strains that are detected	Reference
YO5-1005	AAATTGTACGATGCACGTTCTAGA	O	Schubert et al. (2007)
YO3-2558	AGGCTCATCTAACAGCAACTGTC		
YN5-1780	TCCGAATGGGACAAGAAAACTTG	N	Schubert et al. (2007)
YN3-2,438	TGGTTCATCCAGTAGCAATTGCT		
YN-NA5-116	TTTGATCTTCGTCGTACAAACCG	NA, NA-NTN	Schubert et al. (2007)
YN-NA3-622	CTTGATAAGATGGTTCATTTGTTT		
YN5-1780	TCCGAATGGGACAAGAAAACTTG	O:N, N-Wi, NTN	Schubert et al. (2007)
YO3-2558	AGGCTCATCTAACAGCAACTGTC		
PVYO5-8528	CATTAGACGATGAGTTTGAATTT	O, O:N, N-Wi	Hühnlein et al. (2013)
PVYO3-8651	TATCTTTTCCTTTGTTCGGG		
PVYN5-8639	AAGGTAGCATTCAACCAAATCTC	NTN	Hühnlein et al. (2013)
PVYO3-9497	CCACAATGACGAAATCACCCTG		

The design of PVY^{N-Wi} specific primers was more complicated, since isolates of this strain have either two, three or even four RJs (Fig. 2b, 13). Therefore, two primer pairs were designed amplifying a first 798 bp long product that is located in the HC-Pro and P3 coding sequence, and a second 121 bp long fragment that is located in the NIb and CP coding sequence of the PVY genome (Fig. 13, 14a, f). The first primer pair detects the RJ that is

typical for all PVY^{N-Wi} and, unfortunately, all PVY^{NTN} isolates (Fig. 14a). However, by using the second PVY^O-specific primer pair the detection becomes specific because it is located in the sequence that is of PVY^O-type for PVY^{N-Wi} but of PVY^N-type for PVY^{NTN}. Indeed, the PVY^O-specific primers do also amplify a product from isolates of the PVY^O strain (Fig. 14f). However, if the presence of PVY^O isolates was excluded by PVY^O-specific primers, then 121 bp long products amplified by the second PVY^{N-Wi} primers certainly origin from PVY$^{O:N}$ or PVY^{N-Wi} isolates.
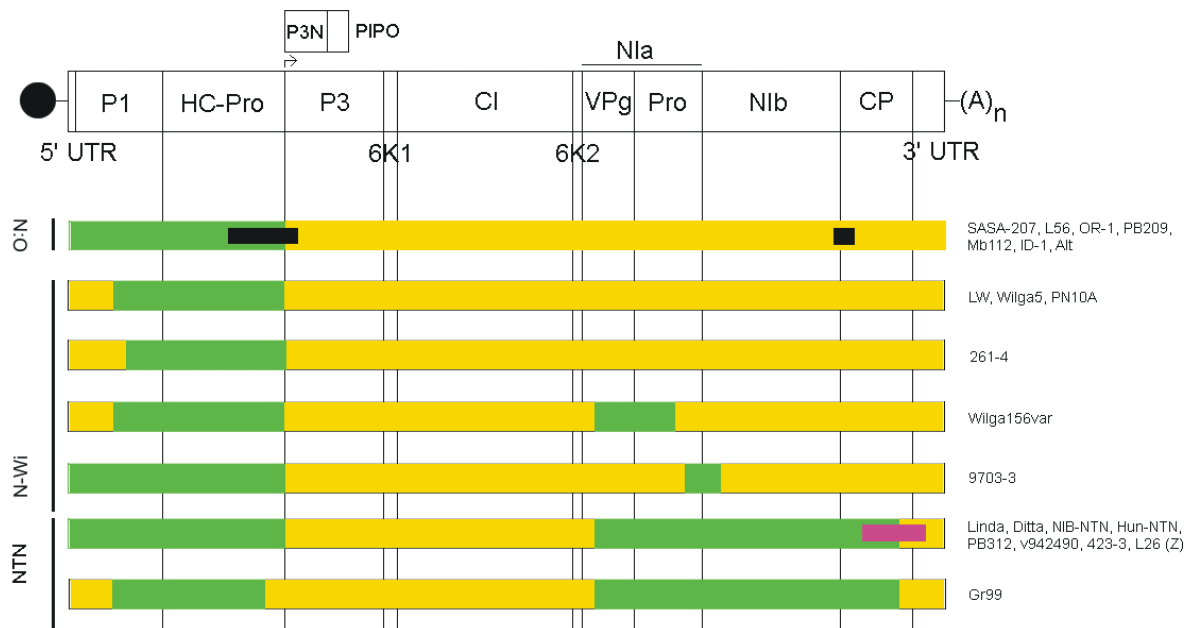
Fig. 13 Location of PCR products amplified by strain specific PVY primers for the detection of PVY$^{O:N/N-Wi}$ or of PVY^{NTN}. Two primer pairs are needed for detection of PVY$^{O:N/N-Wi}$ (black rectangles) and one for the detection of PVY^{NTN} (magenta rectangle). Abbreviations of PVY strains and proteins are in accordance to Fig. 2.
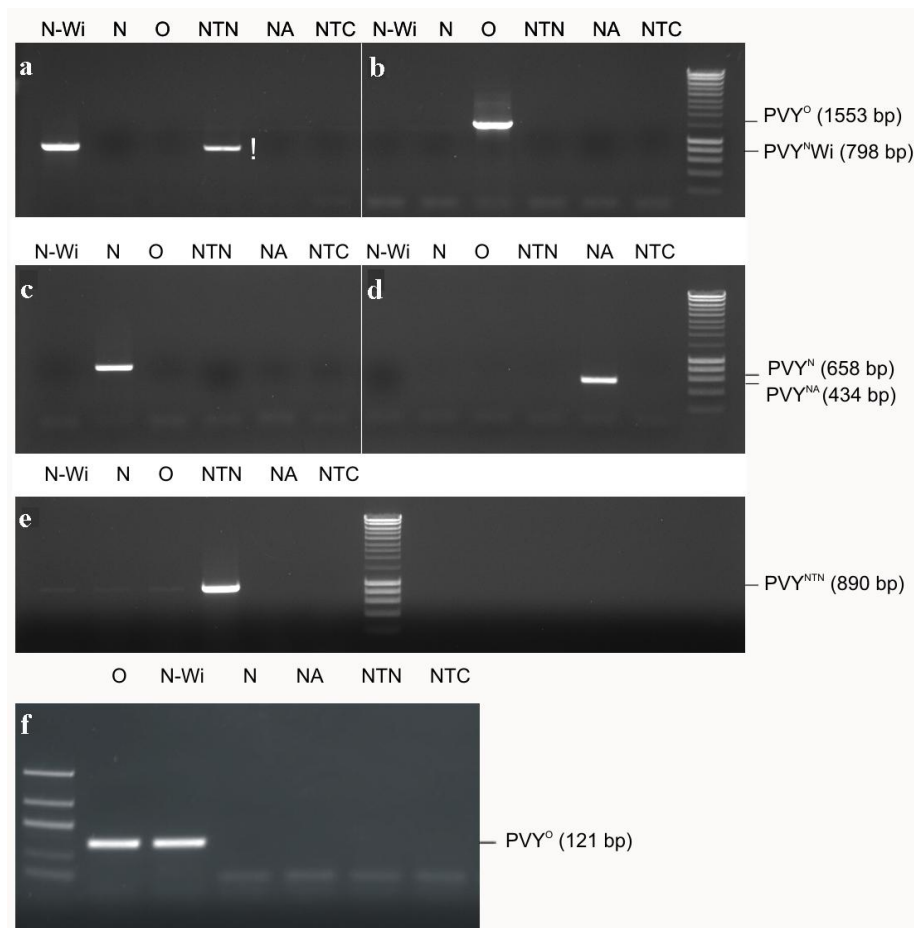
Fig. 14 Detection of different PVY strains by specific PCR primers amplifying either $PVY^{O:N/N-Wi}$ and PVY^{NTN} (**a**), PVY^{O} (**b**), PVY^{N} (**c**), $PVY^{NA/NA-NTN}$ (**d**), PVY^{NTN} (**e**) or PVY^{O} and $PVY^{O:N/N-Wi}$ (**f**). Isolates used were 261-4 (PVY^{N-Wi}), O12 (PVY^{O}), CH605 (PVY^{N}), Nicola (PVY^{NA-NTN}) and Gr99 (PVY^{NTN}).

For the absolute quantification of PVY by using the PVYall5-8911, PVYall3-9090 and MMPVYall5-9194, MMPVYall3-9420 primers, respectively, a RNA standard was prepared. Since the primers anneal to sequences of the PVY CP, the standard was made of an *in vitro* transcribed PCR fragment located between positions 8500 nt and the 3'end of the PVY genome (PVY^{NTN}, isolate Gr99). The amplified fragment was cloned as described in chapter 4.3. The recombinant plasmid DNA was linearized at the 3' end of the PVY cDNA by using the restriction enzyme Sal I (FastDigest®, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After linearization, the DNA was purified by using phenol, Tris-saturated, pH 7/chloroform extraction and precipitated with 0.3 M sodium acetate solution, pH 5.2 and 70% ethanol. Subsequently, the resuspended DNA was transcribed by using the TranscriptAid® T7 High Yield Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After transcription, the template DNA was removed by DNase I digestion. Then, the RNA was purified by using phenol, pH 4.7/chloroform extraction and precipitated with ethanol. Due to the high yield of RNA transcription, the pellet was resuspended in ten times more diethylpyrocarbonat (DEPC)-treated water than recommended

in the manufacturer's protocol. The yield measured by UV absorbance was 224 ng RNA per µl.

A serial dilution was performed after calculation of the RNA copy number. The size of the insert was 1200 bp (8500 nt to 3' end). According to the equation described in chapter 4.1, 677.5 ng RNA were needed to obtain 10^{12} RNA copies. The yield of RNA after transcription was 224 ng RNA per µl. Therefore, 3.02 µl of recombinant RNA were diluted in 96.98 µl DEPC-treated water to obtain 10^{10} RNA copies per µl. Starting from this initial concentration, the RNA was repeatedly diluted 10fold until a final concentration of 10 RNA copies per µl. The dilution series was then amplified in duplicates by RT-qPCR (Fig. 15).

The LOD was determined according to its definition: the lowest quantity of a template that can be distinguished from the non-template control (NTC) in 95% of the cases (Waiblinger et al. 2011). Therefore, the three highest RNA standard dilutions were pipetted to a 96well plate as follows: 6 replicates of 10^3 copies, 30 replicates of 10^2 copies and 60 replicates of 10 copies. By using RT-qPCR the template was amplified by either one of the following primer pairs: PVYall5-8911 and PVYall3-9090 or MMPVYall5-9194, MMPVYall3-9420. The LOD and the LOQ were 10^2 RNA copies by using the first mentioned primer pair. However, by using the second mentioned primer pair, 10^2 RNA copies were not in the linear range of the standard dilution series. Therefore, the LOQ for a RT-qPCR using these primers was only 10^3 RNA copies.
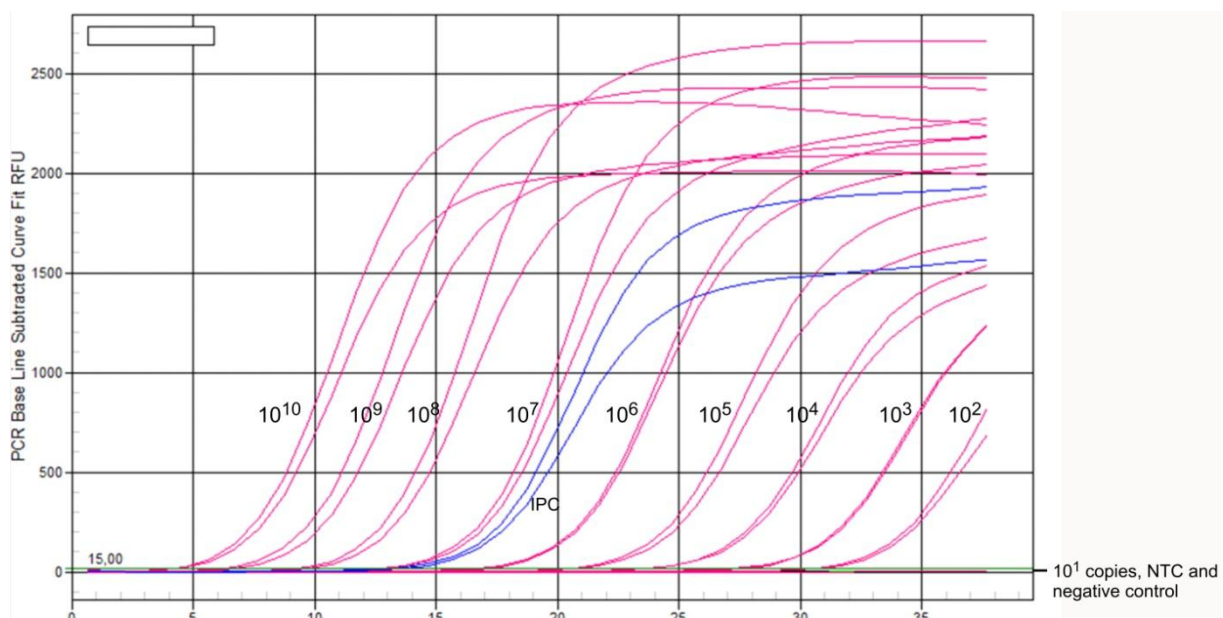
Fig. 15 Dilution series from 10^{10} to 10 copies of recombinant RNA made from the 3' end terminal 1,200 nucleotides of the PVY^{NTN} (isolate Gr99) genome (8500 nt to 3' end). The RNA standard was amplified by using RT-qPCR and primers: MMPVYall5-9194, MMPVYall3-9420. NTC = non-template control, IPC = interplate calibrator. The RT-qPCR in the picture above had an efficiency of 99.8%, a correlation coefficient (R^2) of 0.996, a slope of 3.33 and a y-intercept of 36.61.

4.4.2 PLRV

The genome of PLRV is highly conserved and primers were designed that matched well with the CP and RTD sequences of all PLRV isolates available at the GenBank. However, in a preliminary test it was ascertained that the detected initial amount of PLRV RNA is dependent on which RNA species is used for the amplification with RT-qPCR (Fig. 16).
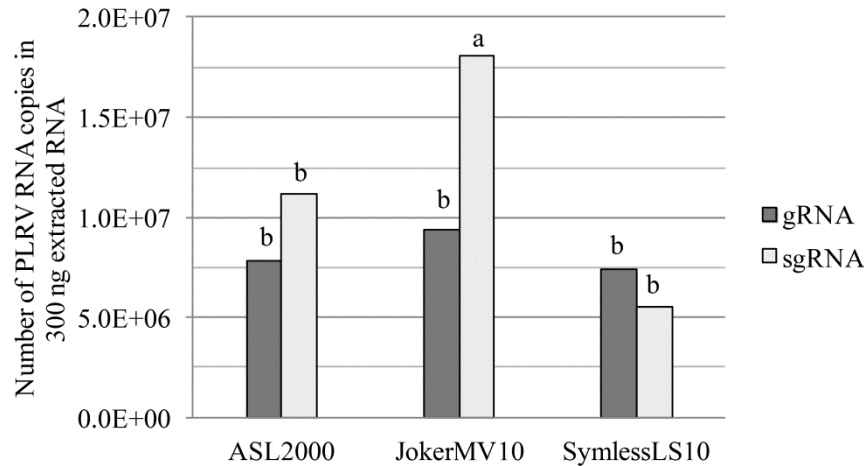
Fig. 16 The absolute numbers of PLRV RNA copies detected in a preliminary test by RT-qPCR. Potato plants originating from *in vitro* cultivation were infected either with PLRV isolate ASL2000, JokerMV10 or SymlessLS10. After three weeks the amount of genomic (g) RNA and sub genomic (sg) RNA was determined by means of two different primer pairs matching either with a sequence in ORF2 (a region from which no sg RNA is produced) or in ORF3 expressing the CP (a region from which the sg RNA1 is produced). The letters "a" and "b" indicate significance. n = 10, $p < 0.01$.

In cells infected with positive-strand RNA viruses that produce sub genomic (sg) RNAs the ratio of genomic (g) RNA and sg RNA is ≤ 1 meaning that usually more sg RNA than g RNA is produced, especially in later stages of infection when structural proteins are needed for encapsidation and movement (Sztuba-Solińska et al. 2011). Although contrasting results exist, sg RNAs of PLRV probably are not encapsidated (Brault et al. 2003; Lee et al. 2005) and the amount of virus particles is overestimated, if primers are used that anneal only to sequences that produce sg RNAs. Therefore, different primers were designed that matched either with a sequence in ORF2 (a region from which no sg RNA is produced) or in ORF3 expressing the CP (a region from which the sg RNA1 is produced) (Fig. 17). Afterwards, the RT-qPCR efficiency was determined. A comparison of the absolute numbers of g RNA and sg RNA copies is only reliable, when the efficiency of both primer pairs is approximately equal (Tab. 5).
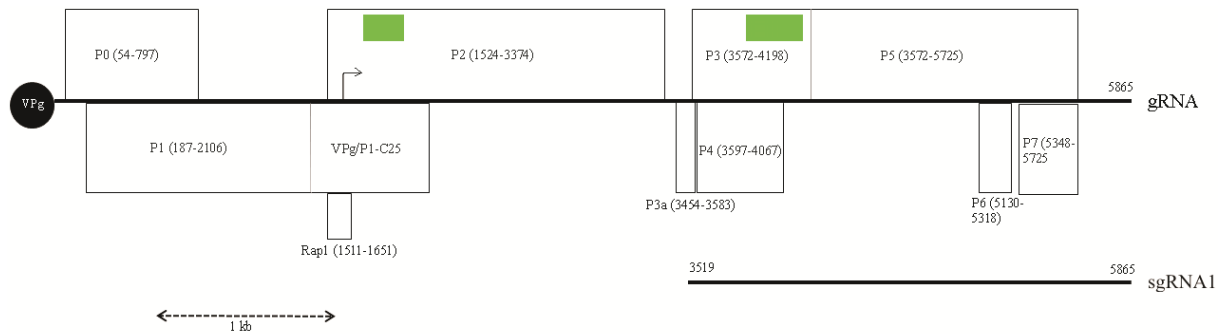
Fig. 17 Position of the PCR products amplified by primers located either in the genomic (left green rectangle) or in the sub genomic RNA-producing region (right green rectangle) of the PLRV genome. Abbreviations of PLRV proteins and RNA species are in accordance to Fig. 4.

Tab. 5 Name, sequence and efficiency of genomic and sub genomic PLRV primers

Names of primers	Sequences of primers	RT-qPCR efficiency	R^2	Slope	Y-intercept
PLRVfw-g	TCCATCAACTTCAGCAGAG	94.1%	0.999	-3.472	34,62
PLRVrev-g	CACTATCTTCTCCTCTATCTCC				
PLRVfw-sg	ATCGCCGCTCAAGAAGAACTG	94.1%	0.998	-3.472	36,76
PLRVrev-sg	GTAGGACTGGAGGGATGATACTTTG				

fw: forward, rev: reverse, g: genomic, sg: sub genomic, R^2: correlation coefficient

The above mentioned primers were used for an experiment, with which the virulence of different PLRV isolates should be examined (Hühnlein et al. 2016b). At the time of sampling, three and a half weeks after infection, isolate JokerMV10 produced significantly more sg RNA than gRNA independent of which cultivar was used. It seemed that the isolates differ in the time to when they switch from an equal production g and sg RNA to a primary production of sg RNA. However, to the present, the mechanism, which is responsible for the accomplishment of the ratio of g and sg RNAs is not known (Sztuba-Solińska et al. 2011). Further results of the above mentioned experiment are published in Hühnlein et al. (2016b, chapter 5.3).

The RNA standard for RT-qPCR was different for the use of primers matching with a sequence in ORF2 and for primers located in the sg RNA-producing sequence. The absolute quantification of a product amplified by the first mentioned primers was done with the aid of a standard that was located between ORF0 and ORF2 (nt 396-2656, according to isolate JokerMV10). The standard that was used for the second mentioned primers was located in ORF3 expressing the CP (nt 3,852-4,133, according to isolate JokerMV10). The production and dilution of the PLRV RNA standards was the same as described for the PVY RNA standard (chapter 4.4.1).

4.5 Efforts to increase the efficiency of the assays

The efficiency of a RT-qPCR is not only dependent on the efficiency of the primers as described in detail in chapter 4.4. For the high-throughput application in laboratories, time and cost efficiency are also indispensable. RNA extraction reagents are expensive if purchased. Furthermore, the throughput of RNA extraction is limited, since the RNA of each sample has to be prepared, individually. That is the reason why ELISA is still the method of choice for virus detection in laboratories with limited funds and equipment, e. g. that of plant protection services. Therefore, the advantages of ELISA (low costs and high throuput) should be combined with the high versatility and sensitivity of RT-qPCR. This was achieved by replacing RNA extraction with the immuno-capture (IC) method. For conventional PCR this method was often used to increase the PCR efficiency and ease (Rowhani et al. 1995; Werner et al. 1997; Chikh-Ali et al. 2013). In contrast, the application in combination with RT-qPCR was rarely documented (Li et al. 2010; Pospieszny et al. 2012). An absolute quantification with IC-RT-qPCR was not yet described.

4.5.1 Detection of PVY using IC-RT-qPCR

Beforehand, it was not clear, if the relationship between the initial and the detected copy numbers of virus would be linear, when amplified by IC-RT-qPCR. The question was, whether the virions are evenly distributed within the mixture of plant sap, so that the number of virions that bind to the coated IgG antibodies is proportional to the number of virions within the plant sap. This issue was addressed by means of a dilution series of plant sap from PVY infected potato leaves.

Two leaf discs, either from healthy or PVY infected potato plants, were punched out with the aid of 1.5 ml reaction tubes and ground in 500 μl extraction buffer [PBS + 2% (w/v) PVP + 0.2% (w/v) dried milk] by means of three 2.5 mm stainless steel beads using a Retsch® mixer mill (Retsch GmbH, Haan, Germany). The plant sap was mixed with sap from a healthy potato plant in different ratios with the result that the sap from PVY infected leaves was diluted from 1:100 to 1:50,000. 25 μl of the dilutions were filled in each of the 96 wells of a normal PCR plate coated with PVY IgG polyclonal antibodies (JKI, Institute for Epidemiology and Pathogen Diagnostics, Quedlinburg, Germany). In order to obtain a high throughput, the plate was washed with the aid of an ELISA washer. Afterwards, a RT was realized within this plate by using the reverse primer with isolate independent efficiency (PVY3-9090). For qPCR 2 μl of the resulting cDNA solution were pipetted into a new PCR plate. The qPCR was accomplished by using forward and reverse primers with isolate independent efficiency (PVY5-8911, PVY3-9090). As it is shown in Fig. 18, the relationship

between the initial and the detected copy numbers of PVY is linear, when amplified by IC-RT-qPCR. Therefore, IC-RT-qPCR can be used also for absolute quantification. However, a standard is needed that consists of virions instead of RNA, because not all virions within the mixture of plant sap and extraction buffer bind to the IgG antibodies. In turn, this is the limiting factor of using IC-RT-qPCR for absolute quantification, because virus purification is expensive in time and lab equipment. Nevertheless, if a relative comparison of the absolute quantity of virus particles is the target of investigation, IC-RT-qPCR using a RNA standard is yet a sufficient method, because the relationship between the initial and the detected copy numbers of PVY is linear (Fig. 18).
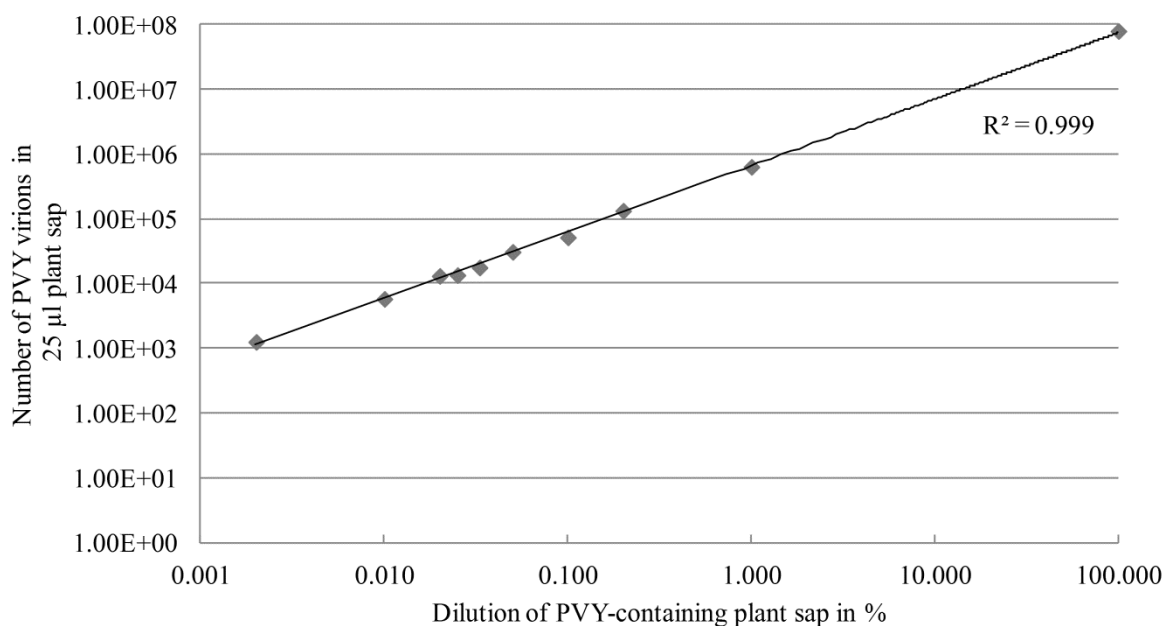
Fig. 18 IC-RT-qPCR of a dilution series of PVY-containing plant sap. The sap was diluted in the ratios of 1:100 (1% PVY-containing plant sap in 99% negatively tested plant sap) to 1:50,000 (0.002% PVY-containing plant sap in 99.998% negatively tested plant sap). The percentage of PVY-containing plant sap in negatively tested plant sap (x axis) as well as the absolute numbers of PVY virions detected in 25 µl of plant sap (y axis) are displayed in a logarithmic scale. A power trendline was included to demonstrate the linear relationship between the logarithmized data on the y and x axes. R^2=correlation coefficient.

Additionally, a dilution series was also used for DAS-ELISA to compare the sensitivity of both detection methods. As it was described for the IC method above, the plant sap of PVY infected and healthy leaf tissues was mixed after grinding in extraction buffer, but in the ratios of 1:20 to 1:500. ELISA plates were coated with the same PVY IgG polyclonal antibodies and 100 µl of the plant sap dilutions were filled in each well.

Tab. 6 Results of DAS-ELISA with different dilutions of plant sap from PVY infected leaf tissues

Dilution level in %	Mean OD value (ELISA)	Result (pos/neg)
Undiluted	0.76	pos
5.00	0.31	pos
3.30	0.26	pos
2.50	0.23	pos
2.00	0.22	pos
1.40	0.19	pos
1.00	0.14	pos
0.70	0.11	pos
0.50	0.09	pos
0.30	0.07	neg*
0.20	0.06	neg*

*The threshold value for negative results (0.08) was determined as follows: mean of negative controls + 3 x the standard deviation of negative controls.

With DAS-ELISA positive values were obtained only until a dilution of 1:200 (0.5% sap of a PVY infected potato plant in 99.5% sap of a healthy potato plant). However, the OD value of the undiluted sample was quite low. Probably, the level of dilution can be increased by using leaf tissue with higher virus titers. Nevertheless, it is clearly shown, that the sensitivity of IC-RT-qPCR is 250times higher than the sensitivity of DAS-ELISA when used with the above mentioned IgG antibodies to detect PVY.

4.5.2 Detection of PLRV using IC-RT-qPCR

If PLRV infected leaf tissue is used for IC-RT-qPCR, then the complete digestion of phloem cell walls is the limiting factor for a sufficient release of PLRV particles into the extraction buffer. As it was described for virus purification, the digestion can be obtained by adding cell wall digesting enzymes. Such enzymes were also tested for the extraction of PLRV infected leaf tissues and usage in IC-RT-qPCR (Tab. 7).

Tab. 7 List of commercial enzyme formulations used for the extraction of PLRV infected leaf tissues

Name of enzyme	Manufacturer	Enzyme activities
Novalin ®	NovaBiotec® Dr. Fechter GmbH, Berlin, Germany	Cellulases and lingnocellulases
Vegazym® HC	Erbslöh Geisenheim GmbH, Geisenheim, Germany	Cellulases, hemicellulases
Fructozym ® P	Erbslöh Geisenheim GmbH, Geisenheim, Germany	Pectinases
Depol™ 793L	Biocatalysts Limited, Wales, UK	Pectinases, hemicellulases, hydrolases

The enzymes were tested at different pH-values, incubation temperatures and times, concentrations and with and without grinding in liquid nitrogen. For the evaluation of the enzyme activity at different pH-values, phosphate citrate buffers were prepared with pH-values from 5 to 7. Afterwards, 5 µl of enzyme was mixed with 10 ml of buffer to obtain an enzyme concentration of 500 ppm. A large quantity of PLRV infected potato leaves was pestled in liquid nitrogen. With the aid of a cap from a 1.5 ml centrifuge tube, 97 mg (+/- 3 mg) grinded plant material was mixed with 1 ml of enzyme/buffer solution by vortexing and was then incubated at 30 °C for 90 min. As controls, grinded plant material was also incubated in extraction buffer (see chapter 4.5.1 for composition) and adjusted to different pH-values. A part of the controls was neutralized after incubation by adding 1M sodium hydroxide (NaOH). All reactions were performed in triplicates. After incubation, the tubes were centrifuged at 8000 rpm and 50 µl of the supernatant was filled into a PCR plate coated with PLRV IgG antibodies (JKI, Institute for Epidemiology and Pathogen Diagnostics, Quedlinburg, Germany). After incubation over night at 4 °C and washing, the RT was accomplished within the PCR plate by using the reverse PLRV primer that anneals in the genomic region of the PLRV genome (PLRVrev-g). 2 µl of cDNA were transferred to a new PCR plate and a qPCR was realized by using the forward and reverse genomic PLRV primers (PLRVfw-g, PLRVrev-g).
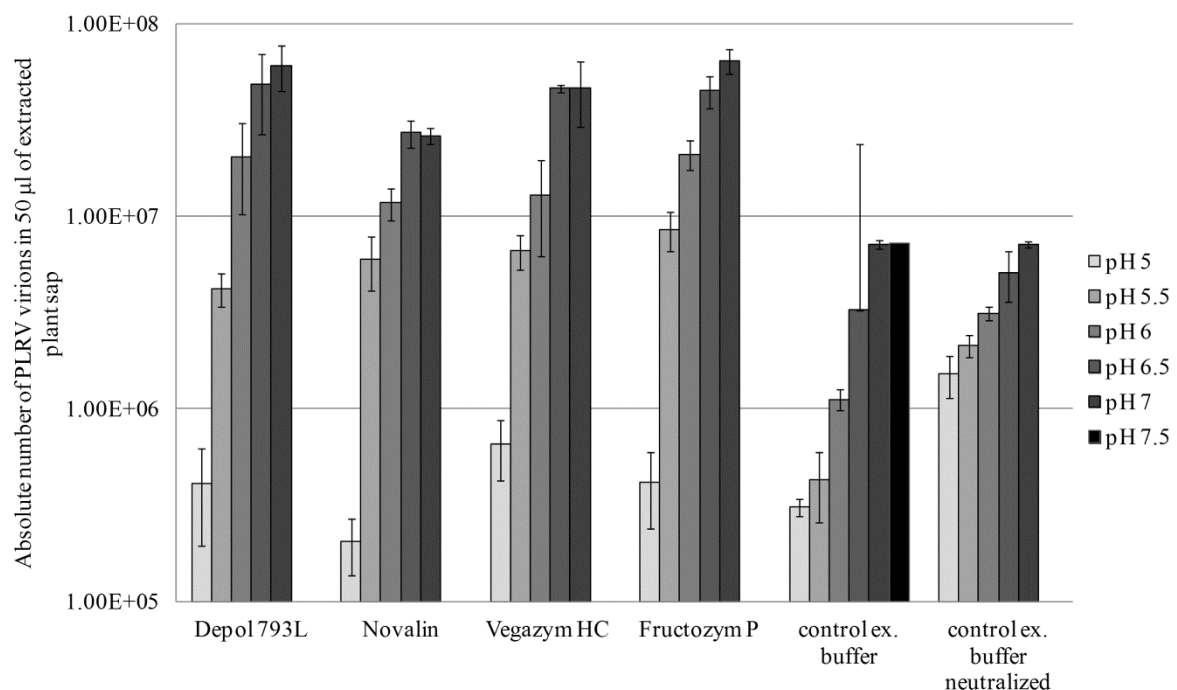
Fig. 19 Absolute number of PLRV particles in 50 µl of plant sap extracted either with a mixture of phosphate citrate buffer and one of four different commercial enzymes or with extraction buffer, solely. The phosphate citrate and the extraction buffer were adjusted to different pH-values prior to incubation. Within the control using neutralized extraction buffer, the mixture was neutralized to pH 7 after incubation. The y-axis was logarithmized. Error indicators were calculated from the standard deviation and represent the variation of the data and not the error in the measurement.

As it is shown in Fig. 19, most PLRV particles were extracted by using a mixture of phosphate citrate buffer and enzyme preparation at pH-values of 7.0 and 7.5. Fewer particles were extracted by using extraction buffer only, independent from the pH-value. However, when the extraction buffer was neutralized by NaOH after an incubation time of 90 min, the number of extracted PLRV particles could be increased at least in the acidic starting preparations. Therefore, the pH-value of the buffer does not only influence the extraction quality and enzyme activity, but also the affinity of particles for binding to IgG antibodies. Thus, the binding of virions to IgG antibodies is decreased by using buffers with low pH-values.

The three best extraction methods using Depol™ 793, Novalin® and Vegazym® HC were used for further optimization. It should be ascertained, which temperature and enzyme concentration would be optimal for the incubation period. The extraction of PLRV infected potato leaves was done as mentioned before. The incubation was also done for 90 min but at different temperatures (15 °C, 20 °C, 25 °C and 30 °C) and concentrations of enzymes (250 ppm, 500 ppm and 1000 ppm).
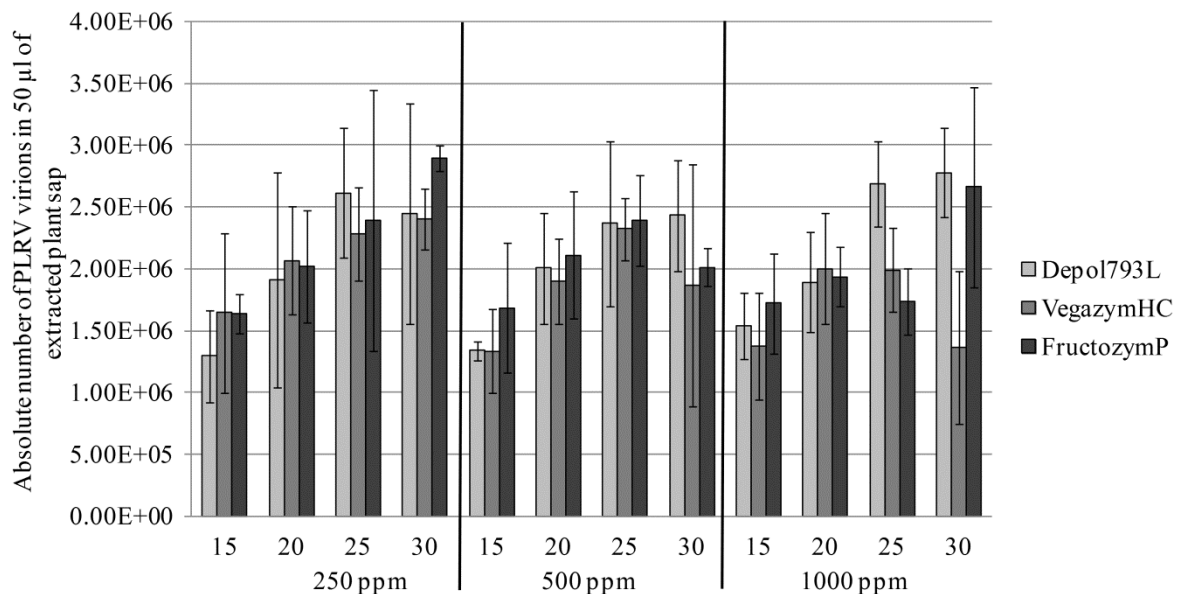
Fig. 20 Absolute number of PLRV particles in 50 µl of plant sap extracted with a mixture of phosphate/citrate buffer and either one of three different commercial enzymes used in a concentration of 250 ppm, 500 ppm or 1000 ppm. The incubation was done for 90 minutes at 15, 20, 25 or 30 °C. Error indicators were calculated from the standard deviation and represent the variation of the data and not the error in the measurement.

Fig. 20 reveals that the incubation temperature, when varied between 15 and 30 °C, has an effect on the efficiency of extraction. In fact, the extraction efficiency seems to increase with increased incubation temperature until 25 °C and remains unchanged when the temperature is further increased to 30 °C. However, by using only triplicates the standard deviation was high

and slight differences between the temperatures used cannot be verified. Since the number of PLRV particles detected by IC-RT-qPCR does not differ between different concentrations of enzymes used for the extraction, the lowest concentration of 250 ppm seems to be sufficient. The incubation time was also varied in order to test, if the extraction efficiency would vary between 30, 60, 90 or 120 minutes. However, no differences were determined between the detected numbers of PLRV particles incubated either for a short or a longer period. Therefore, 30 minutes suffice to extract PLRV from potato leaves.

4.6   Setbacks

During the laboratory work the author of this thesis generated not only desirable results in frequent intervals. There were many obstacles, with which the author was confronted. In published articles of scientific journals, failures and disappointments play, if anything, only a secondary role. Therefore, the author uses this thesis to disclose also the setbacks of her three-year research work.

4.6.1   Contamination of PCR controls

First of all, the contamination of PCR controls was the major difficulty. Several times, when an efficient primer pair was designed and optimized, the negative and non-template controls were contaminated with the amplified PCR product. As an example, contaminations were recorded with primers that detect all isolates of PVY with a high efficiency: PVYall5-8500 (primer sequence: AGGAGGAACTGAAGGCTTTCACTGAAAT) and PVYall3-8610 (primer sequence: TCCTCCTGCATCGATTGTGTCAT). Immediately, all buffers, enzymes, dNTPs and magnesium solutions as well as primers and probes were renewed. A RT-qPCR was done with the renewed reagents on a MiniOpticon® Real-Time PCR System (Bio-Rad Laboratories, Inc.). A positive control was pipetted to the first column of a PCR plate. Non-template controls were placed in different distances to the positive control in order to test a possible contamination by evaporation. As it is shown in Fig. 21, the contaminations still appeared independent from the distance to the positive control.
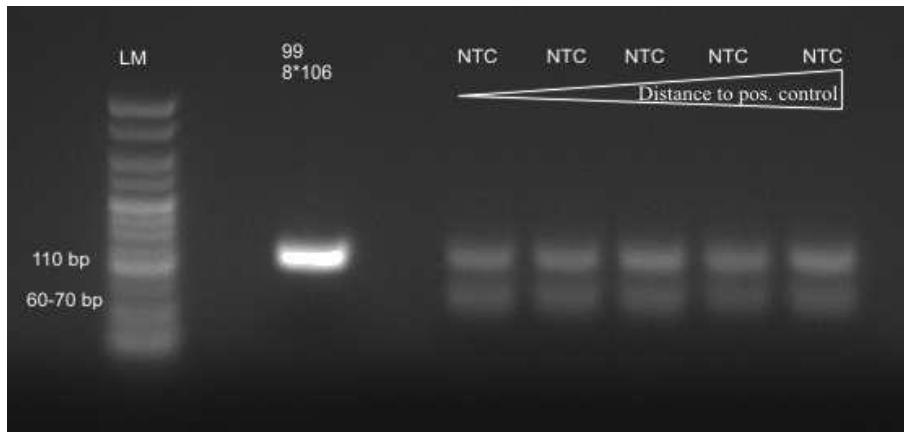
Fig. 21 Gel electrophoresis of qPCR products amplified by PVYall5-8500 and PVYall3-8610 primers. The positive control was a PVY standard of 8×10^6 RNA copies derived from nt 6700-8800 of PVY^{NTN} (isolate Gr99). The non-template controls (NTC) were run on a PCR plate in different distances to the positive control. The expected PCR product has a length of 110 bp, primer dimmers are approximately 60 bp long. LM: DNA ladder.

With the same reagents a second qPCR was done but without any positive control. The second qPCR was realized on different qPCR instruments: MiniOpticon® and iQ5® Real-Time PCR Systems (Bio-Rad Laboratories, Inc.). The iQ5 qPCR instrument was located in a separate laboratory, where no qPCR was realized up to that time using the before mentioned primers. Surprisingly, no contaminations appeared on the gel, regardless of which qPCR instrument was used (Fig. 22).
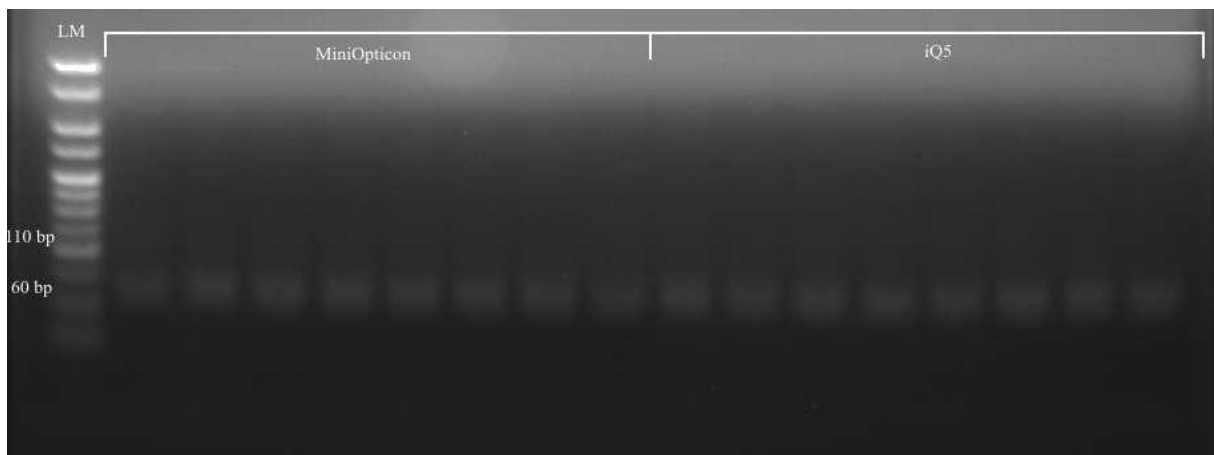
Fig. 22 Gel electrophoresis of a qPCR using PVYall5-8500 and PVYall3-8610 primers. No positive control was pipetted to the wells. The qPCR was run on two different qPCR instruments (MiniOpticon® and iQ5®, both from BioRad Laboratories, Inc.). No contamination is visible. Primer dimmers have a length of approximately 60 bp.

Therefore, the contamination did not originate from a contamination of reagents, lab equipment or qPCR instruments. It was assumed that the PCR product evaporated during the amplification and contaminated the other wells of the plate, although thoroughly sealed. Therefore, positive controls and NTCs were pipetted to a PCR plate and sealed in the case of NTCs and capped with optical cap strips in the case of positive controls. Subsequently, a RT-

PCR was performed using either PVYall5-8500 and PVYall3-8610 primers or PVYall5-8500 and a new 3' primer (PVYall3-8750, primer sequence: GGCATTCTCATTTTGGAC GTGATAG) as an alternative, if the contaminations could not be eliminated. The contamination of NTCs was successfully eliminated as it is shown in Fig. 23. However, the use of optical cap strips is considerably more expensive than using adhesive seals, which leads to a rejection of the PVYall5-8500 and PVYall3-8610 primers.

Therefore, new primers were designed that were described in detail in chapter 4.4.1. PCR products amplified by these primers (PVYall5-8911, PVYall3-9090, PVYall5-9194 and PVYall3-9420) never generated any contaminations. Therefore, the propensity of PCR products to evaporate seems to be dependent on their length and nucleotide sequence.
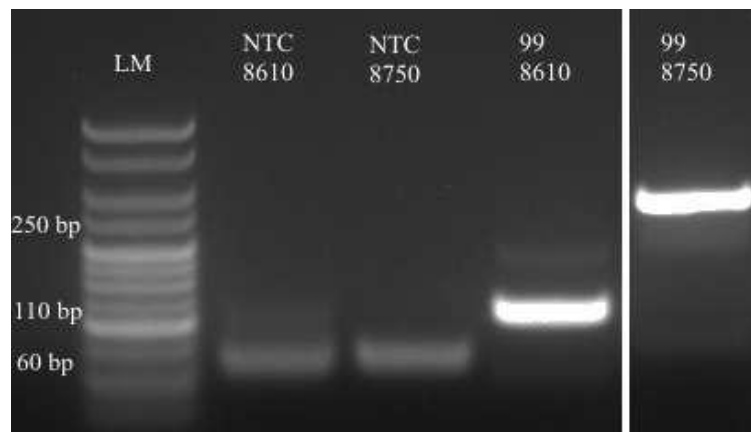
Fig. 23 Gel electrophoresis of PCR products amplified either by PVYall5-8500 and PVYall3-8610 primers or by PVYall5-8500 and PVYall3-8750 primers. The positive control was plasmid DNA with a PVY insert from nt 6700-8800 of PVY^{NTN} (isolate Gr99: "99"). LM = DNA ladder

4.6.2   Low efficiency of fluorophore-labeled TagMan® probes

As it was mentioned in chapter 4.4.1, the fluorescence during qPCR was emitted from Sybr® Green. However, fluorophore labeled TaqMan® probes were also designed for detecting PVY in target cDNA that generates unspecific products when amplified by PCR. This was the case, e. g., when RNA was extracted from the potato cultivar 'Mayan Gold' and amplified by using the PVYall5-9194 and PVYall3-9420 primers. When the fluorescence was emitted from Sybr® Green, the desired and unspecific products could not be distinguished because of similar melting points. Therefore, a FAM-labeled TagMan® probe was designed that annealed at nt position 9330 (PVYFAM-9330, sequence: ACACAGAGAGGCACACCA CCGAGGA). However, a slope of -3.77 and an efficiency of only 84% were not sufficient to quantify PVY RNA copies, absolutely. A previous designed FAM-labeled probe that gave high qPCR efficiencies was used with the PVYall5-8500 and PVYall3-8610 primers. However, this primer-probe combination could not be used further on, because of the above

described contaminations (Fig. 21). In fact, TaqMan® qPCR assays with high efficiencies were generated by using primers that produced short amplicons. However, short amplicons were more often the reason for contaminations than longer ones. Therefore, the author refrained from using fluorophore labeled probes for qPCR. By designing primers generating almost no primer dimers, Sybr® Green was successfully used in high-efficiency qPCR assays. The production of unspecific amplicons, e. g. by using RNA from 'Mayan Gold', was re-examined by other PVY primers, such as PVYall5-8911 and PVYall3-9090. With these primers, no PCR product was amplified indicating that the PVYall5-9194 and PVYall3-9420 primers annealed within the genomic RNA of this cultivar. Unfortunately, the primers that did not generate unspecific products were not as efficient as the primers that did so (see chapter 4.4.1).

4.6.3 Enzymes for the detection of PLRV by using IC-RT-qPCR – no success after all?

As it was shown in chapter 4.5.2, the extraction of PLRV from potato leaves ground in liquid nitrogen was significantly increased by adding cellulase and pektinase enzymes. However, by accident, in one experiment the leaf material was ground in the buffer solutions without prior freezing. The surprising result was that no difference was ascertained between the numbers of quantified PLRV particles extracted either with extraction buffer, solely or with a mixture of buffer and enzyme (Fig. 24). Only by using phosphate citrate buffer, the amount of extracted PLRV was increased by adding an enzyme preparation. The reason for this result was that the leaf material pestled in a mortar at room temperature was more finely ground than with the addition of liquid nitrogen, which made the leaf tissue hard as stone. Therefore, for IC-RT-qPCR the addition of enzymes is not necessary, when an extraction buffer is used consisting of PBS, 2% (w/v) PVP and 0.2% (w/v) dried milk, and when the plant material is finely ground at room temperature.
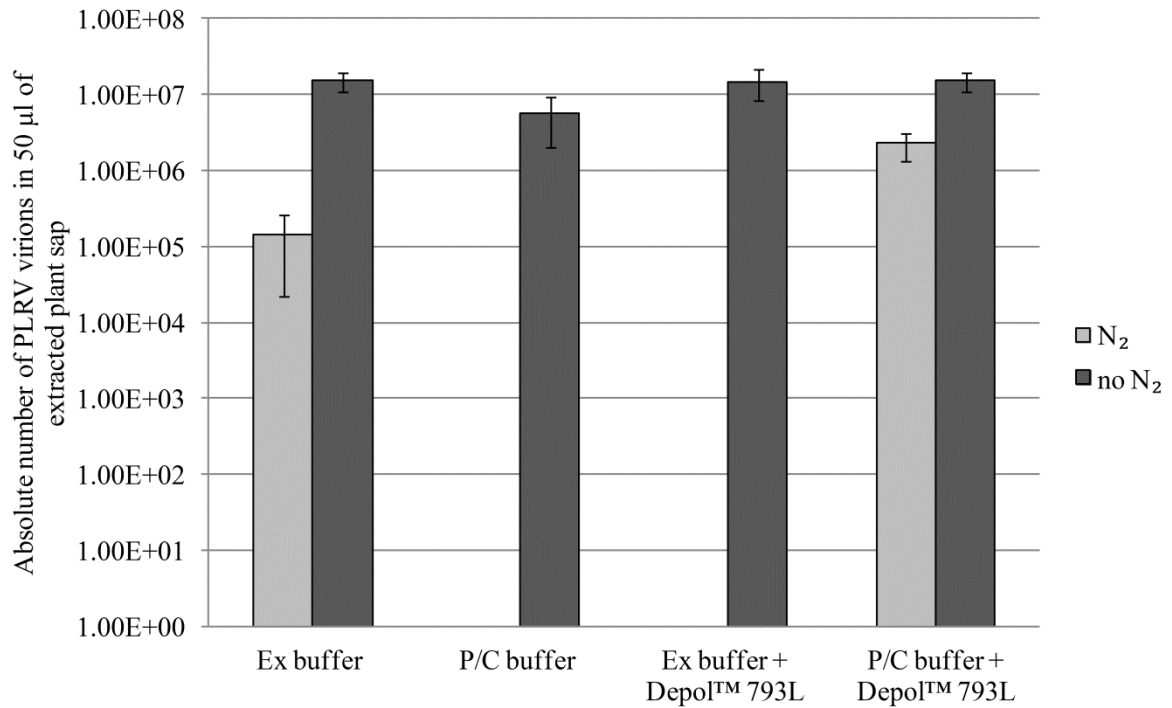
Fig. 24 Absolute number of PLRV particles in 50 µl of plant sap extracted either with extraction (ex) buffer, phosphate/citrate (P/C) buffer or with a mixture of Depol™ 793L and extraction buffer and phosphate/citrate buffer, respectively. Some of the extractions were done with potato leaves that were ground in liquid nitrogen (N_2). The y-axis was logarithmized. Error indicators were calculated from the standard deviation and represent the variation of the data and not the error in the measurement.

5 Applications in potato research and production

The absolute quantification of PVY and PLRV by using the optimized methods described in chapter 4.5 enables several applications in potato research and production. This chapter contains publications of the author that address the utilization of an absolute quantification of PVY for seed potato certification, the examination of PVY and PLRV regarding their virulence and the evaluation of transgenic potato plants regarding their susceptibility to PVY. Furthermore, in chapter 5.2 the validity of RT-qPCR results is discussed in the view of an evaluation of potato progenies regarding their resistance type. Within the four successive publications, the numbers of tables and figures as well as the references are detached from the registers in this thesis.

5.1 Comparison of three methods for the detection of *Potato virus Y* in seed potato certification

Comparison of three methods for the detection of *Potato virus* Y in seed potato certification

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Abstract

Potato virus Y (PVY) is becoming increasingly important in potato growing regions worldwide. The main reason for this is an increase in the incidence of infections with recombinant forms of PVY, such as PVY^{N}Wi and PVY^{NTN}. They are characterized by high virulence and low symptom expression, which is especially true of PVY^{N}Wi. This makes it difficult to detect infected seed potato plants during certification. In Mecklenburg-Western Pomerania (North-East Germany) in 2008 an unusually high incidence of infection with PVY was recorded in fields where seed potatoes were being grown. In this study we examined, which strains of PVY caused these infections. Furthermore, we have developed a reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assay for direct tuber testing, which we compared to direct tuber testing by ELISA and growing-on tests. As a result, we recommend for direct tuber testing by RT-qPCR or ELISA. These methods are less space- and time-consuming and therefore less costly alternatives to conventional ELISA testing of eye cuttings from seed potatoes. Additionally, the RT-qPCR method has a high efficacy, so that even freshly harvested non-dormant tubers can be tested, which makes testing very fast and economical. This is of special interest in cases when tubers shall be exported to the other hemisphere of the world.

Key words: PVY, RT-qPCR, seed potato, tuber

Introduction

Potato virus Y (PVY) is the type member of the *Potyvirus* genus (*Potyviridae* family) and features a single-stranded positive-sense genomic RNA of approximately 9.7 kb. The filamentous particles are mostly 730×11 nm. The RNA encodes a large single polyprotein which is processed into 10 mature protein products by virus-specific proteases. Recently, an overlapping coding sequence was found within the long open reading frame of *Potyviridae* species (Chung et al. 2008). The coding sequence, PIPO (Pretty Interesting Potyviridae ORF), is located in the P3 cistron (see Fig. 1). PVY is one of the most important viruses damaging the production of potatoes worldwide (Valkonen 2007). Especially in years when aphids are abundant the number of primary

infections increases resulting in yield losses and frequent rejection of seed potatoes. Whereas *Potato leafroll virus* (PLRV) can be effectively controlled by application of insecticides, PVY is transmitted by aphid vectors in a non-persistent manner and therefore even a short probe of PVY infected leaves followed by probing of healthy plants is sufficient to transmit the virus. Therefore, PVY cannot be effectively controlled by insecticides. Additionally, insecticides cause an increase of probing activity because they act by impairing the metabolism and activity of the nervous system of insects (Thieme et al. 2009). In contrast to PLRV, the importance of PVY has increased over the last 30 years. It is assumed that this may either be due to earlier and more intense colonization by aphids promoted by climate change combined with the low effect of insecticides on PVY transmission (Zahn 2004) or an alteration that has occurred in the spectrum of strains of PVY (Chrzanowska 1991, Singh et al. 2003, Visser & Bellstedt 2009). Most of the PVY strains detected recently in Europe have sequences that indicate recombination events between PVY^O and PVY^N. They have 1 to 4 recombinant junctions (RJ, Fig. 1). If they react with strain specific monoclonal antibodies like PVY^N they are designated as European tuber necrotic strains (PVY^{NTN}). Recombinant PVY strains that react like PVY^O strains are designated as Wilga-strains (PVY^{N}Wi). Both, PVY^{NTN} and PVY^{N}Wi cause tobacco to develop veinal necrosis and potato tubers to generate necrotic rings (Chikh Ali et al. 2007, Schubert et al. 2007). Today, in European potato growing regions recombinant forms of PVY are more common than the non-recombinant forms (Lindner & Billenkamp 2005, Blanchard et al. 2008). The reasons for this shift in the spectrum of strains are unknown.

In all seed potato producing countries there are sophisticated systems for ensuring the distribution of only virus free seed potatoes. Certification schemes are based on visual inspections of fields and serological testing of eye cuttings from a defined number of tubers. This is a time consuming and expensive procedure. If the seed potatoes are for export this procedure can be especially limiting as some countries request export certificates including tests for the presence of PVY using RT-PCR. Currently, in Germany certification is solely based on field inspections and tests using ELISA.

In Germany plant protection services have recorded a considerable increase in PVY infections relative to PLRV over the last 30 years, too. In 2008 in Mecklenburg-Western Pomerania (M-WP) unusually high levels of infection of
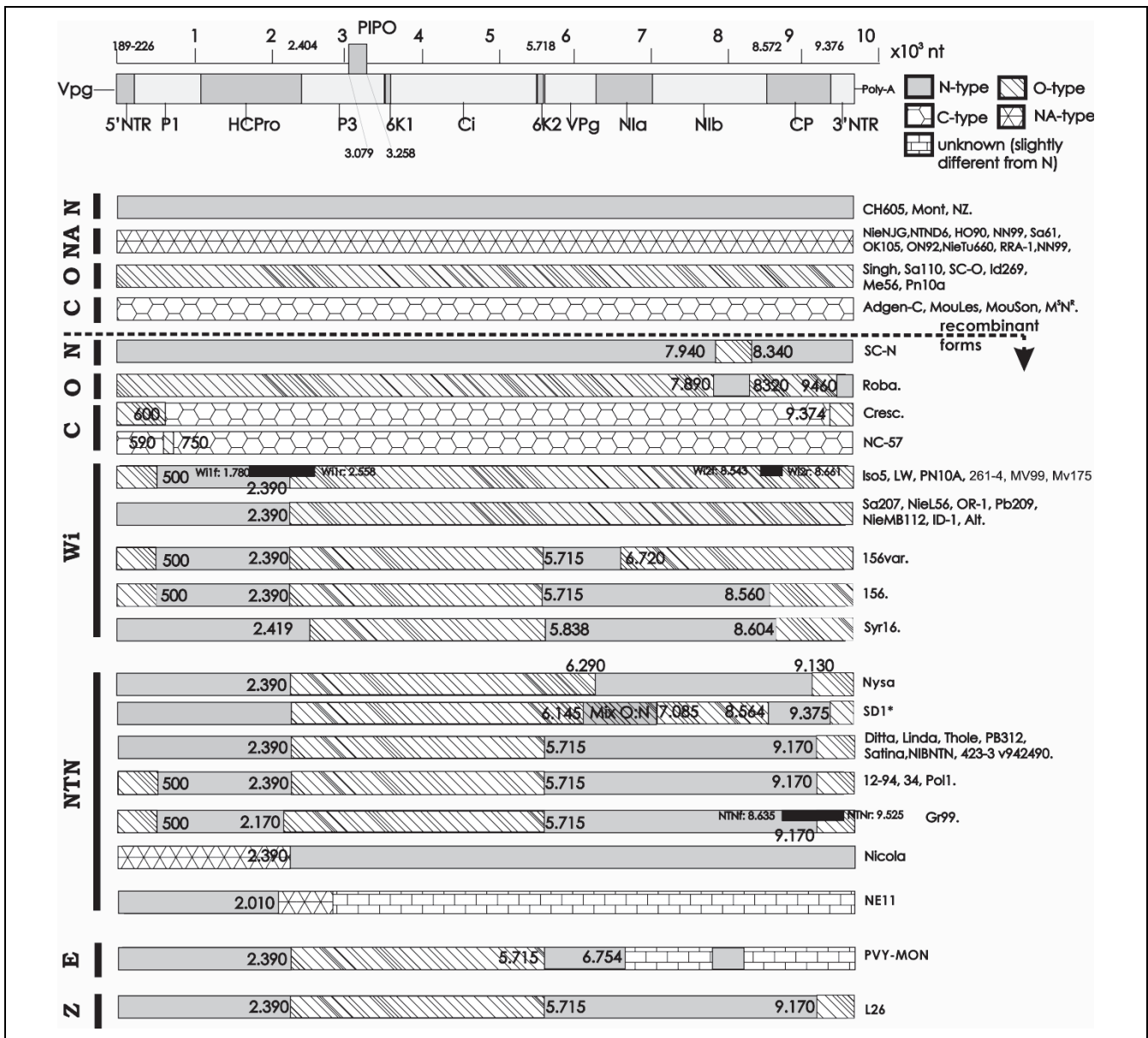
Fig. 1: Genome structure of different isolates assigned to known strains and variants of PVY. VPg = viral genome linked protein, Poly-A = poly-A tail, P1 = P1 protein, HCPro = helper component protease, P3 = P3 protein, Ci = cellular inclusion protein, NIa = nuclear inclusion protein a, NIb = nuclear inclusion protein b, CP = coat protein. The non-translated regions are abbreviated as NTR. The overlapping coding sequence PIPO (Pretty Interesting Potyviridae ORF) within the P3 cistron is indicated (Chung et al. 2008). Designation of strains is given on the left and that of isolates on the right hand side of the structures (N = PVY^N, NA = PVY^{NA}, O = PVY^O, C = PVY^C, Wi = PVY^{N}Wi, NTN = PVY^{NTN}, E = PVY^E, Z = PVY^Z). Positions of RJs are given inside the schematically drawn genomes by switching from one filling pattern to another. Counting of RJs was done from 5' to 3' end. Positions of the fragments detected using RT-PCR specific PVY^{N}Wi and PVY^{NTN} primers are given inside the respective genome structures as black boxes. (*) Isolate SD1 was rectified by the authors since nucleotides 9375 till 9706 were erroneously published in GenBank® in reverse order.

seed potatoes with PVY were recorded during the official certification, thus, leading to high rejection rates of seed potato lots. Especially, the cultivars 'Innovator', 'Elkana', 'Zorba' and 'Donald' were seriously affected. To investigate whether this was caused by new and more virulent isolates, several seed lots were analyzed for the presence of different PVY strains. Two unusual isolates were completely sequenced and their biological characteristics determined. The high incidence of infections might have been caused by unrecognized infection with PVY of lots used for seed potato production. Therefore, it was determined which cultivars do not show visible symptoms when infected with strains of PVY. Furthermore, growing-on tests are currently the routine detection method for PVY during seed potato certification. However, planting of eyeplugs following ELISA is time- and space-consuming. In this study it was evaluated, if direct

tuber testing using ELISA or reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) could be a faster and cheaper alternative to growing-on tests.

Material and methods

Seed potato samples and symptom observation

For post-control in 2010, 184 potato tubers were planted out originating from 25 cultivars of PVY infected plant propagating material growing in different regions of M-WP in 2008 and 2009. In June 2010, ELISA was done on terminal leaflets of each plant. Symptoms on plants were evaluated in July. Afterwards, potato plants were individually harvested and stored. For the estimation of PVY infection of the stored potato tubers, direct tuber testing using ELISA was done in October 2010. Skin scrapings from the same stored tubers were used for RT-qPCR and RT-PCR in May, 2011.

ELISA testing of leaf material and tubers

For direct tuber testing using ELISA, tuber sap was sampled from tuber flesh in the close proximity of a bud within the upper third of the rose end using a modified dentist's drill (Gugerli 1979). Monoclonal antibodies (PVY mono cocktail, Bioreba AG) were used for ELISA of leaf and tuber sap samples. ELISA was done according to the manufacturer's protocol with the following validated modifications: the volume of sap and buffer was 100 µl, washing steps were done with distilled water + Tween 20, and incubation of conjugate was 4 h at 30°C. Ovalbumin was used to minimize unspecific reactions which often occurs when material with high starch content is tested, and the microtiter plates were not kept in the dark during incubation with substrate.

General PVY detection using RT-qPCR

The same 184 potato tubers were tested for PVY using RT-qPCR. Following PVY detection using RT-qPCR the samples that were tested positive were used to detect specific strains of PVY using conventional RT-PCR.

RNA was extracted from small pieces of the tubers. No attention was paid to the part of the tuber sampled as previous studies show that after dormancy breaking PVY concentration hardly differs between heel and rose end of tubers (Treder et al. 2009). Approximately 50 mg skin was taken from each of the tubers using a sterile scalpel blade. Each sample was ground using a pestle in 1.5 ml microcentrifuge tubes containing 500 µl of RNA isolation reagent (Concert™ Plant RNA Reagent, Life Technologies™). Further purification of RNA was done following the manufacturer's protocol. Purified precipitated RNA was redissolved in diethylpyrocarbonate (DEPC)-treated distilled water to a concentration of 200 to 400 ng per µl. Total RNA amount was measured via ultraviolet absorbance by a

NanoDrop 8000 instrument (Thermo Fisher Scientific). The quality of RNA was ensured by further using only samples with an OD 260/280 and OD 260/230 ratio of at least 1.8. However, with samples of cultivars 'Royal' and 'Marena' only low OD 260/230 ratios of extracted RNA could be produced (OD 260/230 < 1.0). For the quantification of PVY RNA using qPCR, RNA standards were prepared consisting of a dilution series of PVY RNA transcripts. The sequence of the transcripts corresponded to PVY RNA from position 8600 (highly conserved region of the coat protein) to the poly-A tail. Reverse transcription was performed using 2 µl purified RNA and 18 µl RT mixture (DEPC-treated water; $1 \times$ RT reaction buffer consisting of 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT; 200 µM of each dNTP; 0.4 µM reverse primer [PVYall3-9090, Hühnlein, unpublished]; 80 U M-MLV RT [Promega] and 4 U RNase inhibitor [RiboLockTM, Thermo Fisher Scientific]). The RT reaction was done in duplicate without denaturation at 42°C for 50 min following 10 min at 72°C for enzyme inactivation. In a second step 2 µl of cDNA reaction mix was combined with 18 µl qPCR reaction mix (distilled water; $1 \times$ reaction buffer consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 and 0.1% Tween 20; 150 µM of each dNTP; 0.3 µM of forward and reverse primers [PVYall5-8911, PVYall3-9090, Hühnlein, unpublished]; 2.5 mM $MgCl_2$; 2 U Taq DNA polymerase [Bioron GmbH] and $1 \times$ Sybr®Green I [Life Technologies™]). PCR reaction was performed in white 96-well plates (Biozym Scientific GmbH) sealed with adhesive clear seals (Thermo Scientific ABsolute™ QPCR Seal) in a MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the following protocol: 2 min at 95°C for initial denaturation followed by 40 cycles with 15 s at 94°C for denaturation, 15 s at 58°C for annealing, 20 s at 72°C for elongation and 15 s at 83°C for melting of nonspecific products. Melting of primer dimers from non-template controls happened at 79°C. Fluorescence was measured after each cycle. Following real-time PCR, the amplicons were melted at 95°C for 1 min and then fully annealed at 55°C for 1 min. For determination of the melting point(s) of the PCR product(s) the temperature was then increased incrementally to 95°C at a rate of 0.5°C every 10 s (as fixed on the MyiQ™2).

The limit of detection (LOD) of the qPCR assay was determined by repeated quantification of highly diluted standard RNA (1000, 100 and 10 RNA transcript copies). Generally, the LOD is expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time. Therefore, each diluted RNA was amplified 100 times, and the RNA dilution which was amplified at least 95times was set as the LOD of the assay. Figure 2 shows exemplarily a standard series from 10^{10} to 10 RNA transcript copies. The LOD for the assay was 100 RNA transcript copies which corresponds to 100 PVY RNAs.

The cDNA of 184 samples was applied in duplicates. Therefore standards were applied only to the first 96-well plate. An interplate calibrator (IPC) consisting of diluted PCR product produced with PVYall primers (Hühlein, unpu-
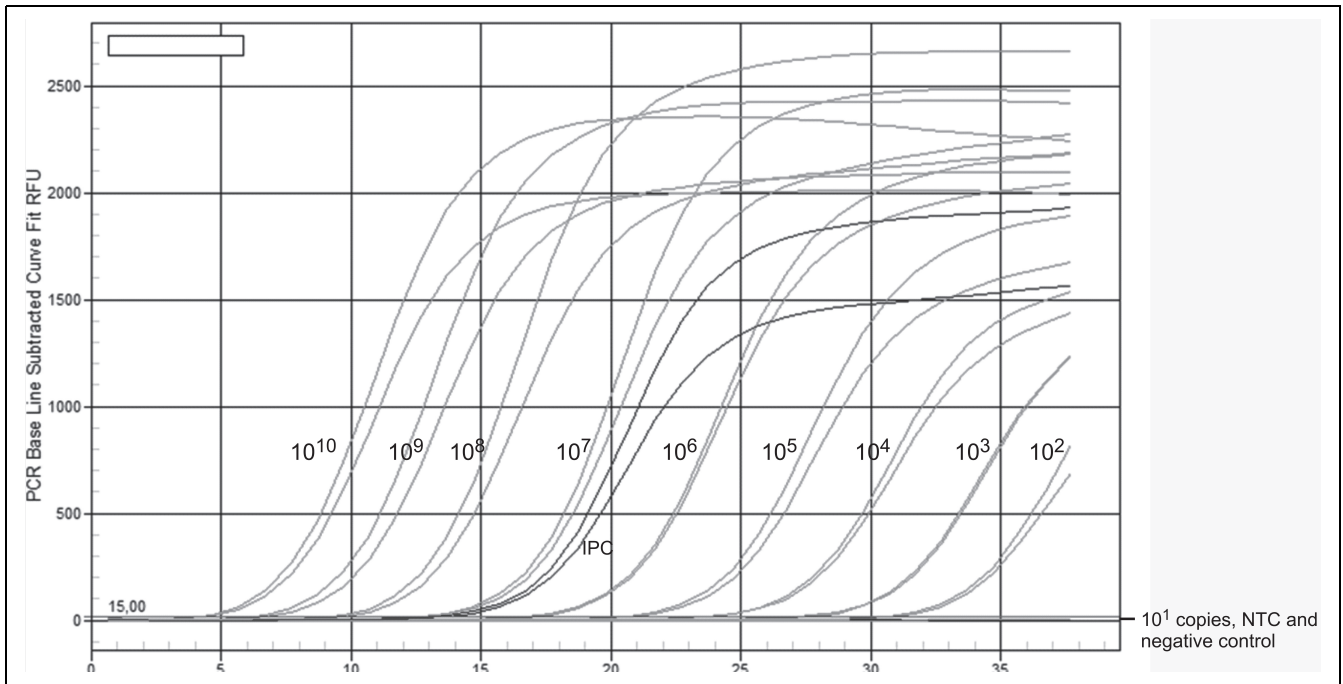
Fig. 2:   Amplification of a dilution series of *in-vitro* RNA transcripts displaying the PVY standard. Ten copies of the standard RNA, negative and non-template control do not exceed the threshold. IPC = interplate calibrator. Efficiency: 99.8, R^2: 0.996, slope: 3.33, intercept: 36.61

bished) was used for normalization and variation compensation of all plates.

To calculate absolute quantities from amplification data, the software GenEx Enterprise (MultiD Analyses AB) was used for reverse calibration. Results and qPCR efficiency were calculated based on a 95% confidence level. The estimated efficiency of the qPCR assay was 93% and the lower and upper confidence limit of efficiency was 89% and 99%, respectively. For each sample the total RNA amount per µl was determined after RNA extraction. Therefore, the absolute quantities of PVY in tubers could be given as copies of PVY per 300 ng total RNA (see Fig. 3).
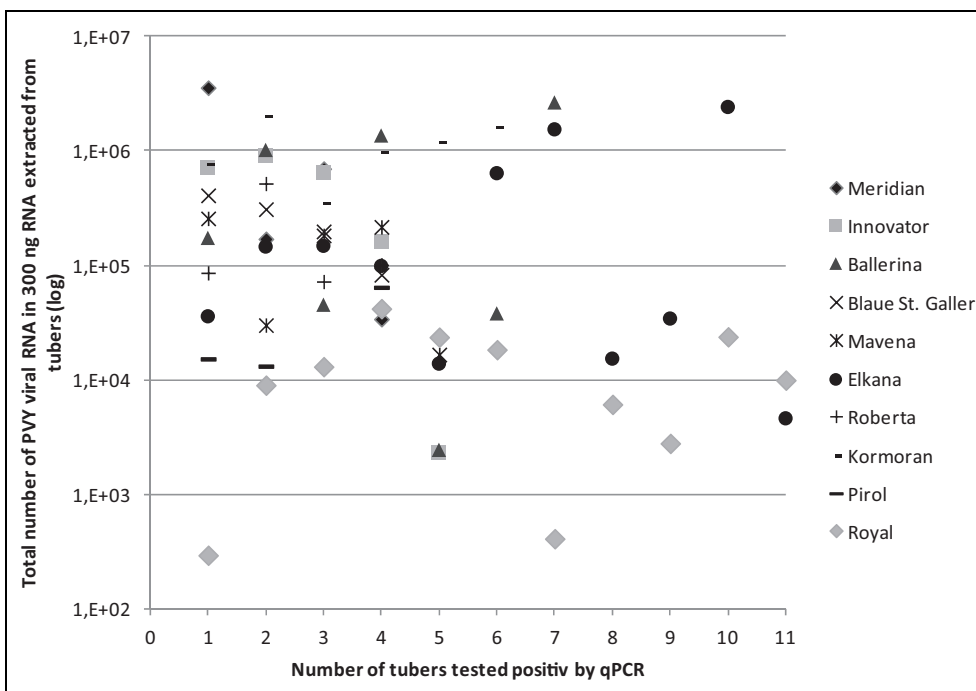
Fig. 3:   The number of each cultivar tested positive using RT-qPCR. Values are logarithmic. Only those cultivars for which at least 4 tubers were found to be positive were included.

Specific detection of strains of PVY using conventional PCR

RNAs from tuber samples that tested positive in RT-qPCR were used for strain specific conventional RT-PCR. For the detection of PVY^O, PVY^N, PVY^C and PVY^{NA} the primers used were those designed by Schubert et al. (2007). However, for the detection of PVY^NWi and PVY^{NTN} the PCR products have a length of 5 052 bp and 3 867 bp, respectively. Since special polymerases are necessary for such large fragments, alternative primer combinations were designed resulting in shorter fragments. They can be used, however, only when the absence of PVY^N or PVY^O was confirmed by PVY^O and PVY^N specific primers. For PVY^{NTN} isolates a larger N-type segment located between the 3rd and 4th RJ (position 5 715 to 9 170) is typical. It was used

to discriminate PVY^{NTN} from PVY^NWi. Primers used in this study for the detection of PVY^{NTN} are located at the 3'-end of the CP sequence generating an 869 bp PCR fragment. For the detection of PVY^NWi two PCR fragments have to be amplified, with a length of 798 (located between HCPro and P3) and 121 bp (located between NIb and CP), respectively (Fig. 1). Thus, the presence of up to two RJs can be determined, which is typically for most of the PVY^NWi isolates so far identified in Central Europe. Two PVY isolates that could not be assigned to any of the PVY strains with primers listed in Table 1 were completely sequenced and are designated as PVY-MV isolates in the text below. For cDNA synthesis RNA was reverse transcribed using Oligo-dT$_{18}$ following the above protocol except the incubation period was 60 min. Samples were tested for PVY strains in parallel reactions

Table 1: Primers used for detecting PVY in general and identifying strains

Primer name	Sequences (5'-3')	Detected PVY strains/applications	Source
MMPVYall5-9194	AGGTCACATCACGAACAC	Primers show missmatches with	(Hühnlein, unpublished)
MMPVYall3-9420	CGGAGAGACACTACATCAC	some PVY strains	(Hühnlein, unpublished)
Oligo-dT-18	Oligo(dT)18	For cDNA synthesis	Thermo Fisher Scientific
YN5-8635	AAGGTAGCATTCAACCAAATCTC	NTN	This study
YO3-9525	CCACAATGACGAAATCACCCTG		
YN-NA5-116	TTTGATCTTCGTCGTACAAACCG	NA	(Schubert et al. 2007)
YN-NA3-622	CTTGATAAGATGGTTCATTTGTTT		
YO5-1005	AAATTGTACGATGCACGTTCTAGA	O	(Schubert et al. 2007)
YO3-2558	AGGCTCATCTAACAGCAACTGTC		
YN5-1780	TCCGAATGGGACAAGAAAACTTG	N	(Schubert et al. 2007)
YN3-2438	TGGTTCATCCAGTAGCAATTGCT		
YN5-1780	TCCGAATGGGACAAGAAAACTTG	Wilga	(Schubert et al. 2007)
YO3-2558	AGGCTCATCTAACAGCAACTGTC		
YC5-125	ATATTGAAAACCGTCTTAGTTCGTT	C	(Schubert et al. 2007)
YC3-460	GCAGCCATCTGAAAGTAGTGC		
PVY5end(3)	AAATTAAAACAACTCAATACAAC	For cloning of complete sequences	This study
PVYHC3-1500	CATTCAYGAGCTGTATYTTCYTTTCC		
PVYHC5-1465	AARGARAATACAGCTCRTGAATGGC	For cloning of complete sequences	This study
PVYHC3-2150	CCAAGCTTYGGCACACACATGTC		
PVYHC5-2130	GAGGARGATGCAAAGGAYTTCAC	For cloning of complete sequences	This study
PVY3-3060	AAGAAYGCTTGYGGTGATATGTTG		
PVY5-3000	CAACATGGYAYTCATACARAGCAAA	For cloning of complete sequences	This study
PVY3-4360	GTTTTWGARCCTTGYGCATCAACA		
PVY5-2700	GRGTGTCAGCRGCAGAAACACTC	For cloning of complete sequences	This study
PVY3-4360	GTTTTWGARCCTTGYGCATCAACA		
PVYCI5-4300	GTKTCAGCYACTCCAGTGGGAAG	For cloning of complete sequences	This study
PVYCI3-5750	CTTYAADGCTTGRATTCTTTTGGAT		
PVYCI5-5650	RTTGCWRTYGGTGGWATAGGRCT	For cloning of complete sequences	This study
PVYNA3-7000	AGYTTYARYGGRCCCCACAACAC		
PVY5-6570	ACARAARYTRCRYTTCCGAGCTCC	For cloning of complete sequences	This study
PVYNIb3-8800	ACGTATCAAACTGTGATTGAGTTGC		
PVY5-6570	ACARAARYTRCRYTTCCGAGCTCC	For cloning of complete sequences	This study
PVY3-8730	GGCATTCTCATTTTRGACGTRATAGC		
PVYNIb5-8600	ACAAGSAAATGACACAATYGATGC	For cloning of complete sequences	This study
PVY3end(3)	TTTTTTTTTTTTTTTGTCTCCTGATTG		

with one primer pair per reaction. The reaction mix for PCR was the same as for general PVY detection but without Sybr®Green. The PCR protocol was: 3 min at 96°C for initial denaturation and 35 cycles of 30 s at 96°C for denaturation, 30 s at 62°C for annealing and 1:40 min at 72°C for elongation followed by a final extension for 10 min at 72°C. PCR products were separated on 1% TAE-agarose gels (EDTA concentration reduced to 1 mM) with 0.1 µg ml^{-1} ethidium bromide.

Cloning, sequencing and sequence analysis of PVY isolates

cDNAs of two of the PVY-MV isolates that could not be assigned to any PVY strains were cloned as overlapping fragments of about 2 300 bp and completely sequenced. Only the 3'end of the genome was sequenced directly from the PCR fragment. The reaction protocol was identical to that described above for RT-PCR but using the cloning primers listed in Table 1. Bands of correct size were purified from gel (Ultrafree®-DA Gel Extraction Kit, Millipore, Carl Roth GmbH + Co. KG), ligated into pGEM®-T easy vector (Promega Cooperation) and transformed into NEB Turbo Competent *Escherichia coli* (New England BioLabs® Inc.). Clones containing the specific insert were sequenced on a

GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter®) using specific, M13 reverse and universal primers (GenomeLab™ DTCS-Quick Start Kit, Beckman Coulter®). In order to validate sequences at least two clones were sequenced in parallel. The Megalin program (Lasergene 8, DNA Star Inc.) was used with default settings to align complete genome sequences of the PVY-MV isolates with several other isolates representative of all PVY strains from GenBank®. Phylogenetic analysis was performed using MEGA 5.05 (Tamura et al. 2011). Alignments were analyzed for recombination using RDP software (Martin et al. 2010).

Results

Symptoms shown by potato plants and serological analysis

Potato plants showing symptoms in the field all gave positive results in ELISA (Table 2). Symptoms on potato plants varied depending on the cultivar ranging from a light mosaic to a pronounced rugose mosaic. Some cultivars, such as 'Opal', 'Donald', 'Zorba', 'Roberta', 'Sommergold', 'Krone' or 'Innovator', exclusively develop light mosaics which makes selection difficult. Only when climatic condi-

Table 2: Serological analysis of leaves and symptom expression of positive tested potato plants, post-control growing, Gülzow 2010

Cultivar (year of harvest)	OD-value (ELISA)	OD-value (ELISA) negative control	Symptomes
Krone (2009)	0.692–1.195	0,034	Mosaic, light
Meridian (2009)	1.325–2.156	0,034	Rugose mosaic, heavy
Innovator (2009)	1.973	0,032	Rugose mosaic, heavy
Sommergold (2008)	0.563–2.289	0,033	Mosaic, light
Patrona (2009)	1.610	0,032	Mosaic, light
Satina (2008)	1.236, 2.412	0,035	Rugose mosaic, heavy
Desirée (2009)	0.966, 2.296	0,032	Rugose mosaic, heavy
Cindy (2009)	2.396, 2.413	0,036	Rugose mosaic, heavy
Caruso (2009)	0.155–1.262	0,035	Rugose mosaic, heavy
Opal (2008)	2.506, 2.530, 2.571	0,033	Scarce mosaic, light
Ballerina (2009)	1.070–2.642	0,034	Rugose mosaic, heavy
Bernadette (2009)	0.109	0,033	Mosaic, light
Zorba (2008)	2.532–2.764	0,033	Mosaic, light
Karlena (2009)	1.525, 1.711	0,032	Rugose mosaic, heavy
Blaue St. Galler (2009)	1.847–2.195	0,034	Rugose mosaic, heavy
Marena (2009)	0.438–2.068	0,038	Rugose mosaic, heavy
Bellinda (2009)	0.155, 0.189	0,033	Rugose mosaic, heavy
Elkana (2009)	0.494–2.503	0,034	Rugose mosaic, heavy
Verdi (2009)	0.563–1.330	0,034	Mosaic, light
Roberta (2009)	1.385–2.602	0,033	Scarce mosaic, light
Kormoran (2009)	0.326–1.871	0,034	Rugose mosaic, heavy
Pirol (2008)	2.307	0,032	Rugose mosaic, heavy
Sava (2009)	0.308–2.449	0,032	Rugose mosaic, heavy
Royal (2009)	0.332–2.425	0,032	Mosaic, light
Donald (2009)	2.325, 2.343	0,032	Scarce mosaic, light

tions facilitate symptom expression as was the case in 2010, 'Innovator' shows strong mosaic patterns.

General detection of PVY using RT-qPCR

The sensitivity of the assay was determined by testing potato plants infected with different strains of PVY under equal conditions using RT-qPCR (Fig. 7). It is clearly shown that the differences of virus concentration vary between potato lines but not between the strains. This indicates an equal efficacy of the assay for different strains of PVY. Primers with mismatches in the sequence to some strains of PVY result in different efficacies for the strains of PVY (Fig. 8) and were therefore rejected. The limit of detection of the qPCR assay was 100 PVY RNA copies in 2 μl cDNA reaction mix with an error probability of 5%. Therefore, results showing less than 100 copies were classified as negative. Comparative results for PVY testing are given in Table 3. Altogether, 86 out of the 184 tubers tested were positive for PVY when analyzed using RT-qPCR. When analyzed using ELISA and leaf samples from field potato plants, 95 samples tested positive. The difference in number of positives for leaves and tubers is because PVY is not evenly distributed in all tubers of a plant (Rusetsky & Blotskaya 2001). Furthermore, virus concentration in tubers is often lower than in leaf material (Kogovšek et al. 2011). Comparison of the results for direct testing of tubers using ELISA and skin scrapings from tubers using RT-qPCR reveals no advantage of a PCR based method over ELISA. When comparing tubers of cultivars tested using both ELISA and RT-qPCR, 67 tubers were tested positive for PVY using ELISA and 69 tubers were shown to be positive using RT-qPCR. Virus concentration determined by RT-qPCR varied greatly between samples originating from one cultivar (Fig. 3). At first glance it appears that it is not possible to draw any conclusions about the susceptibility to PVY of the studied cultivars using this method. However, only 18 or fewer tubers of

Table 3: Results of the comparison of using ELISA and RT-qPCR to detect PVY in leaves and tubers.

Potato cultivar	Total number of tested samples	ELISA		qPCR	Strain specific PCR[c]		
		Leaves[a]	Tubers[b]	Tubers[c]	PVY[NTN]	PVY[N]Wi	unknown
Krone	7	3	3	3	–	3	–
Meridian	8	4	4	4	–	4	–
Innovator	7	3	n.t.	4	3	1	–
Sommergold	6	3	n.t.	3	3	–	–
Patrona	6	3	1	2	–	1	1
Satina	4	1	0	0	–	–	–
Desirée	4	2	1	2	1	–	1
Cindy	4	2	2	1	1	–	–
Caruso	6	3	2	2	–	2	–
Opal	6	2	1	1	–	–	1
Ballerina	13	7	7	7	3	4	–
Bernadette	2	1	n.t.	0	–	–	–
Zorba	6	3	2	3	3	–	–
Karlena	8	4	3	4	–	1	3
Blaue St. Galler	7	3	n.t.	4	1	3	–
Marena	12	5	5	5	–	2	3
Bellinda	4	2	n.t.	1	1	–	–
Elkana	14	10	9	11	7	5 (1 mixed)	–
Verdi	2	1	n.t.	1	1	–	–
Roberta	8	4	4	4	–	2	2
Kormoran	12	6	6	6	–	6	–
Pirol	10	6	2 (4 n.t.)	4	4	–	–
Sava	8	2	3	2	–	2	–
Royal	18	14	13	11	–	1	10
Donald	2	1	1	1	1	1 (mixed)	–
Sum	184	95	69 (16 n.t.)	86	30	38	21

[a] Leaves collected from field plants in June 2010

[b] Direct tuber testing using ELISA from field plants tested 8 weaks after harvesting on 28th October 2010

[c] Tubers from field plants tested after storage in April 2011

n.t. – not tested

each cultivar were tested using RT-qPCR. If a higher number of tubers had been tested then it would have been possible to carry out a statistical analysis that might have shown some differences.

Strain specific detection of PVY using conventional PCR

Amplifications were performed in parallel reactions with strain specific and PVYall primers to monitor RNA quality. In addition to all samples that tested positive using RT-qPCR, samples of healthy tubers, non-template controls and positive controls for each strain were tested also by using each primer pair. In this way, sample contamination was excluded and the accuracy of the RT-PCR reaction validated. During the primer optimization process strain specific primers published by Schubert et al. (2007) gave a clear single band for PVY^O, PVY^N and PVY^{NA}. A unique product was also amplified using the new specific PVY^{NTN} primers (Fig. 4A). For the detection of PVY^{N}Wi two primer pairs were used. A positive signal with the second primer pair excludes the presence of a second RJ (Fig. 4B-C; Fig. 1). The combination of both primer pairs guarantees a specific detection of most of PVY^{N}Wi isolates without the need for special amplification enzymes. The presence of PVY^O in the sample can be analyzed by a parallel testing of all samples with PVY^O specific primers. Compared to other PVY^{N}Wi isolates the isolates 156 and Syr-NB-16 exhibit two further RJs N:O between positions 5 715 and 8 560 and between positions 5 838 and 8 604, respectively. Therefore, no PCR product is amplified with the second PVY^O strain specific PVY^{N}Wi primer pair. However, the use of PVY^{NTN} specific primers also does not result in any amplification product since the PVY^{NTN}-specific forward primer is based on a PVY^N-type sequence and anneals only at position 8.635, which in both isolates is within the PVY^O-type segment.

In the samples where only the first PVY^{N}Wi primer pair gave a positive signal the isolates turned out to be PVY^{NTN}. This was verified with PVY^{NTN} specific primers. Thus, it can be excluded that among samples tested there are PVY^{N}Wi strains with genome structures like isolates 156 or Syr-NB-16, or novel PVY molecular genotypes such as isolate NE-11 (Lorenzen et al. 2008) or strain PVY^E (Galvino-Costa et al. 2012). These genotypes cannot be detected using the specific primers mentioned above. However, among the PVY^{NTN} isolates detected the presence of PVY^Z cannot be excluded since it is not molecularly distinguishable from PVY^{NTN}. As shown in Table 3, in all the 86 samples that tested positive, PVY^{NTN} was detected 30 times and PVY^{N}Wilga 38 times. Strains of PVY^O, PVY^N or PVY^{NA} were not detected. However, a considerable number of PVY isolates could not be assigned to any PVY strain. These samples were tested a second time for the presence of above mentioned strains, and a third time with PVY^C primers. None of the PCR reactions resulted in amplification products. Conspicuously, isolates that could not be assigned to any strain were more frequent in the cultivars 'Marena' and 'Royal'. Therefore, two isolates found in these cultivars were completely sequenced.
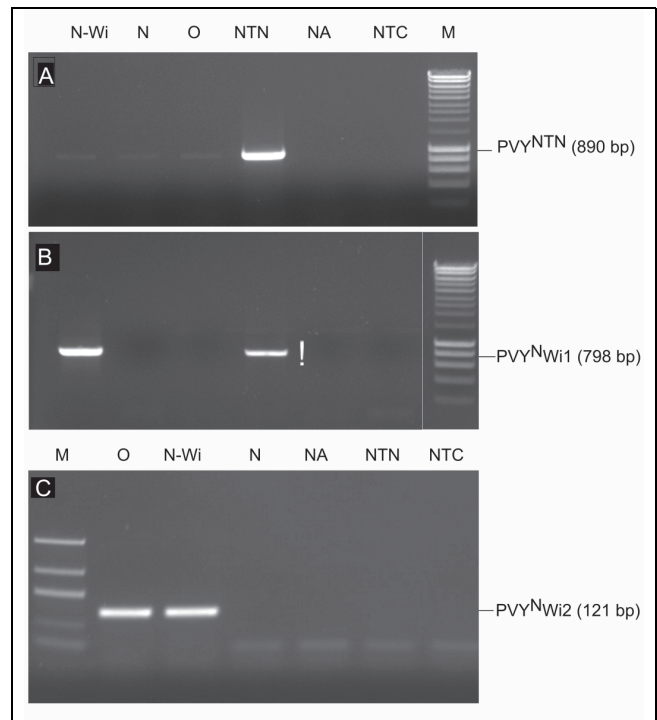
Fig. 4: RT-PCR of different PVY isolates (N-Wi = PVY^{N}Wi, N = PVY^N, O = PVY^O, NTN = PVY^{NTN}, NA = PVY^{NA}, NTC = non template control). A. Strain specific primers for the detection of PVY^{NTN} (product: 890 bp). B. Strain specific primers for the detection of PVY^{N}Wi (first product: 798 bp). Primers also amplify PVY^{NTN} (!). C. Strain specific primers for the detection of PVY^{N}Wi (second product: 121 bp). M = DNA Marker A, B: HyperLadderTM I (Bioline GmbH), DNA Marker C: 5 bands from 50 to 500 bp (self-produced).

Sequence analysis of PVY isolates

In this study some isolates could not be detected by strain specific primers. Therefore, the sequencing of two of these isolates should reveal the reason for this failure. The isolates found in cultivars 'Marena' and 'Royal' were named MV99 and MV175 and their sequences have the GenBank® acession numbers HE608963 and HE608964, respectively.

Phylogenetic analysis of complete sequences revealed that both, MV99 and MV175, cluster together with PVY^{N}Wi isolates of previously identified recombination types LW, 261-4 and Isol5 (Fig. 5). The difference between both these clusters is a recombination event O:N at nucleotide position ~500. Both clusters lack a second introgression of a PVY^N-strain sequence in the region of VPg/NIa-NIb characteristic of several isolates described from Germany and Syria (Chikh Ali et al. 2007, Schubert et al. 2007).

The result of a recombination analysis with RDP4 is shown in Fig. 6. Several full length sequences with a similar recombination structure are included. It illustrates that the parental sequence of PVY^O differs from the PVY^O-specific part of PVY^{N}Wi isolates. However, these differences are only marginal as shown in Table 4. The highest difference is 1.9% (SCRI_O, MV175, LW, Isol5) compared to 18% for a PVY^N
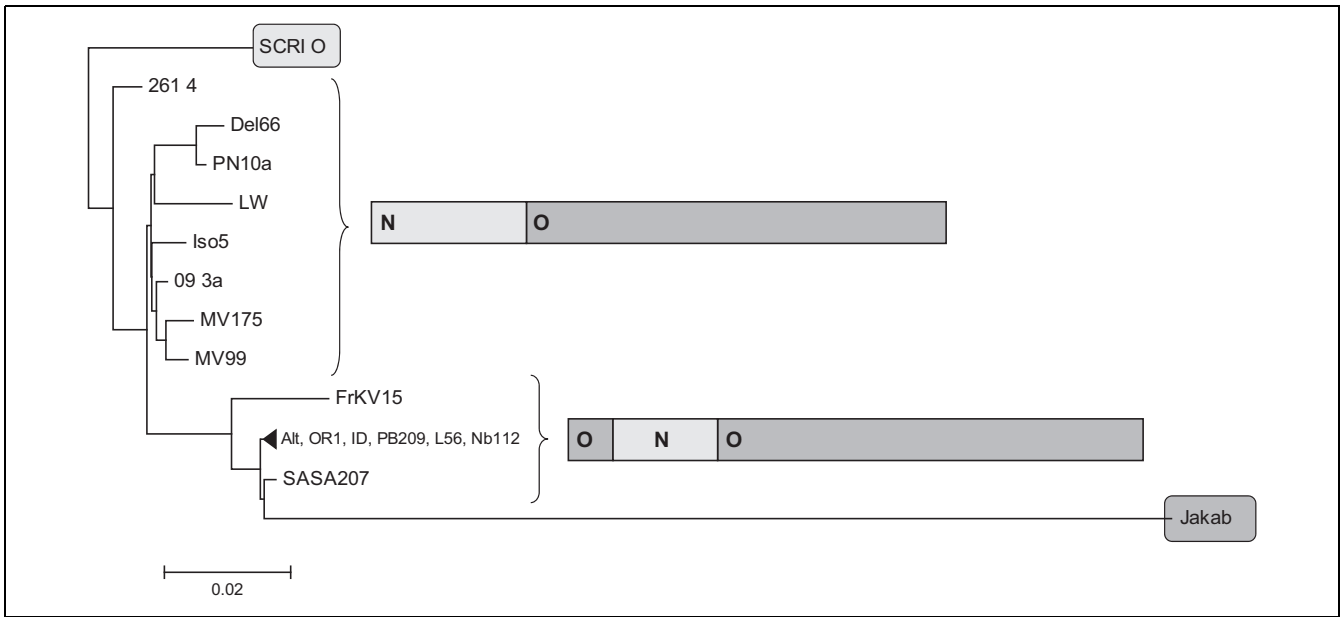
Fig. 5:   Molecular phylogenetic analysis of 18 structurally related PVY^{N}Wi-isolates (lacking the O:N:O recombination in the 3'-region of the sequence) by maximum likelihood (ML) method. The evolutionary history was inferred by using the ML method based on the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (-22686.6274) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branches of six closely related sequences have been collapsed. All positions containing gaps and missing data were eliminated. Molecular pattern of both recombinant clusters is shown on the right hand side.
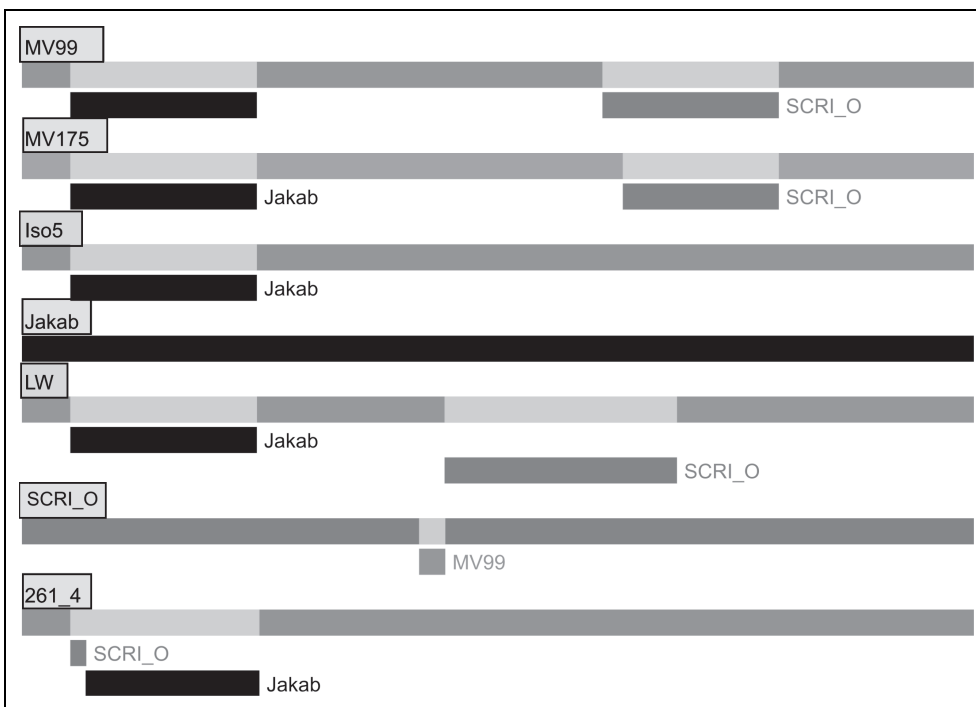
Fig. 6:   Illustration of recombination sites for isolates MV99 and MV175 detected by RDP3.
Parental isolates were the PVY^O strain, isolate SCRI-O (AJ585196) and the PVY^N strain, isolate CH605/Jakab (X97895). The structurally similar isolates 261-4 (AM11 3988), Isol5 (AJ890350) and LW (AJ890359) were included for comparison. A windows size of 100, step size of 10 and replicate number for bootstraps of 100 was chosen. Random seed number was 3, cutoff percentage 70 and highest p-Value 0.1. Designation of different isolates boxed in black.

strain (CH605←→LW). At the beginning of this century the severe infections in Central Germany were due to isolates of type 261-4 (Schubert, unpublished). None of the virulent isolates MV99, MV175 and 261-4 have either the Asn25Ile or Glu68Lys mutation (Moury & Simon 2011) in their coat protein amino acid sequence. These mutations are thought

to influence the concentration of virus in potato or change efficiency of aphid transmission.

Since there are only minor molecular differences between MV99, MV175 and previously sequenced PVY^{N}Wi isolates, it is difficult to account for why specific PVY^{N}Wi primers did not result in any PCR product although RNA quality and
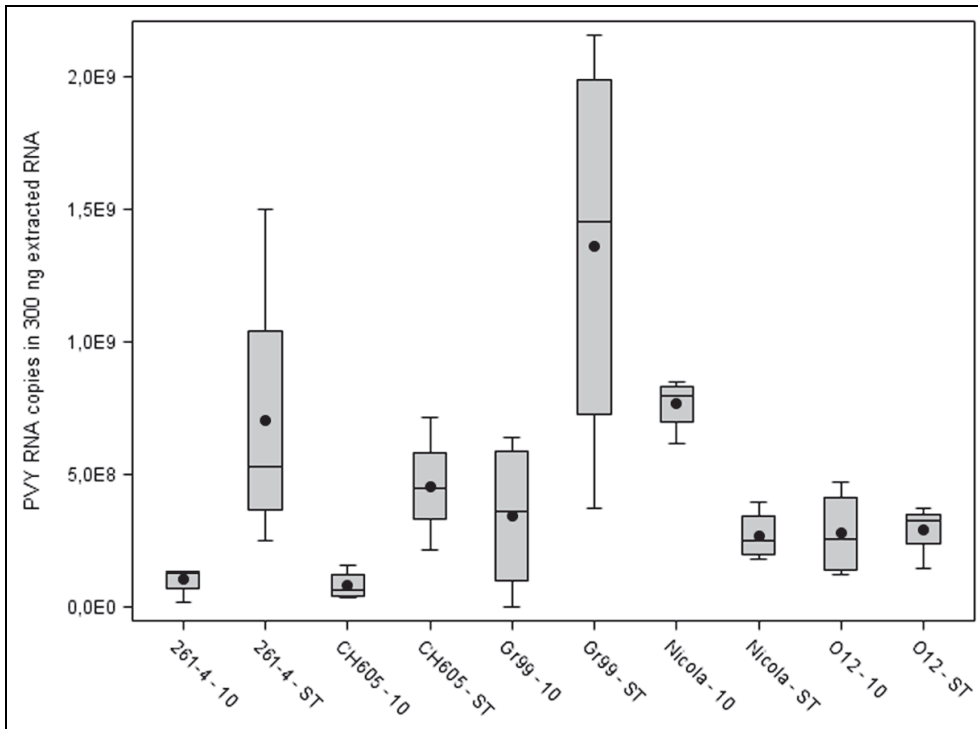
Fig. 7: Box-and-whisker plot of accumulation of PVY RNA copies of five PVY isolates in two different potato lines ("10" and "ST") susceptible to PVY (n = 4). RT-qPCR was done with primers showing no mismatches in the sequence to all currently known strains of PVY. 261-4 = isolate of PVY^{N}Wi, CH605 = isolate of PVY^{N}, Gr99 = isolate of PVY^{NTN}, Nicola = isolate of PVY^{NA-NTN} and O12 = isolate of PVY^{O}. Black dots indicate means.
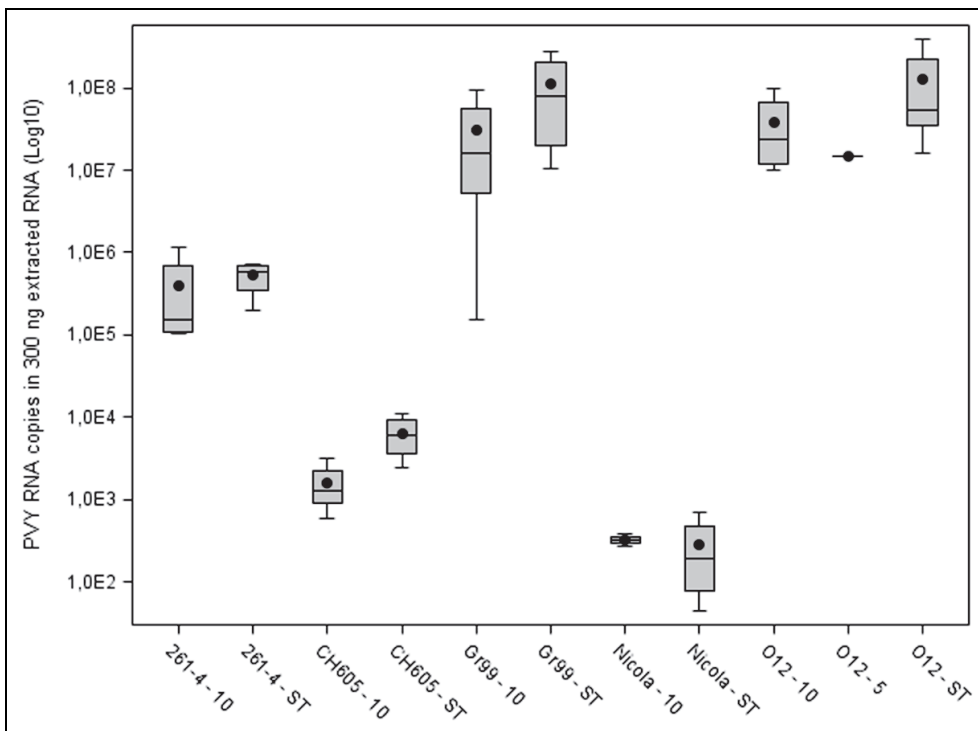
Fig. 8: Box-and-whisker plot of accumulation of PVY RNA copies of five PVY isolates in two different potato lines ("10" and "ST") susceptible to PVY (n = 4). RT-qPCR was done with primers showing mismatches in the sequence to PVY^{N} and PVY$^{NA/NTN}$ isolates of PVY. 261-4 = isolate of PVY^{N}Wi, CH605 = isolate of PVY^{N}, Gr99 = isolate of PVY^{NTN}, Nicola = isolate of PVY^{NA-NTN} and O12 = isolate of PVY^{O}. Black dots indicate means.

accuracy of the RT-PCR assay were validated. This phenomenon is discussed below.

Discussion

The increase of PVY infections detected during field inspections and certification of seed potato over the last 30 years worldwide has resulted in a large number of extensive studies that attempt to clarify possible reasons for the phenomenon. It is considered that the basic cause is a shift in the isolates towards recombinant forms of PVY such as PVY^{N}Wi and PVY^{NTN} (Singh et al. 2003, Lindner & Billenkamp 2005, Yin et al. 2012). It is suggested that the positive selection for the recombinant isolates is due to a milder symptom expression especially of the PVY^{N}Wi isolates, which results in more false negative scorings during seed potato field inspections (Kerlan 2004). However, in this

Table 4: Similarity/divergence of sequences of the 3'-part (from nt 2 430) of several isolates of PVY^{N}Wi, cluster Isol5.

SCRI_O	261-4	CH605	Isol5	LW	MV99	MV175	***
***	98.3	84.5	98.2	98.1	98.3	98.2	SCRI_O
1.7	***	84.8	99.0	98.3	99.0	98.9	261-4
17.9	17.5	***	84.8	84.5	84.8	84.6	CH605*
1.9	1.0	17.6	***	98.1	98.9	98.8	Isol5
1.9	1.7	18.0	1.9	***	98.2	98.1	LW
1.8	1.0	17.6	1.1	1.8	***	99.3	MV99
1.9	1.1	17.8	1.2	1.9	0.7	***	MV175

* – N-strain

study samples infected with PVY^{N}Wi often showed severe mosaic symptoms. Symptom expression is heavily dependent on cultivar and climatic conditions, which favored PVY symptoms in the year 2010 in M-WP. Whitworth et al. (2012) demonstrated that in some cultivars especially PVY^{N}Wi is unevenly distributed within tubers which could cause lower detection rates. However, in this study cultivars infected exclusively with PVY^{N}Wi (e. g. 'Krone'; 'Meridian' and 'Kormoran') did not show any differences in the number of positive leaf and tuber samples which leads us to the assumption that in our study the detection rate of PVY^{N}Wi was not reduced.

In this study it was demonstrated that direct tuber testing using RT-qPCR has no advantage over ELISA direct tuber testing for seed potatoes. However, ELISA from tubers was done 8 weeks after harvest when dormancy was not yet broken. RT-qPCR was done 33 weeks after harvest using dormant potato tubers. Fox et al. (2005) demonstrated that the reliability of ELISA from tuber samples drastically decreases when tubers start sprouting. Therefore, we assume that using non-dormant tuber samples RT-qPCR is more reliable than ELISA. Furthermore, direct tuber testing is faster and less space-consuming than growing-on tests and therefore represents a cheaper method for detecting PVY, especially since no greenhouse is needed. However, the use of single tubers can lead to a lower reliability of the test, since not all tubers of an infected plant carry the virus (Fox et al. 2005). This phenomenon is clearly shown by our results (Table 2). When comparing samples tested using ELISA from leaves and tubers as well as samples tested using RT-qPCR from tuber skin scrapings, the number of PVY positive tuber samples often was lower than the number of positive leaf samples. RT-qPCR is a very sensitive detection method, and bulking of samples would prevent the problem of uneven distribution of PVY in plant organs. False positives in this study produced by the RT-qPCR method could be caused by tuber storage, when PVY carrying tubers lay next to healthy tubers. Primer dimers as cause for false positives can be excluded since fluorescence was measured at high temperatures, when dimers were melted. Although the distribution of PVY within tubers hardly differs between heel and rose end, little differences of quantities around the LOD can lead to false positives and false negatives, respectively. False negatives cannot be prevented, completely, since the LOD of the RT-qPCR assay is higher than one virus copy. Furthermore, in this study a comparison is only possible with the ELISA methods, which can also produce false positives and false negatives in the range of optical density (OD) threshold. Diligence during sample storage and preparation avoiding contamination, as well as the right sampling time before dormancy braking, can reduce the problem of false positives and false negatives.

In this study it was shown that PVY^{N}Wi and PVY^{NTN} isolates were detected in samples of potato tubers harvested from inspection fields in M-WP. Since the strain specific primers used did not detect some PVY isolates in tubers, two of these isolates were sequenced and compared with all currently published sequences of PVY isolates. Typical genome organizations of PVY strains are presented in Fig. 1. Since Schubert et al. (2007) determined 3 PVY^{NTN} and 5 PVY^{N}Wi recombination variants, the number of variants of PVY^{NTN} sequenced has increased. Currently, 7 different variants of PVY^{NTN} have been characterized. N Nysa (accession number FJ666337) has a shorter recombination fragment with the 3rd RJ closer to the 3'-end. The PVY^N-type segment between the second and third RJ of isolate SD1 (accession number EU182576) is even shorter with a length of only 811 bp. Instead, it has a region between nucleotides 6145 and 7095 with mixed PVY^O- and PVY^N-type sequences. NE-11 (accession number DQ157180) is characterized as a PVY^N isolate causing PVY^{NTN}-like symptoms. It is intermediate between PVY^N and PVY^{NA-N} isolates. Sections from NE-11 can be found in PVY-MON (accession number FJ204165), which is an isolate of strain PVY^E. Therefore, recombinants can be parents for further recombinant genotypes (Galvino-Costa et al. 2012). The genome of isolate L26 displays typical features of PVY^{NTN}-type isolates with 3 RJs and was classified as PVY^{NTN} by Hu et al. (2009). However, due to a single nucleotide change at nucleotide 1627 (A to G), resulting in a single amino acid change (D to G) in the HC-Pro, this isolate does not cause vein necrosis in tobacco. Therefore, it was later assigned to PVY^Z by Kerlan et al. (2011).

The results of the sequence analysis showed that the two sequenced isolates are nearly identical to previously sequenced PVY^{N}Wi isolates 261-4 and Isol5. PVY^{N}Wi-isolates Isol5 and 261-4 are highly virulent under field conditions. Isol5 overcame transgenic PVY resistance (Schubert

et al. 2004) and 261-4 caused heavy infections of seed potato in fields in Saxony-Anhalt, Germany, in 2003 (Schubert, unpublished). Since Isol5, 261-4 and MV99 and MV175 are homologous, the heavy virus infections recorded in some cultivars in the years 2008 and 2009 in M-WP could be due to their higher transmission rates due to the higher virus concentrations in plants. Comparison of virus concentration of 261-4 with that of other common strains in potato (Fig. 7) revealed that recombinant N:O strains do not reach higher concentrations than the parental N- or O-strain or the recombinant NA-NTN strain. An explanation of the reasons for the high virulence in given PVY[N]Wi isolates cannot be presented.

Our strain specific primers for PVY[N]Wi did not detect isolates of type MV99 and MV175. The reason for that is not clear, especially, since the highly homologous isolate 261-4 was utilized for PCR optimization and shows exact matches in primer regions with isolates MV99 and MV179. Conspicuously, PVY isolates that could not be classified using the strain specific assay were found especially in cultivars 'Marena' and 'Royal'. One can speculate that some components of the solution of RNA extracted from tubers had an inhibiting effect on the strain specific PCR assay but not on the non-specific assay. 'Royal' and 'Marena' are cultivars used for processing and have a high starch content. The optical density (OD) ratio 260/230 of extracted RNA was rather low for these two cultivars (OD 260/230 < 1.0) which among others is an indication of contamination with polysaccharides. A further phenol purification step during the RNA isolation or the design of other PVY[N]Wi or PVY[NTN] primers may resolve this problem. However, for seed potato certification it is not important to be able to differentiate between the different strains of PVY. Therefore, it is essential to develop primers that match with as many PVY isolates as possible. The primers we designed for the general detection of PVY are, based on alignments, compatible with at least 60 different PVY isolates. Even novel PVY molecular genotypes, such as isolate NE-11 or strain PVY[E], did not show mismatches between the primer and respective gene sequences. Recent publications about the quantitative detection of PVY described assays with primers showing one or more mismatches in the sequence of some strains of PVY (Agindotan et al. 2007, Kogovšek et al. 2008). By using such primers (MMPVYall5-9194 and MMPVYall3-9420, see table 1) for RT-qPCR no equal efficacy over all isolates can be guaranteed (compare Fig. 7 and Fig. 8).

In 2008 in M-WP especially the cultivars, 'Innovator', 'Elkana', 'Zorba' and 'Donald' were seriously affected by rejection of seed potato lots. In this study we did not find a reason for this incident. Indeed, 'Innovator', 'Zorba' and 'Donald' normally develop mild symptoms when infected with recombinant PVY isolates, which might have been an indication for positive selection. Furthermore, 'Elkana' showed a heavy rugose mosaic, which refutes the hypothesis of high rejection rates due to a positive selection because of mild symptoms. The seriously affected cultivars were infected with recombinant isolates as were the other cultivars, and no new strain of PVY was found. Therefore, in the future it is necessary to observe symptom expression under diverse climatic conditions and test seed potato lots randomly, regardless of symptom development. Only large numbers of tested plants guarantee a low rejection rate of seed potato lots in the following year. With our developed assay for direct tuber testing using RT-qPCR a high throughput can be obtained with low costs since no greenhouse is necessary as it is for growing-on tests. Further experiments have to be done, evaluating bulking of tubers for a higher reliability of the test.

In previous experiments in which a limited number of tubers were used (Hühnlein, unpublished) we showed that PVY can be detected even in freshly harvested tubers using the more sensitive RT-qPCR method. A comparison of ELISA and RT-qPCR using freshly harvested tubers needs to be done. Furthermore, it was also possible to detect PLRV using the same total RNA extracts and the RT-qPCR assay with PLRV specific primers. If further primers can be designed for the detection of other potato viruses such as *Potato virus* X, *Potato virus* M or *Potato virus* S, the RT-qPCR method could be used for the official certification analysis of seed potatoes. The detection system used in this study is based on using low priced enzymes, which drastically reduces the costs of the test.

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References

Agindotan BO, Shiel PJ & Berger PH, 2007. Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan® real-time RT-PCR. J Virol Meth 142, 1-9.

Blanchard A, Rolland M, Lacroix C, Kerlan C & Jacquot E, 2008. *Potato virus* Y: a century of evolution. Curr Top Virol 7, 21-32.

Chikh Ali M, Maoka T & Natsuaki KT, 2007. The occurrence and characterization of new recombinant isolates of PVY displaying shared properties of PVY[N]W and PVY[NTN]. J Phytopathol 155, 409-415.

Chrzanowska M, 1991. New isolates of the necrotic strain of *Potato virus* Y (PVY[N]) found recently in Poland. Potato Res 34, 179-182.

Chung BY-W, Miller WA, Atkins JF & Firth AE, 2008. An overlapping essential gene in the *Potyviridae*. Proc Natl Acad Sci 105, 5897-5902.

Fox A, Evans F & Browning I, 2005. Direct tuber testing for *Potato virus* Y potyvirus by real-time RT-PCR and ELISA: reliable options for post-harvest testing? EPPO Bulletin 35, 93-97.

Galvino-Costa SBF, Figueira AdR, Camargos VV, Geraldino PS, Hu X-J, Nikolaeva OV, Kerlan C & Karasev AV, 2012. A novel type of *Potato virus* Y recombinant genome, determined for the genetic strain PVY[E]. Plant Pathol 61, 388-398.

Gugerli P, 1979. Le test immuno-enzymatique (ELISA) et son application pour le diagnostic rapide des viroses de la pomme de terre. Reveue Swiss Agric 11, 253-260.

Hu X, Meacham T, Ewing L, Gray S & Karasev AV, 2009. A novel recombinant strain of *Potato virus* Y suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Res 143, 68-76.

Kerlan C, 2004. Evolution in *Potato virus* Y: from recombination in the genome to emergence and spreading of variants. In: Giblot-Ducray D (Ed.), EAPR Virology 2004, Abstracts of the 12th European Association for Potato Research Virology Section Meeting, Rennes, France, 13−19 June 2004, Wageningen, Netherlands, European Association for Potato Research 23-27.

Kerlan C, Nikolaeva OV, Hu X, Meacham T, Gray SM & Karasev AV, 2011. Identification of the Molecular Make-Up of the *Potato virus* Y Strain PVY(Z): Genetic Typing of PVY(Z)-NTN. Phytopathology 101, 1052-1060.

Kogovšek P, Gow L, Pompe-Novak M, Gruden K, Foster GD, Boonham N & Ravnikar A, 2008. Single-step RT real-time PCR for sensitive detection and discrimination of *Potato virus* Y isolates. J Virol Meth 149, 1-11.

Kogovšek P, Kladnik A, Mlakar J, Tušek Žnidarič M, Dermastia M, Ravnikar M & Pompe-Novak M, 2011. Distribution of *Potato virus* Y in Potato plant organs, tissues, and cells. Phytopathology 101, 1292-1300.

Lindner K & Billenkamp N, 2005. Veränderungen im Stammspektrum des Kartoffelvirus Y: Eine Ursache für die Zunahme der Virusanfälligkeit von Kartoffel- und Tabaksorten? Journal für Kulturpflanzen 57, 245-353.

Lorenzen J, Nolte P, Martin D, Pasche JS & Gudmestad NC, 2008. NE-11 represents a new strain variant class of *Potato virus* Y. Arch Virol 153, 517-525.

Martin DP, Lemey P, Lott M, Moulton V, Posada D & Lefeuvre P, 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26, 2462-2463.

Moury B & Simon V, 2011. dN/dS-based methods detect positive selection linked to trade-offs between different fitness traits in the coat protein of *Potato virus* Y. Mol Biol Evol 28, 2707-2717.

Rusetsky NV & Blotskaya ZV, 2001. Distribution and accumulation of PVM and PVY, PVX in infected potato plants. Arch Phytopathol Plant Protect 34, 275-282.

Schubert J, Matousek J & Mattern D, 2004. Pathogen-derived resistance in potato to *Potato virus* Y − aspects of stability and biosafety under field conditions. Virus Res 100, 41-50.

Schubert J, Fomitcheva V & Sztangret-Wiśniewska J, 2007. Differentiation of *Potato virus* Y strains using improved sets of diagnostic PCR-primers. J Virol Meth 140, 66-74.

Singh RP, McLaren DL, Nie X & Singh M, 2003. Possible escape of a recombinant isolate of *Potato virus* Y by serological indexing and methods of its detection. Plant Dis 87, 679-685.

Tamura K & Nei M, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial-DNA in humans and chimpanzees. Mol Biol Evol 10, 512-526.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

Thieme T, Hoffmann U & Heimbach U, 2009. Efficacy of plant protection substances against virus transmission by aphids infesting potato. Journal für Kulturpflanzen 61, 21-30.

Treder K, Przewodowski W & Barnyk A, 2009. Factors influencing detection of *Potato leafroll virus* and *Potato virus* Y in potato tuber extracts. Plant Breeding and Seed Science 59, 65-74.

Valkonen JP, 2007. Viruses: Economical losses and biotechnological potential. In: Vreugdenhil D, Bradshaw J, Gebhardt C, Govers F, McKarren DKL, Taylor MA & Ross HA (Eds.). Potato biology and biotechnology − Advances and perspectives, Amsterdam, Elsevier 619-641.

Visser JC & Bellstedt DU, 2009. An assessment of molecular variability and recombination patterns in South African isolates of *Potato virus* Y. Arch Virol 154, 1891-1900.

Whitworth JL, Hamm PB & Nolte P, 2012. Distribution of *Potato virus* Y strains in tubers during the post-harvest period. Am J Potato Res 89, 136-141.

Yin Z, Chrzanowska M, Michalak K, Zagórska H & Zimnoch-Guzowska E, 2012. Recombinants of PVY strains predominate among isolates from potato crop in Poland. Journal of Plant Protection Research 52, 214-219.

Zahn V, 2004. Altered occurrence of viruses. Results of potato virus testing by LWK Hannover. Kartoffelbau 55, 394-397.

5.2 **The use of RT-qPCR to evaluate the resistance of *Solanum* species and progenies of somatic hybrids to *Potato virus Y***

The use of RT-qPCR to evaluate the resistance of *Solanum* species and progenies of somatic hybrids to *Potato virus Y*

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Abstract

Extreme resistance (ER) to *Potato virus Y* (PVY) in wild species of potato is a valuable trait. The introgression of *Ry* genes into cultivated potato via crossing, transgenesis or somatic hybridization leads to potato plants that remain symptomless and almost free of virus after inoculation with PVY. However, ER is not only recorded for species with *Ry* genes, such as *S. stoloniferum* and *S. chacoense*, but also in wild potatoes for which the mechanism and the location of resistance genes on the chromosomes is currently unknown. To identify ER in these plants, mechanical inoculation or grafting trials are conducted following the verification of the absence of PVY using ELISA. However, recently very sensitive detection methods were developed that can detect extremely low amounts of virus, which make it more difficult to discriminate between susceptible and resistant genotypes. In this study the accumulation of PVY was determined in different species of wild potato and progenies of somatic hybrids using RT-qPCR. The numbers of PVY RNA copies detected in the different genotypes only differed at a very low level. There was slightly more virus RNA in an accession of *S. etuberosum*, previously defined as extremely resistant using ELISA, than in the other genotypes. Therefore, the quantification of PVY RNA using RT-qPCR may be a good way of evaluating resistance in breeding programs. However, since in theory a single copy of a virus can be found using this method, it is necessary to define a threshold for standardized conditions, up to which a plant is defined as resistant. Furthermore, several intermediate stages of susceptibility could be defined based on the ranking of potato cultivars, as for instance, used by the Federal Plant Variety Office in Germany.

Introduction

Potato virus Y (PVY) is one of the most damaging potato viruses, particularly in non-developed countries, where seed certification measures are not yet introduced (Valkonen 2007). Species of wild potato, especially those with an endosperm balance number (EBN) of 1, are a valuable source of resistance to PVY (Bradeen and Kole 2011). Some of these species that carry a *Ry* gene are extremely resistant to PVY, which means that plants that are inoculated with PVY do not develop any symptoms and the virus titer in their leaves remains extremely low (Valkonen et al. 1996; Hämäläinen et al. 1998). A further advantage is the monogenic nature of *Ry*-derived resistance, which can be easily inherited. *Ry* genes are described and mapped for *Solanum tuberosum* ssp. *andigena* on chromosome XI (Hämäläinen et al. 1998), *S. stoloniferum* on chromosome XII (Flis et al. 2005; Song et al. 2005), *S. chacoense* on chromosome IX (Sato et al. 2006), *S. phureja* on chromosome IX (Al-Abedy et al. 2012; Torrance et al. 2012) and *S. tarnii* on chromosomes III, V and XII (Janine König 2016, JKI, Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany; personal communication). However, crosses of 1EBN species, such as *S. cardiophyllum, S. etuberosum, S. pinnatisectum or S. tarnii* with cultivated *S. tuberosum* (4EBN) are incompatible because of endosperm failure (Johnston and Hanneman 1980). Endosperm failure results in a collapse of the endosperm causing the embryo to degenerate (Dinu et al. 2005). Crossing barriers can be overcome using several methods, including ploidy and EBN manipulations (Watanabe 2015), using bridging species (Jansky 2006) or embryo rescue (Singsit and Hanneman 1991). However, the crossing of distant EBN species often results in odd ploidies that hamper further breeding (Watanabe 2015). With the aid of transgenesis sexual reproduction can be circumvented and single genes can be introduced into the potato genome.

Another method that overcomes all sexual barriers is the symmetric somatic hybridization via protoplast fusion, because no meiotic segregation is required (Fish et al. 1988). With the aid of this technique the transfer of both, mono- and polygenic traits between sexually incompatible species is possible (Thieme et al. 2008). In the past, protoplast fusion was used primarily to fuse 1EBN and 2EBN species in order to utilize the fusion products as bridge species for crosses with cultivated potato. The introgression of PVY resistance is achieved by somatic hybridization, e. g., of *S. etuberosum* and S. *berthaultii* (Novy et al. 2002), *S. brevidens* and dihaploid *S. tuberosum* (Valkonen et al. 1994) or *S. etuberosum* and dihaploid *S. tuberosum* (Gavrilenko et al. 2003; Tiwari et al. 2010). In order to shorten the breeding

process, protoplast fusion is also used for 1EBN species of wild potato and the 4EBN cultivated potato. *S. tarnii* protoplasts are fused with protoplasts of the cv. Delikat. The somatic hybrids (SHs) and their fertile first backcross generation (BC$_1$) are very resistant to PVY and late blight (Thieme et al. 2008). Resistance genes can be detected using molecular markers, such as RYSC3 for the detection of *Ry*$_{adg}$ (Kasai et al. 2000), YES3-3A/B for the detection of *Ry-f*$_{sto}$ (Song and Schwarzfischer 2008) or the RAPD marker 38–530 for the detection of *Ry*$_{chc}$ (Sato et al. 2006). However, for several other PVY resistant species of wild potato there is no physiological or molecular information on resistance genes. Their type of resistance is assessed by mechanical inoculation, grafting and field trials (Lindner et al. 2011). For example, *S. etuberosum* is categorized as extremely resistant by Gavrilenko et al. (2003) and Thieme et al. (2004), since no virus was detected by ELISA after mechanical inoculation or grafting.

Quantitative reverse transcription real time PCR (RT-qPCR) is a very sensitive method for detecting and quantifying the virus titer in plant tissues (Rizza et al. 2009; Debreczeni et al. 2011; Ferriol et al. 2011; Angel Herrera-Vasquez et al. 2015). Dependent on the antibodies used in the ELISA, RT-qPCR can be several hundred times more sensitive than ELISA (Kogovsek et al. 2008; Gawande et al. 2011; De Francesco et al. 2015). As a consequence, very low virus titers can be detected and quantified by RT-qPCR. Extreme resistance is defined as the absence of symptoms combined with extremely low amounts of virus in plants grafted on infected rootstocks (Valkonen et al. 1996). Potato plants that are tolerant to a PVY infection remain symptomless, but can contain considerable amounts of virus. Based on these two definitions, the question arises where the threshold is between resistant and susceptible/tolerant potato plants in terms of their virus titer after inoculation with PVY.

In this study we determined the number of PVY RNA copies in resistant and susceptible accessions of wild species of potato and the progenies of somatic hybrids between *Solanum* species and potato cultivars generated by protoplast fusion. The advantages of using RT-qPCR to quantify the amount of PVY in plant tissue in order to define the relationship between resistance and virus titer is discussed.

Material and methods

Plant material

Genbank accessions of species of wild potato and progenies of SHs and their resistance to PVY are listed in Tab. 1. This material was provided by the JKI, Institute for Breeding Research on Agricultural Crops, Groß Lüsewitz, Germany. *S. tuberosum* cv. Hermes was used

as the rootstock and PVY-donor in grafting experiments. All plants were maintained as *in vitro* plants on MS5 medium (Murashige and Skoog 1962) at 20 °C and 16 h illumination.

Tab.1: Description of wild potato species and progenies of somatic hybrids used in the grafting experiments

Solanum species/ Fusion combination	Ploidy/ EBN	Genebank Accession/ Generation and Genotype No.	Code	Resistance to PVY	Reference for PVY tests
S. cardiophyllum Lindl.	2n=2x, 1	GLKS 30108	*cph*	ER	Thieme et al. 2010
S. etuberosum Lindl.	2n=2x, 1	VIR k-9141	*etb*	ER	Gavrilenko et al. 2003
S. pinnatisectum Dun.	2n=2x, 1	GLKS 31607	*pnt*	ER	Thieme et al. 2009
S. stoloniferum Schltdl. et Bouché	2n=4x, 2	GLKS 30071	*sto*	ER	Cockerham 1943
S. tarnii Hawkes et Hjerting	2n=2x, nd (1)	GLKS 32870	*trn*	ER	Thieme and Thieme 1998, Thieme et al. 2008
S. tarnii Hawkes et Hjerting	2n=2x, nd (1)	GLKS 35385	*trn* (S)	S	Nachtigall 2010 unpublished
cph (+) cv. Agave	2n=nd	BC1 1186/5/2	BC$_1$ *cph* (+) Agave	ER	Thieme et al. 2010
etb (+) clone T67	2n=4x, nd	BC1 39/1	BC$_1$ *etb* (+) T67	nd	Thieme 2000 unpublished
etb (+) clone T67	2n=4x, nd	BC3 39/1/2/40	BC$_3$ *etb* (+) T67	nd	Thieme 2000 unpublished
pnt (+) cv. Rasant	2n=5x, nd	BC1 2044/1/2	BC$_1$ *pnt* (+) Rasant clone 1	ER	Thieme et al. 2009
pnt (+) cv. Rasant	2n=5x, nd	BC1 2044/2/5	BC$_1$ *pnt* (+) Rasant clone 2	ER	Thieme et al. 2009
trn (+) cv. Delikat	2n=5x, nd	BC1 7/53	BC$_1$ *trn* (+) Delikat	ER	Nachtigall 2011 unpublished
trn (+) cv. Delikat	2n<4x, nd	BC2 7/53/3	BC$_2$ *trn* (+) Delikat	ER	Nachtigall 2011 unpublished
trn (+) cv. Delikat	2n<4x, nd	BC3 7/53/3/23	BC$_3$ *trn* (+) Delikat	ER	Nachtigall 2011 unpublished

GLKS – Gross Lüsewitz Potato Collection of the IPK Gene Bank, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany; VIR – Vavilov Institute of Plant Industry, St. Petersburg, Russia; ER – extreme resistant; S – susceptible; BC – back-cross generation; nd – not determined

Grafting experiments

In vitro plantlets of *S. tuberosum* cv. Hermes, progenies of SHs and species of wild potato were transferred to substrate-filled pots. Four days after acclimation, plants of cv. Hermes were mechanically inoculated either with PVY[N-Wi] (isolate 261-4, accession number AM113988) or PVY[NTN] (isolate Gr99, accession number AJ890343). For the mechanical inoculation of these plants, 2 g of PVY infected leaves of *N. tabacum* cv. Samsun NN were ground in 10 ml of 0.1 M Na-phosphate buffer, pH 8. Afterwards, the tobacco buffer mixture was rubbed onto silicon carbide powdered plantlets. The plants were rinsed with tap water 10 min after inoculation. Two weeks after inoculation the plants were tested for PVY infection using DAS-ELISA and IgG polyclonal antibodies (JKI, Institute for Epidemiology and Pathogen Diagnostics, Quedlinburg, Germany). 98% of the plants of cv. Hermes were successfully infected with PVY. At least 40 plants of each progeny of SHs and of each species of wild potato, respectively, were grafted onto PVY infected rootstocks of cv. Hermes using grafting clips (Bato Plastics B.V., Zevenbergen, The Netherlands) (Fig. 1). Three weeks after grafting, samples were taken from the scions and rootstocks. By using the lid of a 1.5 ml centrifuge tube, three leaf discs per scion and rootstock, respectively, were punched out. These samples were immediately frozen in liquid nitrogen.

RNA extraction and quantitative reverse transcription real-time PCR (RT-qPCR)

Each sample was homogenized in an 1.5 ml microcentrifuge tube containing 500 µl of RNA isolation reagent (Concert[TM] Plant RNA Reagent, Life Technologies[TM]) and three 2.5 mm stainless steel beads using a Retsch[®] mixer mill (Retsch GmbH, Haan, Germany). Further purification of the RNA was done following the manufacturer's protocol. The purified precipitated RNA was re-dissolved in diethylpyrocarbonate (DEPC)-treated distilled water to a concentration of 200 to 400 ng per µl. The total RNA amount was measured in terms of ultraviolet absorbance using a NanoDrop 8000 instrument (Thermo Fisher Scientific). For the quantification of PVY RNA, a previously described RT-qPCR assay was used (Hühnlein et al. 2013; Hühnlein et al. 2016a). RNA standards were prepared consisting of a dilution series of PVY RNA transcripts. The sequence of the transcripts correspond to PVY isolate Gr99 from nucleotide position 8600, which is a highly conserved region of the coat protein, to the poly-A tail. Reverse transcription was performed using 2 µl purified plant RNA and 18 µl RT mixture (DEPC-treated water; 1 x RT reaction buffer consisting of 50mM Tris-HCl [pH 8.3], 75mM KCl, 3mM $MgCl_2$ and 10mM DTT; 200 µM of each dNTP; 0.4 µM reverse primer [PVYall3-9420, primer sequence: CGGAGAGACACTACATCAC (Hühnlein et al. 2013)]; 80 U M-MLV RT [Promega] and 4 U RNase inhibitor [RiboLockTM, Thermo Fisher Scientific]). The RT reaction was done in duplicate without denaturation at 42 °C for 50 minutes following

10 minutes at 72 °C for enzyme inactivation. In a second step 2 µl of cDNA reaction mixture was combined with 18 µl qPCR reaction mixture (distilled water; 1 x reaction buffer consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 and 0.1% Tween 20; 150 µM of each dNTP; 0.3 µM of forward and reverse primers [PVYall5-9194, primer sequence: AGGTCACATCACGAACAC (Hühnlein et al. 2013) and PVYall3-9420]; 2.5 mM $MgCl_2$; 2 U Taq DNA polymerase [Bioron GmbH] and 1 x Sybr®Green I [Life Technologies™]). PCR reaction was carried out in white 96-well plates (Biozym Scientific GmbH) sealed with adhesive clear seals (Thermo Scientific ABsolute™ QPCR Seal) in a MyiQ™2 Two-Colour Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the following protocol: 2 min at 95 °C for initial denaturation followed by 40 cycles of 15 s at 94 °C for denaturation, 15 s at 58 °C for annealing, 20 s at 72 °C for elongation and 15 s at 85 °C for melting of non-specific products. Fluorescence was measured after each cycle. Following real-time PCR, the amplicons were melted at 95 °C for 1 min and then fully reannealed at 55 °C for 1 min. For determining the melting point(s) of the PCR product(s) the temperature was then increased incrementally to 95°C at a rate of 0.5°C every 10 s (as fixed on the MyiQ™2). Melting of amplicons generated with PVY primers happened at 87.5 °C whereas the low abundant primer dimers melted at 82 °C.

Statistics

There were duplicates of the cDNAs of the samples and standards. Standards were used only for the first 96-well plate. An inter-plate calibrator (IPC) consisting of a diluted PCR product generated using PVY amplification primers was used for normalization and variation compensation of all plates. To calculate absolute quantities from amplification data, the software GenEx Enterprise 5 (MultiD Analyses AB, Sweden) was used for reverse calibration. Results and qPCR efficiency determination were calculated based on a 95% confidence level. The average efficiency of the qPCR assay was 99.5% and the lower and upper confidence limits of efficiency were 95% and 102%, respectively. The estimated efficiency for all qPCR plates was corrected to100% using GenEx software. Since 2 µl of plant RNA was used for RT, the absolute quantities of PVY RNA could be calculated for 300 ng of extracted plant RNA (Fig. 3). Confidence intervals (CI) were calculated from the standard deviation of means and sample size. The CIs were adjusted to 87%, resulting in error bars in a graph that touch at a *p*-value of 0.05 (Krzywinski and Altman 2013). A Pearson correlation coefficient and its *p*-value were calculated from the absolute quantities of PVY RNA copies in the susceptible *S. tarnii* (S) genotype and in the respective rootstock of cv. Hermes.

Results

Symptoms of the grafted scions

The success of grafting was equal for most of the wild species of potato and also for progenies of SHs. Between 75% and 80% of the scions developed a vascular connection with the rootstock. However, scions of *S. cardiophyllum* often failed to develop, which decreased the percentage of successful grafts to only 45% for this genotype. This was due to the fragile habit of *S. cardiophyllum* plants, whose thin shoots could only be attached to rootstocks with difficulty (Fig. 1k). Chlorosis or mottling of young leaves, which are typical symptoms of a PVY infection, were not detected in wild species of potato, progenies of SHs or in the susceptible accession *S. tarnii* GLKS 35385 (S) three weeks after grafting. The scions of the wild potato *S. pinnatisectum*, described as extremely resistant to PVY (Thieme et al. 2009), had a great number of necrotic lesions, which is typical of a HR (Fig. 1o). However, this was only the case for scions inoculated with isolate 261-4 and not recorded in progenies of SHs between *S. pinnatisectum* and cv. Rasant.
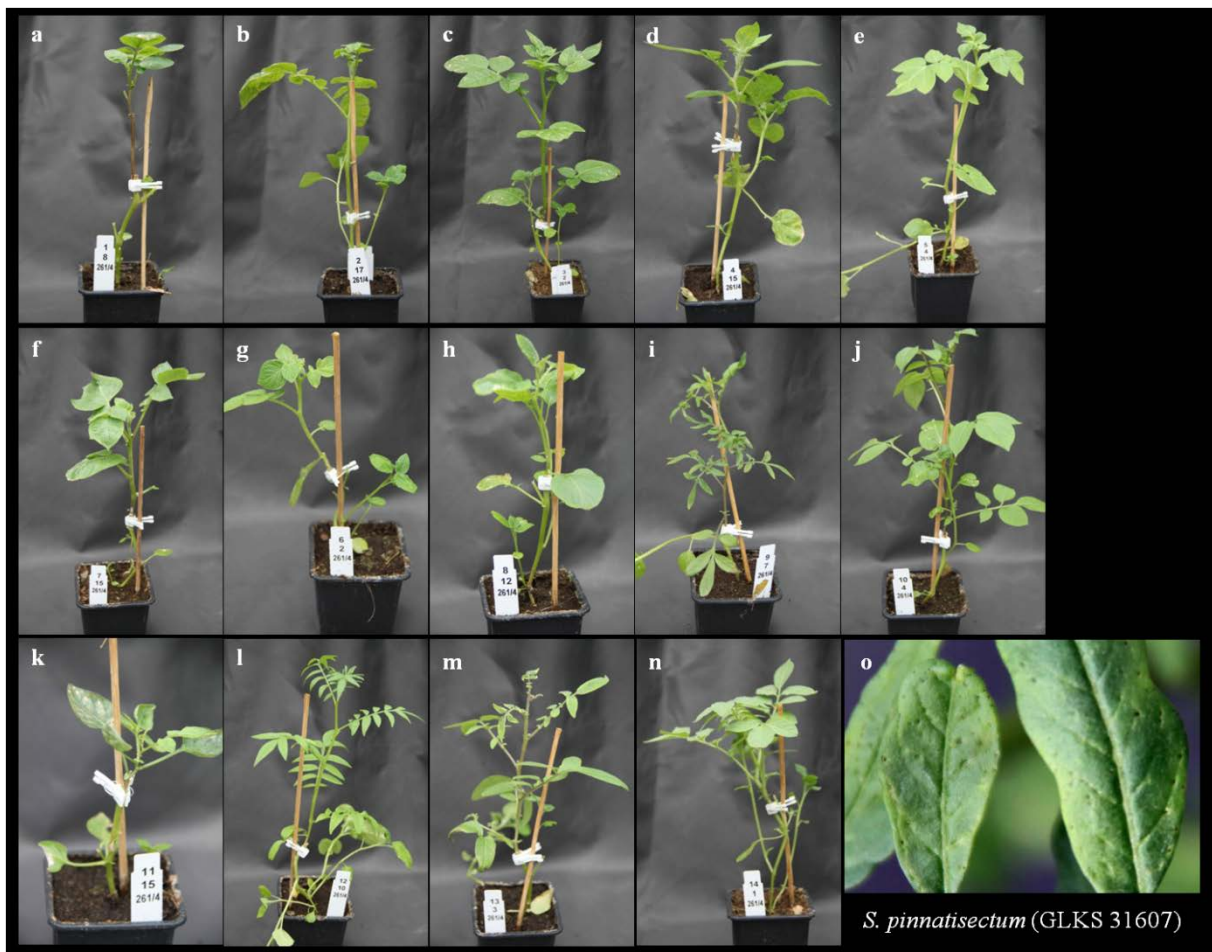
Fig. 1: Species of wild potato and progenies of somatic hybrids grafted onto PVY^{N-Wi} (isolate 261-4) infected rootstocks of cv. Hermes. Scions were BC$_1$ of *cph* (+) cv. Agave (**a**), BC$_1$ of *pnt* (+) cv. Rasant, clone 1 (**b**), BC$_1$ of *pnt* (+) cv. Rasant, clone 2 (**c**), BC$_1$ of *trn* (+) cv. Delikat (**d**), BC$_2$ of *trn* (+) cv. Delikat (**e**), BC$_3$ of *trn* (+) cv. Delikat (**f**), BC$_1$ of *etb* (+) T67 (**g**), BC$_3$ of *etb* (+) T67 (**h**), *S. tarnii* (S)(**i**), *S. stoloniferum* (**j**), *S. cardiophyllum* (**k**), *S. pinnatisectum* (**l**), *S. tarnii* (**m**), *S. etuberosum*

R (**n**). The pictures were taken three weeks after grafting. *S. pinnatisectum* developed several necrotic lesions on the leaves (**o**).

Number of PVY RNA copies in the scions

The PVY primers PVYall5-9194 and PVYall3-9420 matched a 100% the sequences of PVY isolates 261-4 and Gr99, with which the rootstocks of cv. Hermes were inoculated. In order to amplify the standard and samples with equal efficiencies, the standard contained the recombinant RNA of the coat protein sequence of isolate Gr99. The RT-qPCR efficiency of the standard PVY RNA was 99.5% (Fig. 2). However, standard quantities below 100 PVY RNA copies were not in the linear range of the standard curve. Therefore, the limit of quantification (LOQ) was set to 100 PVY RNA copies. All quantities detected below this limit are not reliable.
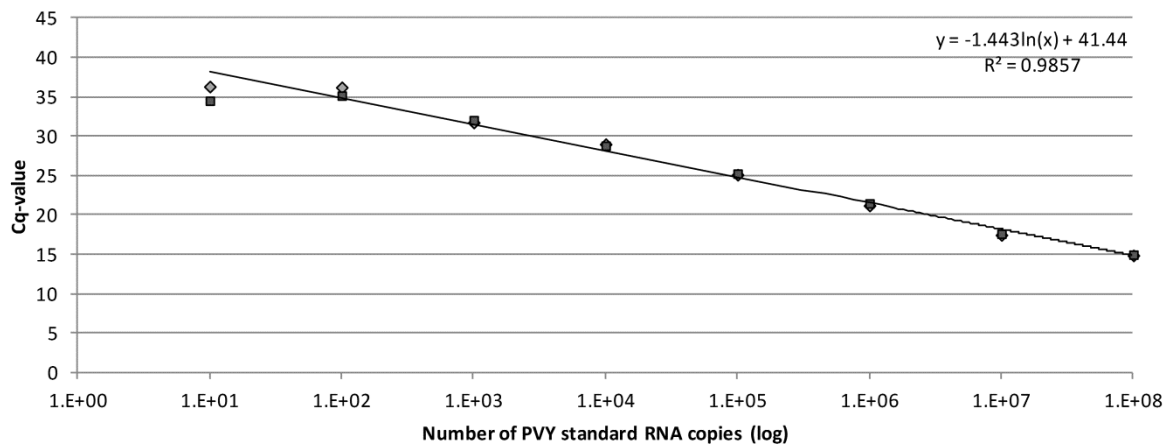
Fig. 2: The Cq-value plotted against log quantity of PVY standard RNA copies. The linear range for the standard consists of a 10 fold dilution series from 10^2 to 10^8 PVY RNA copies. Recombinant RNA comes from nt 8600 to the polyA-tail of PVY^{NTN} isolate Gr99 (accession number AJ890343). The RT-qPCR efficiency is 99.5%.

All plants that were infected with isolate Gr99 had higher numbers of PVY RNA copies than plants infected with isolate 261-4, regardless of whether the scion or rootstock was sampled (Fig. 3). Therefore, the numbers of PVY RNA copies of isolate Gr99 are all within the reliable range of quantification. According to the European Cultivated Potato Database (ECPD 2015), cv. Hermes is highly resistant to PVY. Nevertheless, nearly all plants were infected by mechanical inoculation. The virus titer of those plants with 300 ng of extracted RNA, was at a medium level, compared to highly susceptible cultivars (results not shown) and ranged between 110,000 and 420,000 PVY RNA copies (Gr99) and 14,000 and 230,000 PVY RNA copies (261-4). The number of PVY RNA copies detected in the susceptible accession of *S. tarnii* (S) was similar to those detected in plants of cv. Hermes indicating a similar level of susceptibility. Moreover, the number of PVY RNA copies in the scions of *S.*

tarnii (S) were significantly correlated with the PVY copy numbers in their respective rootstocks with a Pearson correlation coefficient of 0.73 and a *p*-value of <0.01.

Among the scions of other accessions and the progenies of SHs, either no or only very low virus titers were detected with dissimilar results for the two PVY isolates (Fig. 3). For example, more than 700 PVY RNA copies were detected in clone 2 of BC_1 *pnt* (+) cv. Rasant inoculated with isolate Gr99, but no virus was detected in those scions inoculated with isolate 261-4. Conspicuously, scions of *S. etuberosum*, on average, were always infected, regardless of whether this species was used as an accession from the VIR gene bank or as progeny from a somatic hybridization. Moreover, although not significant, the quantity of virus recorded was second highest after that detected in the susceptible *S. tarnii* (S). Furthermore, the percentage of PVY-infected scions originating from *S. etuberosum* was almost always higher than that of scions from other accessions (see crossed black boxes in Fig. 3a,b). No virus was detected in the wild species of potato *S. pinnatisectum*, which indicates an efficient restriction of the PVY infection. However, no local lesions were visible in the BC progenies and low amounts of PVY (Gr99) were recorded in the BC_2.
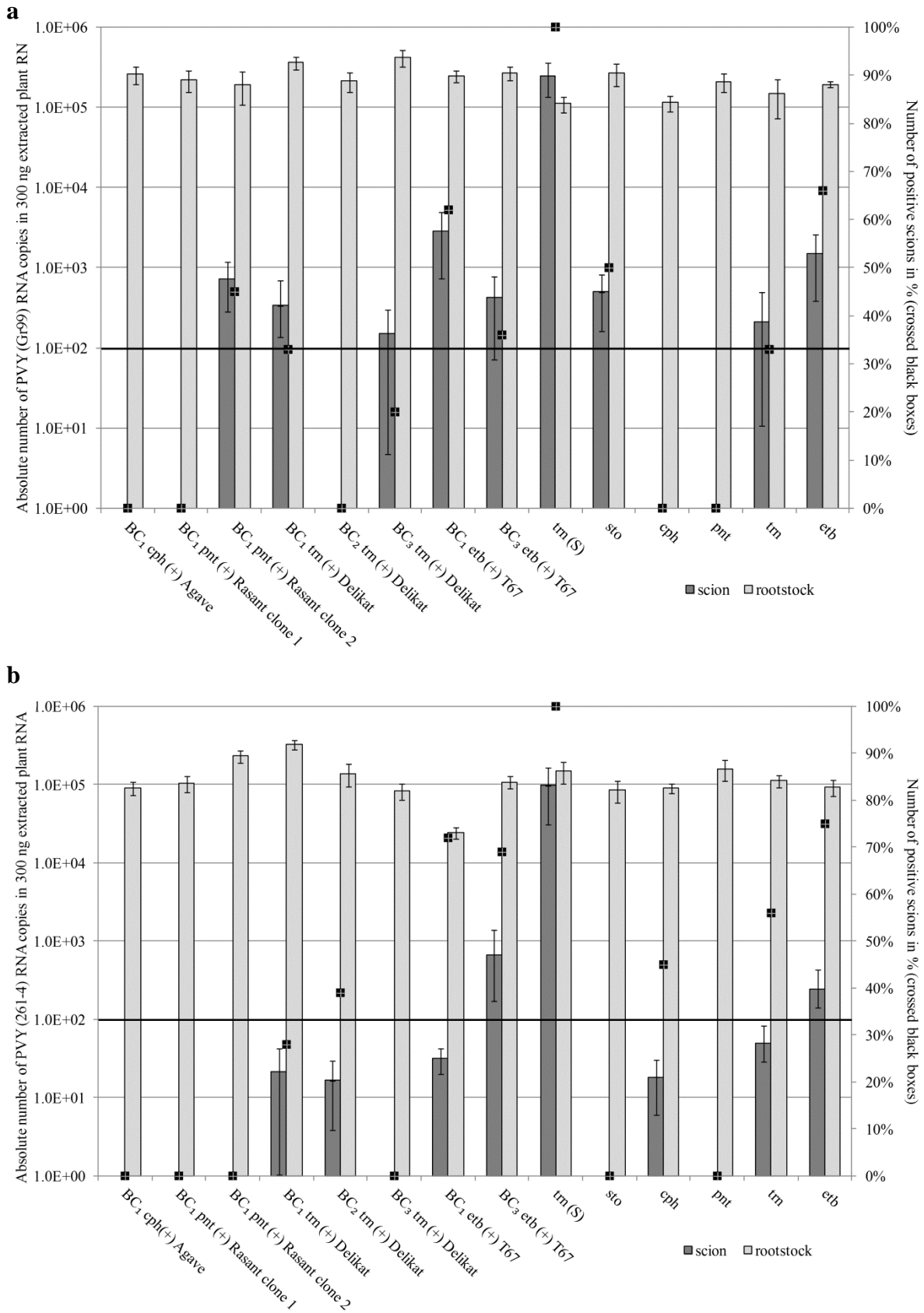
Fig. 3: Mean quantities of PVY RNA copies detected in 300 ng of plant RNA extracted from scions that were inoculated either with isolate Gr99 (**a**) or isolate 261-4 (**b**). Error indicators on each bar are calculated from confidence intervals of 87%, for which the bars touch at *p*=0.05 (Krzywinski and Altman 2013). The black horizontal line emphasizes the limit of quantification (LOQ).

Discussion

In this study the accumulation of PVY was determined in different species of wild potato and progenies of SHs between species of wild potato and *S. tuberosum* cultivars. With one exception, all clones were previously categorized as extremely resistant. After grafting the clones onto PVY-infected rootstocks their virus titer was determined using RT-qPCR. Although not significant, the wild potato *S. etuberosum* and the progenies of SHs between *S. etuberosum* and a haploid *S. tuberosum* contained higher numbers of PVY RNA copies and the percentage of infected plants was mostly higher than in the other plants tested. This indicates there are more resistance genes in *S. etuberosum* than in the other species of wild potato tested, which accumulated either less or no virus RNA. Novy and Helgeson (1994) assessment of the resistance to PVY^O of SHs of *S. etuberosum* and *S. tuberosum* x *S. berthaultii* hybrids found none that were as resistant as the *S. etuberosum* parent. They conclude that the expression of resistance by *S. etuberosum* may not always be dominant. However, Gavrilenko et al. (2003) report that the frequency of the alleles that originated either from *S. etuberosum* (E-genome) or the susceptible hybrid parent (A-genome), may have an effect on the expression of virus resistance, resulting in the extreme resistance of hexaploid fusion hybrids with AAEEEE genomes and the susceptibility of hybrids with AAAAEE genomes. Recently, it was shown that the resistance to PVY in *S. etuberosum* originates from a novel variant of the eukariotic initiation factor eE-1 (eIF4E-1), found and named *Eva1* by Duan et al. (2012), which does not bind to VPg, a viral protein required for infectivity. However, they assume the involvement of additional, as yet unknown, factors in PVY resistance in *S. etuberosum*, because the common eIF4E-1 of *S. tuberosum* (SteIF4E-1) is still expressed in this species of wild potato. To sum up, there is a need for further research on the mechanism of resistance in *S. etuberosum,* including that of the BC$_2$ and BC$_3$ hybrids, which will be useful for localizing the virus resistance genes on specific chromosomes (Gavrilenko et al. 2003). Based on our results we cannot confirm that the accession of *S. etuberosum* tested is extremely resistant to PVY.

 S. pinnatisectum is primarily known for its resistance to late blight caused by *Phytophthora infestans* (Polzerova et al. 2011). Resistance to PVY is described for *S. pinnatisectum*, accession number USDI PI253214, and takes the form of a hypersensitive response (HR) (Whitworth et al. 2009). HR is characterized by the development of necrotic lesions on inoculated leaves, which localizes the infection (Valkonen et al. 1996). Thieme et al. (2009) found the accession GLKS 1607 of *S. pinnatisectum* is extremely resistant to a PVY infection. In this study, the same accession produced obvious local lesions when inoculated with PVY^{N-Wi} isolate 261-4, indicating a HR. Thieme et al. (2009) mechanically inoculated test plants with PVY^{N-Wi} isolate LW (accession number AJ890349). However, the isolates LW and 261-

4 are very similar in their sequences with a divergence of only 1.7% (Hühnlein et al. 2013). Therefore, the differences in resistance recorded for *S. pinnatisectum* may result from different growing conditions or different inoculation methods. The virus pressure on graft-inoculated plants is greater and lasts longer. Therefore, local lesions may be more abundant on these plants. Furthermore, in contrast to mechanical sap-inoclulation, grafting of accessions of interest onto virus infected rootstocks (or *vice versa*) is a more reliable method of assessing virus resistance, because a null response to sap-inoculation could be attributable either to ER or to a failure of inoculation (Solomon-Blackburn and Barker 2001). Interestingly, local necrotic lesions were only recorded on the wild species *S. pinnatisectum* and not on the BC progenies of SHs between *pnt* (+) cv. Rasant. Possibly, the genes that are responsible for the HR were already outcrossed in the BC lines. Nevertheless, the virus was also successfully restricted in BC_1 (Fig. 3). Therefore, a further resistance mechanism seems to be active in *S. pinnatisectum*, which is inherited at least by BC_1. Since no virus was detected in this BC generation, *S. pinnatisectum* may also harbour ER gene(s). This would be in accordance with the results of Whitworth et al. (2009), who positively tested *S. pinnatisectum* using CAPS markers that are linked to the *Ry* gene of *S. tuberosum* ssp. *andigena* (Sorri et al. 1999). Therefore, *S. pinnatisectum* may harbour resistance genes for both HR and ER.

If no molecular markers are available, the (virus) resistance of a potato plant can be determined by using mechanical inoculation or grafting experiments. Previously, a plant was assessed to be extremely resistant, when the plant remained symptomless and no virus was detected using ELISA (Gavrilenko et al. 2003; Thieme et al. 2004; Lindner et al. 2011). However, recently several highly sensitive PCR-based detection methods were developed that are able to detect and quantify very low quantities of virus (Balaji et al. 2003; Rizza et al. 2009; Debreczeni et al. 2011; Ferriol et al. 2011; Zhou et al. 2014). Therefore, virus RNA can also be detected in plants that previously were categorized as extremely resistant. In a parallel experiment we determined the PVY titers in different susceptible and resistant potato cultivars using RT-qPCR and revealed amounts of virus ranging from zero to up to 80,000,000 PVY RNA copies in 300 ng of plant RNA, independent of the development of symptoms (results not shown). Therefore, the severity of symptoms should not be used for assessing resistance, especially as the appearance of symptoms is dependent on environmental conditions (Balachandran et al. 1997; Hühnlein et al. 2016b).

Balaji et al. (2003) and Ferriol et al. (2011) suggest the quantification of virus RNA in plants should be used for assessing resistance or tolerance in breeding programs. However, a threshold needs to be determined up to which a plant can be defined as resistant. Furthermore, since the range of virus titers in virus infected plants is wide, several intermediate stages

could be defined for each virus, based on the susceptibility ranking of potato cultivars by the Federal Plant Variety Office in Germany (2014), which ranges from highly susceptible (9) to highly resistant (1). However, since the virus titer in a plant is also dependent on environmental conditions, such evaluations in breeding programs need to be standardized.

One could suggest retaining ELISA tests for determining ER, because it is a threshold up to which a virus can be detected. This is not the case for RT-qPCR, for which in theory a single copy of a virus can be detected. However, by using ELISA some plants may be categorized as extremely resistant, although they are possibly not. This could lead to surprising results in potato breeding, when the desired resistance trait is suddenly lost in a back crossing step, because of its potential polygenic nature (Novy et al. 2007). Therefore, RT-qPCR may be a reliable alternative to ELISA tests, if it is used with defined thresholds determined under standardized conditions. Such a threshold could be based on cultivars whose ER genes have been identified and localized. *S. stoloniferum* is a typical representative of ER (Flis et al. 2005; Valkonen et al. 2008) and could serve as a threshold for ER. However, the threshold has to be determined for different PVY strains since, as shown in this study, virus accumulation differs between strains. Slight differences in viral accumulation between the genotypes were not significant in this study. Therefore, the threshold needs to be calculated based on a greater number of grafted plants than used in this study. Finally, resistance thresholds could also be utilized for genotypes with polygenic resistance in order to test BC generations for possible out crossing of quantitative trait loci (QTL), which would cause a quantitative decline in virus resistance detectable by an increase in the number of copies of RNA virus in plants.

Acknowledgements

References

Al-Abedy, A., Cowan, G., McLean, K., Bryan, G., MacFarlane, S., & Torrance, L. (2012). Genetic analysis of Potyvirus resistance in *Solanum tuberosum* group *Phureja*. *The Dundee Conference. Crop Protection in Northern Britain, 2012, Dundee, UK, 28-29 February 2012*, 291-295.

Angel Herrera-Vasquez, J., Rubio, L., Alfaro-Fernandez, A., Elvira Debreczeni, D., Font-San-Ambrosio, I., Falk, B. W., et al. (2015). Detection and absolute quantitation of *Tomato torrado virus* (ToTV) by real time RT-PCR. *Journal of Virological Methods, 221*, 90-94, doi:10.1016/j.jviromet.2015.04.029.

Balachandran, S., Hurry, V. M., Kelley, S. E., Osmond, C. B., Robinson, S. A., Rohozinski, J., et al. (1997). Concepts of plant biotic stress. Some insights into the stress physiology of virus-infected plants, from the perspective of photosynthesis. *Physiologia Plantarum, 100*(2), 203-213, doi:10.1034/j.1399-3054.1997.1000201.x.

Balaji, B., Bucholtz, D. B., & Anderson, J. M. (2003). *Barley yellow dwarf virus* and *Cereal yellow dwarf virus* quantification by real-time polymerase chain reaction in resistant and susceptible plants. *Phytopathology, 93*(11), 1386-1392, doi:10.1094/phyto.2003.93.11.1386.

Bradeen, J. M., & Kole, C. (2011). *Genetics, genomics and breeding of potato* (Genetics, genomics and breeding of crop plants). Enfield, New Hampshire, USA: Science Publishers.

De Francesco, A., Costa, N., Plata, M. I., & Garcia, M. L. (2015). Improved Detection of *Citrus psorosis virus* and Coat Protein-Derived Transgenes in Citrus Plants: Comparison Between RT-qPCR and TAS-ELISA. *Journal of Phytopathology, 163*(11-12), 915-925, doi:10.1111/jph.12392.

Debreczeni, D. E., Ruiz-Ruiz, S., Aramburu, J., Lopez, C., Belliure, B., Galipienso, L., et al. (2011). Detection, discrimination and absolute quantitation of *Tomato spotted wilt virus* isolates using real time RT-PCR with TaqMan (R) MGB probes. *Journal of Virological Methods, 176*(1-2), 32-37, doi:10.1016/j.jviromet.2011.05.027.

Dinu, II, Hayes, R. J., Kynast, R. G., Phillips, R. L., & Thill, C. A. (2005). Novel inter-series hybrids in *Solanum*, section Petota. *Theoretical and Applied Genetics, 110*(3), 403-415, doi:10.1007/s00122-004-1782-x.

Duan, H., Richael, C., & Rommens, C. M. (2012). Overexpression of the wild potato eIF4E-1 variant Eva1 elicits *Potato virus Y* resistance in plants silenced for native eIF4E-1. *Transgenic Res, 21*(5), 929-938, doi:10.1007/s11248-011-9576-9.

ECPD (2015). ECPD: The European Cultivated Potato Database. The European Cultivated Potato Database. http://www.europotato.org. Accessed 22nd June 2015.

Federal Plant Variety Office of Germany (2014). *Descriptive Variety Lists - Potato*. Hannover, Germany: Federal Plant Variety Office of Germany.

Ferriol, I., Ruiz-Ruiz, S., & Rubio, L. (2011). Detection and absolute quantitation of *Broad bean wilt virus* 1 (BBWV-1) and BBWV-2 by real time RT-PCR. *Journal of Virological Methods, 177*(2), 202-205, doi:10.1016/j.jviromet.2011.08.003.

Fish, N., Karp, A., & Jones, M. G. K. (1988). Production of somatic hybrids by electrofusion in *Solanum*. *Theoretical and Applied Genetics, 76*(2), 260-266.

Flis, B., Hermig, J., Strzelczyk-Zyta, D., Gebhardt, C., & Marezewski, W. (2005). The *Ry*-f(sto) gene from *Solanum stoloniferum* for extreme resistance to *Potato virus Y* maps to potato chromosome XII and is diagnosed by PCR marker GP122(718) in PVY resistant potato cultivars. *Molecular Breeding, 15*(1), 95-101.

Gavrilenko, T., Thieme, R., Heimbach, U., & Thieme, T. (2003). Fertile somatic hybrids of *Solanum etuberosum* (+) dihaploid *Solanum tuberosum* and their backcrossing

progenies: relationships of genome dosage with tuber development and resistance to *Potato virus Y. Euphytica, 131*(3), 323-332, doi:10.1023/a:1024041104170.

Gawande, S. J., Shukla, A., Chimote, V. P., Kaushal, N., Kaundal, P., Garg, I. D., et al. (2011). Development of PCR-based techniques for the detection of immobilised *Potato virus Y* virions. *Journal of Plant Pathology, 93*(1), 127-132.

Hämäläinen, J. H., Sorri, V. A., Watanabe, K. N., Gebhardt, C., & Valkonen, J. P. T. (1998). Molecular examination of a chromosome region that controls resistance to potato Y and A potyviruses in potato. *Theoretical and Applied Genetics, 96*(8), 1036-1043, doi:10.1007/s001220050836.

Hühnlein, A., Drechsler, N., Steinbach, P., Thieme, T., & Schubert, J. (2013). Comparison of three methods for the detection of *Potato virus Y* in seed potato certification. *Journal of Plant Diseases and Protection, 120*(2), 57-69.

Hühnlein, A., Schubert, J., & Thieme, T. (2016a). Evaluation of the substantial equivalence of genetically modified potatoes regarding their susceptibility to *Potato virus Y. unpublished.* (in preparation)

Hühnlein, A., Schubert, J., Zahn, V., & Thieme, T. (2016b). Examination of an isolate of *Potato leaf roll virus* that does not induce visible symptoms in the greenhouse. *European Journal of Plant Pathology*, doi:10.1007/s10658-016-0872-3.

Jansky, S. (2006). Overcoming hybridization barriers in potato. *Plant Breeding, 125*(1), 1-12, doi:10.1111/j.1439-0523.2006.01178.x.

Johnston, S. A., & Hanneman, R. E. (1980). Support of the endosperm balance number hypothesis utilizing some tuber-bearing *Solanum* species. *American Potato Journal, 57*(1), 7-14, doi:10.1007/bf02852750.

Kasai, K., Morikawa, Y., Sorri, V. A., Valkonen, J. P. T., Gebhardt, C., & Watanabe, K. N. (2000). Development of SCAR markers to the PVY resistance gene *Ry(adg)* based on a common feature of plant disease resistance genes. *Genome, 43*(1), 1-8, doi:DOI 10.1139/gen-43-1-1.

Kogovsek, P., Gow, L., Pompe-Novak, M., Gruden, K., Foster, G. D., Boonham, N., et al. (2008). Single-step RT real-time PCR for sensitive detection and discrimination of *Potato virus Y i*solates. *Journal of Virological Methods, 149*(1), 1-11, doi:10.1016/j.jviromet.2008.01.025.

Krzywinski, M., & Altman, N. (2013). Error bars. *Nature Methods, 10*(10), 921-922.

Lindner, K., Behn, A., Schwarzfischer, A., & Song, Y. (2011). Extreme resistance to *Potato Virus Y* in potatoes of the German variety list. *Journal für Kulturpflanzen, 63*(4), 97-103.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497, doi:10.1111/j.1399-3054.1962.tb08052.x.

Novy, R. G., Gillen, A. M., & Whitworth, J. L. (2007). Characterization of the expression and inheritance of *Potato leafroll virus* (PLRV) and *Potato virus Y* (PVY) resistance in three generations of germplasm derived from *Solanum etuberosum. Theoretical and Applied Genetics, 114*(7), 1161-1172, doi:10.1007/s00122-007-0508-2.

Novy, R. G., & Helgeson, J. P. (1994). Resistance to *Potato virus Y* in somatic hybrids between *Solanum etuberosum* and *S. tuberosum* x *S. berthaultii* hybrid. *Theoretical and Applied Genetics, 89*, 783-786.

Novy, R. G., Nasruddin, A., Ragsdale, D. W., & Radcliffe, E. B. (2002). Genetic Resistances to *Potato leafroll virus, Potato virus Y*, and green peach aphid in progeny of *Solanum etuberosum. American Journal of Potato Research, 79*(1), 9-18.

Polzerova, H., Patzak, J., & Greplova, M. (2011). Early characterization of somatic hybrids from symmetric protoplast electrofusion of *Solanum pinnatisectum* Dun. and *Solanum*

tuberosum L. *Plant Cell Tissue and Organ Culture, 104*(2), 163-170, doi:10.1007/s11240-010-9813-6.

Rizza, S., Nobile, G., Tessitori, M., Catara, A., & Conte, E. (2009). Real time RT-PCR assay for quantitative detection of Citrus viroid III in plant tissues. *Plant Pathology, 58*(1), 181-185, doi: 10.1111/j.1365-3059.2008.01941.x.

Sato, M., Nishikawa, K., Komura, K., & Hosaka, K. (2006). *Potato virus Y* resistance gene, *Ry(chc)*, mapped to the distal end of potato chromosome 9. *Euphytica, 149*(3), 367-372, doi:10.1007/s10681-006-9090-y.

Singsit, C., & Hanneman, R. E. (1991). Rescuing abortive inter-EBN potato hybrids through double pollination and embryo culture. *Plant Cell Reports, 9*(9), 475-478.

Solomon-Blackburn, R. M., & Barker, H. (2001). Breeding virus resistant potatoes *(Solanum tuberosum)*: a review of traditional and molecular approaches. *Heredity, 86*, 17-35, doi:10.1046/j.1365-2540.2001.00799.x.

Song, Y. S., Hepting, L., Schweizer, G., Hartl, L., Wenzel, G., & Schwarzfischer, A. (2005). Mapping of extreme resistance to PVY (*Ry(sto)*) on chromosome XII using anther-culture-derived primary dihaploid potato lines. *Theoretical and Applied Genetics, 111*(5), 879-887, doi:10.1007/s00122-005-0010-7.

Song, Y. S., & Schwarzfischer, A. (2008). Development of STS markers for selection of extreme resistance (*Ry (sto)*) to PVY and maternal pedigree analysis of extremely resistant cultivars *American Journal of Potato Research, 85*(5), 392-393, doi:10.1007/s12230-008-9044-0.

Sorri, V. A., Watanabe, K. N., & Valkonen, J. P. T. (1999). Predicted kinase-3a motif of a resistance gene analogue as a unique marker for virus resistance. *Theoretical and Applied Genetics, 99*(1-2), 164-170, doi:10.1007/s001220051221.

Thieme, R., Darsow, U., Rakosy-Tican, L., Kang, Z., Gavrilenko, T., Antonova, O., et al. (2004). Use of somatic hybridisation to transfer resistance to late blight and *Potato virus Y* (PVY) into cultivated potato. *Plant Breeding and Seed Science, 50*, 113-118.

Thieme, R., Rakosy-Tican, E., Gavrilenko, T., Antonova, O., Schubert, J., Nachtigall, M., et al. (2008). Novel somatic hybrids (*Solanum tuberosum* L. (+) *Solanum tarnii*) and their fertile BC$_1$ progenies express extreme resistance to *Potato virus Y* and late blight. *Theoretical and Applied Genetics, 116*(5), 691-700.

Thieme, R., Schubert, J., Nachtigall, M., Hammann, T., Truberg, B., Heimbach, U., et al. (2009). *Wild potato species of the series Pinnatisecta - progress in their utilisation in potato breeding* (Crop plant resistance to biotic and abiotic factors: current potential and future demands. Proceedings of the 3rd International Symposium on Plant Protection and Plant Health in Europe, Julius Kuhn-Institut, Berlin-Dahlem, Germany, 14-16 May 2009).

Tiwari, J. K., Poonam, Sarkar, D., Pandey, S. K., Gopal, J., & Kumar, S. R. (2010). Molecular and morphological characterization of somatic hybrids between *Solanum tuberosum* L. and *S. etuberosum* Lindl. *Plant Cell Tissue and Organ Culture, 103*(2), 175-187, doi:10.1007/s11240-010-9765-x.

Torrance, L., Cowan, G., McLean, K., Al-Abedy, A., MacFarlane, S., & Bryan, G. (2012). Novel broad-spectrum resistance to potato potyviruses. *Phytopathology, 102*(7), 120-120.

Valkonen, J. P. (2007). Viruses: Economical Losses and Biotechnological Potential In D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D. K. L. McKarren, M. A. Taylor, et al. (Eds.), *Potato Biology and Biotechnology - Advances and Perspectives* (pp. 619-641). Amsterdam: Elsevier.

Valkonen, J. P. T., Jones, R. A. C., Slack, S. A., & Watanabe, K. N. (1996). Resistance specificities to viruses in potato: Standardization of nomenclature. *Plant Breeding, 115*(6), 433-438, doi:10.1111/j.1439-0523.1996.tb00952.x.

Valkonen, J. P. T., Wiegmann, K., Hamalainen, J. H., Marczewski, W., & Watanabe, K. N. (2008). Evidence for utility of the same PCR-based markers for selection of extreme resistance to *Potato virus Y* controlled by *Ry(sto)* of *Solanum stoloniferum* derived from different sources. *Annals of Applied Biology, 152*(1), 121-130, doi: C:\Dokumente und Einstellungen\Anja.Huehnlein\Eigene Dateien\Literatur\PVY

Valkonen, J. P. T., Xu, Y. S., Rokka, V. M., Pulli, S., & Pehu, E. (1994). Transfer of resistance to *Potato leafroll virus*, *Potato virus Y* and *Potato virus X* from *Solanum brevidens* to *Solanum tuberosum* through symmetrical and designed asymmetric somatic hybridization. *Annals of Applied Biology, 124*(2), 351-362, doi:10.1111/j.1744-7348.1994.tb04139.x.

Watanabe, K. (2015). Potato genetics, genomics, and applications. *Breeding Science, 65*(1), 53-68, doi:10.1270/jsbbs.65.53.

Whitworth, J., Novy, R., Hall, D., Crosslin, J., & Brown, C. (2009). Characterization of broad spectrum *Potato Virus Y* resistance in a *Solanum tuberosum* ssp. *andigena*-derived population and select breeding clones using molecular markers, grafting, and field inoculations. *American Journal of Potato Research, 86*(4), 286-296, doi:10.1007/s12230-009-9082-2.

Zhou, T., Du, L., Lan, Y., Sun, F., Fan, Y., & Zhou, Y. (2014). Development of SYBR Green I-Based One-Step Real Time RT-PCR Assay for Quantifying Southern rice black-streaked dwarf virus in Rice. *Journal of Phytopathology, 162*(1), 26-32, doi:10.1111/jph.12152.

5.3 Examination of an isolate of *Potato leaf roll virus* that does not induce visible symptoms in the greenhouse

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Examination of an isolate of *Potato leaf roll virus* that does not induce visible symptoms in the greenhouse

Anja Hühnlein · Jörg Schubert · Volker Zahn · Thomas Thieme

Abstract Over the last 30 years the importance of *Potato leaf roll virus* (PLRV) in commercial potato and seed potato production has decreased considerably. Since PLRV is transmitted by aphids in a persistent manner it can be controlled by applying a systemic insecticide. However, the development of insecticide resistance in the main vectors of PLRV *Myzus persicae*, *Aulacorthum solani, Rhopalosiphoninus latysiphon, Aphis fabae, A. nasturtii, A. frangulae* and *Macrosiphum euphorbiae,* and the development of isolates of PLRV that do not induce visible symptoms in some potato cultivars may lead to a resurgence in the significance of PLRV. Isolates of this type were found repeatedly during growing-on tests in Lower Saxony, Germany. In this study we examined such a symptomless isolate. The visible symptoms induced by this isolate in different potato cultivars were compared with those induced by isolates causing typical symptoms of a PLRV infection. By using quantitative real-time PCR the quantifiable amount of viral RNA was determined. Under climate chamber conditions all the isolates tested induced similar symptoms and did not differ in viral RNA content. Complete sequences for the tested isolates were obtained and used in a phylogenetic analysis. All the PLRV isolates compared were very similar at the molecular level. Several motifs that could play a role in symptom expression were analyzed, but none of them were correlated with the absence of symptoms in potato plants during growing-on tests. The discrepancy between the observations recorded in the growing-on tests and our experiments are discussed.

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Introduction

Potato leaf roll virus (PLRV) is the type member of the genus *Polerovirus* (family *Luteoviridae*). It is an isometric virus approximately 24 nm in diameter (Peters 1967). The genome consists of a single-stranded, positive-sense RNA of about 5800 nt in length (Loebenstein 2001). The structure of the genome is depicted in Fig. 1a. Currently ten open reading frames (ORFs) are identified, which encode 11 proteins. Five proteins are translated from the genomic (g) RNA: P0, which is a

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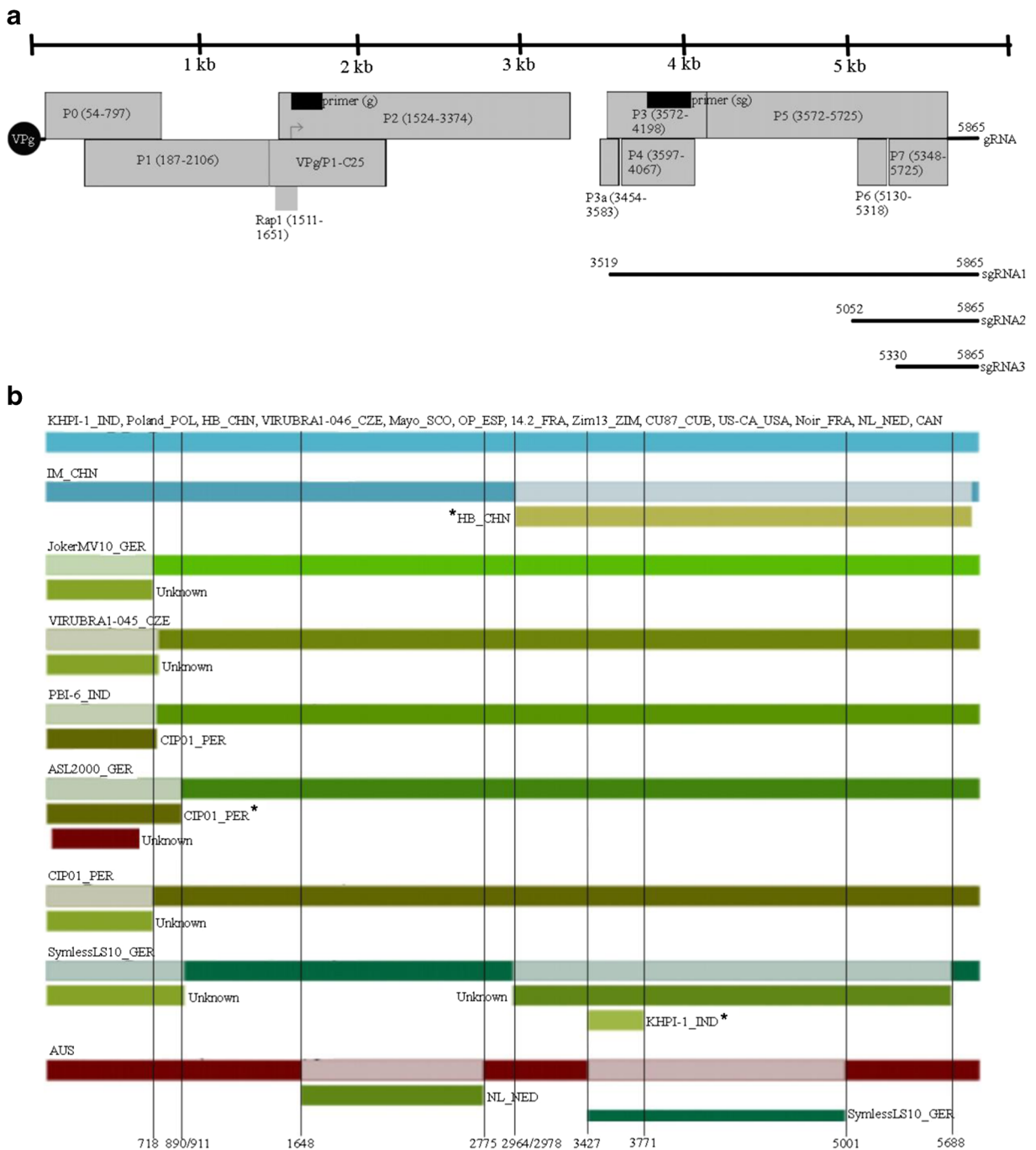
Fig. 1 Schematic diagram of the structure of the genome of PLRV isolate JokerMV10 (JQ346191). **a** Position of PCR primers, ORFs, genomic RNA (g RNA) and sub genomic RNA1, 2 and 3 (sg RNA1, sg RNA2, sg RNA3). The viral genome-linked protein (VPg) is indicated by a *black circle* at the 5′ end. The *arrowhead* in the lower left corner of *oblong* P2 indicates the approximate position of a programmed ribosomal frame shift. The two *black rectangles* signify the position of specific primers for the quantification of genomic (g) and sub genomic (sg) RNA. **b** Schematic representation of the recombination events of selected full-length

PLRV isolates. Seven different recombination events were detected in the analyzed PLRV genomes. *Black vertical lines* mark approximate breakpoints specified by respective nucleotide numbers. The recombination detection programs used for the detection of recombination events were as follows: RDP, GeneConv, BootScan, MaxiChi, Chimaera, SciScan and 3Seq, all available in RDP4 (Martin et al. 2015). The highest acceptable p-value for all programs was adjusted to 0,05. *Asterisks*: Supported by less than 3 programs embedded in the RDP4 software

RNA silencing suppressor (Bortolamiol et al. 2007; Pazhouhandeh et al. 2006; Zhuo et al. 2014), the polyprotein P1, which contains a proteinase and produces the viral genome-linked protein (VPg) by self-proteolysis (Prüfer et al. 1999), P2, which is translated by −1 ribosomal frame shifting from ORF1 and functions as RNA-dependent RNA polymerase (RdRp) (Kamer and Argos 1984) and the replication associated protein (Rap1) (Jaag et al. 2003). The proteins P3, 3a, 4 and 5 are translated from sub genomic (sg) RNA1. P3 is the coat protein and P5 the read through extension of P3 that enables it to be transmitted by aphids (Tacke et al. 1990; Brault et al. 1995). P4 is known as the viral movement protein (Miller and Mayo 1991) and the recently identified P3a is required for long-distance movement and its translation is initiated at a non-AUG codon (Smirnova et al. 2015). P6 and 7 are translated from sg RNA2. The role of P6 is still unknown. It is speculated, that it has a minor supporting role in replication (Mohan et al. 1995). P7 is known to have nucleic acid binding properties (Taliansky et al. 2003; Rohde et al. 1994). Recently a third sg RNA was investigated of which only ORF 7 is expressed and which has a regulatory role within the PLRV genome (Hwang et al. 2013). The two protein clusters either translated from g RNA or sg RNA1 are separated by a 94 nt long intergenic region containing the sg leader sequence, which is thought to maintain a 1:1 ratio in the synthesis of P3 and 4 (Juszczuk et al. 2000).

Symptoms in potato plants consist of chlorosis, upright growth, upward rolling of young leaves and stunting of the shoots. In some cultivars phloem tissue in upper parts of plants becomes necrotic. Furthermore, the authors often observed necrosis of leaf tips on older leaves (Fig. 2a). Net necrosis of tubers is typical for cultivars such as 'Russet Burbank' or 'Green Mountain'. PLRV is described as one of the most important potato viruses worldwide (Robert 1999; Solomon-Blackburn and Barker 2001; Rowhani and

Stacesmith 1979; Loebenstein 2001). This is mainly due to the damage it causes with yield losses of up to 50 % due to reduced tuber size (Domier and D'Arcy 2008; Loebenstein 2001). However, since the middle of the 80ies of the last century the importance of PLRV decreased remarkably, whereas the relative incidence of infections with *Potato virus Y* increased (Schenk 1991; Valkonen 2007; Zahn 2014). This is presumably due to the use of rapid-acting systemic insecticides (Zahn 2004), since PLRV is transmitted by aphids in a persistent manner. Furthermore, in many countries statutory schemes exist for the production of healthy seed potatoes (European European Union 2002), so secondary infections with PLRV are scarce.

However, an underestimating of a PLRV infestation may arise from very mild strains that do not cause typical symptoms in some cultivars, especially if they are infected late in the season (Barker and Woodford 1987; Bradley 1978; Wright et al. 1967). This is a risk for the certification of seed potatoes, because in Germany seed potatoes from certified lots ("Z-Partien") have not necessarily to be tested for virus infections by ELISA and infected but symptomless seed potatoes may be not recognized during field inspections. In the years from 2006 till 2008 unusual high numbers of PLRV infectios were registred by plant protection services in Lower Saxony and Mecklenburg-Western Pomerania, which led to 20–25 % de-classification of basic seed lots (Volker Zahn and Peter Steinbach 2015, personel communication). In growing-on tests in the greenhouse several PLRV-infected plants did not show any symptoms, not even if the plants were maintainted in the greenhouse for further eight weeks. Particularly, this was observed on cultivars 'Agria', 'Pirol', 'Gala' and 'Kuras' (Fig. 2b and c).

In this study isolates differing in the severity of the symptoms they induce in various potato cultivars were investigated. This was done to determine whether the isolate inducing no symptoms during growing-on tests

Fig. 2 Plants infected with PLRV. **a** Cultivar 'Princess' grown in the field showing typical PLRV symptoms such as upright growth, yellowing and upward rolling of young leaves and necrosis on leaf tips. Cultivars 'Agria' (**b**) and 'Kuras'(**c**) grown in the greenhouse

from eye-plugs during a growing-on test accomplished in 2007 in Lower Saxony. The left plant on picture b and c is healthy and the right plant is infected with PLRV. No typical PLRV symptoms are visible

in Lower Saxony belongs to a new strain of PLRV that is less virulent and induces less severe symptoms. Furthermore, we sequenced this isolate and looked for motifs that might be responsible for the reduced virulence.

Materials and methods

Isolates of PLRV investigated, virus transmission and sampling

Three isolates of PLRV were investigated (Tab. 1). Isolate JokerMV10 induces severe symptoms in potato cultivar 'Joker' (stunting and leaf rolling when reared in a greenhouse), isolate ASL2000 induces slight symptoms with slight stunting of plants of cultivar 'Linda' (recorded in a greenhouse) and isolate SymlessLS10 did not induce any symptoms on the cultivar 'Gala' during a growing-on test in a greenhouse accomplished in autum 2007 in Lower Saxony.

All three isolates were maintained on in vitro potato plants and transmitted by means of *M. persicae* to healthy potato cultivars 'Hermes' (low susceptibility to PLRV), 'Saturna' (medium susceptibility) and 'Desirée' (high susceptibility) (Federal Plant Variety Office of Germany 1977–2014; ECPD 2015). Plants of these three cultivars were also propagated in vitro free from any viruses and endogenous bacteria.

For the transmission of PLRV, five apterous female aphids carrying the virus were placed on each healthy potato plant one week after transplanting them from in vitro conditions into substrate-filled pots. Per cultivar and PLRV isolate up to 24 plants were analyzed. In addition, five plants of each cultivar were mock-inoculated using aphids carrying no virus. Plants with settled aphids were maintained at a constant 60 % relative humidity, 20 °C and 16 h illumination at 10,000 lux in a climate chamber, illuminated with EYE-Metal Halide lamps that emit a high portion of UV-A radiation.

After four days all plants were treated with Confidor (0.035 % solution, active agent: imidacloprid, Bayer CropScience GmbH, Leverkusen, Germany) to kill the aphids. After three further weeks the plants were sampled by using the lid of a 1.5-ml microcentrifuge tube to punch out three leaf discs per plant from the upper fully developed leaves. These leaf discs were immediately frozen in liquid nitrogen.

RNA extraction and reverse transcriptase-quantitative PCR (RT-qPCR)

Each sample was homogenized in a 1.5-ml microcentrifuge tube containing 500 μl of RNA isolation reagent (Concert Plant RNA Reagent, Thermo Fisher Scientific, Waltham, USA) and three 2.5 mm stainless steel beads, using a mixer mill (Retsch, Haan, Germany). Further purification of RNA was done following the manufacturer's protocol. Purified precipitated RNA was re-dissolved in diethylpyrocarbonate (DEPC)-treated distilled water to a concentration of 200 to 400 ng per μl. Total RNA amount was measured based on ultraviolet absorbance in a NanoDrop 8000 instrument (Thermo Fisher Scientific). Only samples with an OD 260 nm/280 nm and OD 260 nm/230 nm ratio of at least 1.8 were processed further. For the quantification of PLRV RNA by RT-qPCR, RNA standards were prepared consisting of a dilution series of two different PLRV RNA transcripts. One was located between nt 396–2656 (g region) and the other between nt 3480–4772 (sg region) of the PLRV genome. The nucleotide numbers are based on the sequence of isolate JokerMV10 (JQ346191). Parallel quantification of different genome segments was done since the replication rate of g and sg RNA may vary between PLRV strains. Reverse transcription (RT) was performed using a mixture of 2 μl purified RNA and 18 μl RT (DEPC-treated water; 1 x RT reaction buffer consisting of 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$ and 10 mM

Table 1 Origin and characteristics of the isolates of PLRV compared

Name of isolate	Origin	Accession No.	Symptoms
SymlessLS10	Collected 2007 in Lower Saxony, Germany	JQ346189	Cultivars 'Gala' and 'Kuras' were symptomless in growing-on tests in 2006 and 2009
ASL2000	Collected 2000 in Saxony-Anhalt, Germany	JQ346190	Weak symptom expression (slight stunting)
JokerMV10	Collected 2009 in Mecklenburg-Western Pomerania, Germany	JQ346191	Heavy symptom expression (stunting, leaf rolling)

DTT; 200 μM of each dNTP; 0.4 μM reverse primer [PLRVrev-g and PLRVrev-sg, respectively, see Table 2]; 80 U M-MLV RT [Promega, Madison, USA] and 4 U RNase inhibitor [RiboLock, Thermo Fisher Scientific]). The RT reaction was done in duplicates at 42 °C without previous denaturation for 50 min following 10 min at 72 °C for enzyme inactivation. In a second step 2 μl of cDNA reaction mix was combined with 18 μl qPCR reaction mix (distilled water; 1 x reaction buffer consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 and 0.1 % Tween 20; 150 μM of each dNTP; 0.3 μM of forward and reverse primers [PLRVfw-g, PLRVrev-g and PLRVfw-sg, PLRVrev-sg, respectively, see Table 2]; 2.5 mM $MgCl_2$; 2 U Taq DNA polymerase [Bioron GmbH, Ludwigshafen, Germany] and 1 x SybrGreen I mix [Thermo Fischer Scientific]). The qPCR reaction was performed in white 96-well plates (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) sealed with adhesive clear seals (ABsolute QPCR Seal, Thermo Fischer Scientific) in a MyiQ2 Two-Colour Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA) with the following protocol identical for both primer pairs: 2 min at 95 °C for initial denaturation followed by 40 cycles with 15 s at 94 °C for denaturation, 15 s at 58 °C for annealing, 20 s at 72 °C for elongation and 15 s at 85 °C for melting of nonspecific products. Fluorescence was measured during each cycle after the melting step. Following real-time PCR, the amplicons were melted at 95 °C for 1 min and then fully re-annealed at 55 °C for 1 min. For determination of the melting point(s) of the PCR product(s) the temperature was then increased incrementally to 95 °C at a rate of 0.5 °C every 10 s (as fixed in the MyiQ2). Melting of amplicons originating from the g region happened at 89 °C whereas primer dimers had a melting temperature of 75 °C. Amplicons originating from the sg region melted at 87 °C and unspecific products had a weak melting temperature peak at 79 °C.

The cDNA of 204 samples was used to quantify g and sg RNA in duplicates. Standards were used only in the first 96-well plate. An inter plate calibrator (IPC) consisting of a diluted PCR product produced with the PLRV amplification primers was used for normalization and variation compensation of all plates. To calculate absolute quantities from amplification data, the software GenEx Enterprise 5 (MultiD Analyses AB, Göteborg, Sweden) was used for reverse calibration. Results and qPCR efficiency determination were calculated based on a 95 % confidence level. The estimated efficiency over all qPCR plates was corrected to 100 % using GenEx software. The lower and upper confidence limits of efficiency were 92 % and 107 %, respectively. For each sample the total RNA per μl was determined after RNA extraction. Therefore, the absolute quantities of PLRV RNA could be given as copies of PLRV RNA per 300 ng total RNA. The calculated absolute quantities were subjected to an analysis of variance (ANOVA) followed by a Tukey-Kramer test (Tukey 1949).

Table 2 Position of primers for cloning and quantification of genomic (g) and sub genomic (sg) PLRV RNA

Primer	Nucleotide position*	Application
PLRVfw-g	1605–1624	Forward primer for the quantification of g RNA
PLRVrev-g	1782–1804	Reverse primer for the quantification of g RNA
PLRVfw-sg	3732–3753	Forward primer for the quantification of sg RNA
PLRVrev-sg	3988–4013	Reverse primer for the quantification of sg RNA
PLRVfw122	1–25	Forward primer for the cloning of PLRV isolates
PLRVrev1709	1564–1584	Reverse primer for the cloning of PLRV isolates
PLRVfw1338	1318–1344	Forward primer for the cloning of PLRV isolates
PLRVrev2808	2665–2687	Reverse primer for the cloning of PLRV isolates
PLRVfw2391	2270–2289	Forward primer for the cloning of PLRV isolates
PLRVrev3878	3737–3757	Reverse primer for the cloning of PLRV isolates
PLRVfw3480	3419–3439	Forward primer for the cloning of PLRV isolates
PLRVrev4772	4633–4651	Reverse primer for the cloning of PLRV isolates
PLRVfw4525	4404–4428	Forward primer for the cloning of PLRV isolates
PLRVrev5986	5846–5865	Reverse primer for the cloning of PLRV isolates

*Nucleotide numbers according to PLRV isolate JokerMV10 (JQ346191)

Cloning, sequencing and sequence analysis of isolates of PLRV

The cDNA synthesis was done from purified RNA of samples from plants each infected with one of the isolates of PLRV described in Table 1. The reaction protocol was identical to that described above for RT-qPCR but using the reverse cloning primers listed in Table 2 in parallel reactions. PCR followed with 2 µl of cDNA and the same reaction mix as mentioned above but with the respective forward and reverse cloning primers (Tab. 2) and without Sybr Green. Overlapping fragments were generated of about 1500 bp each but lacking 121 bp from 5′ end yet sequenced only by Mayo et al. (1989). The amplification of the 5′ ends by 5′ RACE was inefficient and therefore skipped. Bands of correct size were purified from gel (Ultrafree-DA Gel Extraction Kit, MILLIPORE, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), ligated into pGEM-T easy vector (Promega) and transformed into NEB Turbo Competent *Escherichia coli* cells (New England BioLabs, Ipswich, USA). Clones containing the specific insert were sequenced on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter, Brea, USA) using M13 reverse and universal primers and gene specific primers with the GenomeLab DTCS-Quick Start Kit (Beckman Coulter). Since the first 121 bp from the 5′ end were not cloned, sequences start with the 122nd nt. In order to validate sequences at least two clones were sequenced in parallel. The primary alignment was done using the MUSCLE program (Edgar 2004) realized by the MEGA software version six with default parameters (Tamura et al. 2013). By utilizing the CLC Sequence Viewer 7.6 (CLC Bio, Aarhus, Denmark), phylogenetic trees were calculated and drawn using the Neighbour-Joining method and a 1000 replicate bootstrap analysis. The complete PLRV nucleotide sequences were used for a recombination detection using RDP4 software (Martin et al. 2015).

Results

Symptoms of potato plants infected with PLRV

The PLRV-infected potato plants of cultivars 'Hermes', 'Saturna' and 'Desirée' showed typical symptoms of a PLRV infection, such as stunting and chlorotic and necrotic areas on leaves three weeks after infection (Fig. 3). Plants infected with isolate SymlessLS10 also showed typical symptoms. However, the severity of symptoms hardly varied between the isolates and cultivars and was ascertained to be medium. Altogether, the symptoms of plants infected with isolate JokerMV10 were slightly more severe (more chlorotic leaves) than those of plants infected with the other two isolates. This situation remained the same for plants kept in a climate chamber for a further two weeks.

Comparison of the number of PLRV RNA copies in infected potato plants

The RNA concentration of PLRV was determined 18 dpi when infection was well established and symptoms clearly visible. For the absolute quantification of PLRV RNA two RT-qPCR assays were developed and optimized. The limit of detection (LOD) of both qPCR assays was determined using repeated quantification of highly diluted standard RNAs (1000, 100 and 10 RNA transcript copies). Generally, the LOD is defined as the lowest amount of analyte that is still detected by the analytical method in at least 95 % of all reactions. Therefore, each diluted RNA was amplified 100 times and the RNA dilution that was amplified at least 95 times was set as the LOD of the assay. Figure 4a and b show examples of the standard series from 10^{10} to 100 RNA transcript copies within the g and sg region of the PLRV genome. The efficiencies of both assays were identical. The LOD for both assays was 100 RNA transcript copies. A graphical presentation of the number of PLRV RNA copies found in the plants infected with one of the three PLRV isolates is shown in Fig. 5.

In comparison to the other cultivars, the lowest number of PLRV RNA copies were recorded in 'Hermes', regardless of the isolate the plants were infected with. Although the differences between the effects of the different isolates of PLRV on 'Hermes' were obvious they were not significant. This was due to the lower infection rate of 'Hermes', which is characterized as highly resistant to PLRV (Ummad-ud-Din et al. 2011). The success of infection was always low and only infected plants were used for statistical calculations. All test plants of 'Saturna' and 'Desirée' were infected and had PLRV RNA copy numbers between 10^6 and 10^8. The PLRV isolates did not differ substantially in terms of the RNA copy numbers recorded. Plants infected with isolate SymlessLS10 did not

Fig. 3 Symptoms of potato plants either mock-inoculated (mock contr.) or infected with one of the three different PLRV isolates indicated at the bottom picture. Photographs were taken immediately after sampling (holes punched in leaves). Cultivars pictured are 'Hermes'(**a**), 'Saturna' (**b**) and 'Desirée' (**c**)

mock contr.   JokerMV10   SymlessLS10   ASL2000
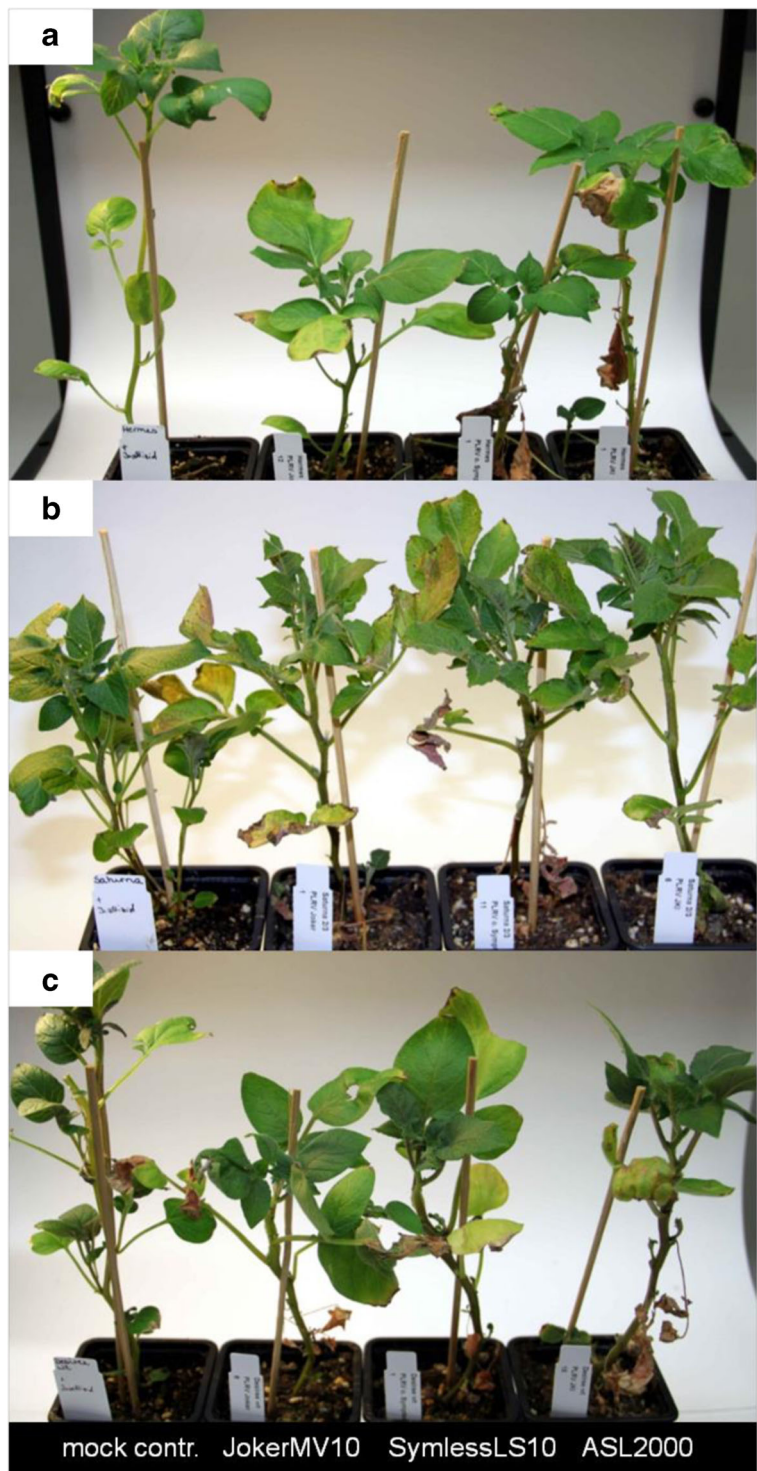
have the lowest number of PLRV RNAs. However, the number of sg RNA copies in 'Saturna' and 'Desiree' plants infected with isolate JokerMV10 was significantly higher than in plants infected with the other two isolates. This was reflected in the visible symptoms that were slightly more severe on

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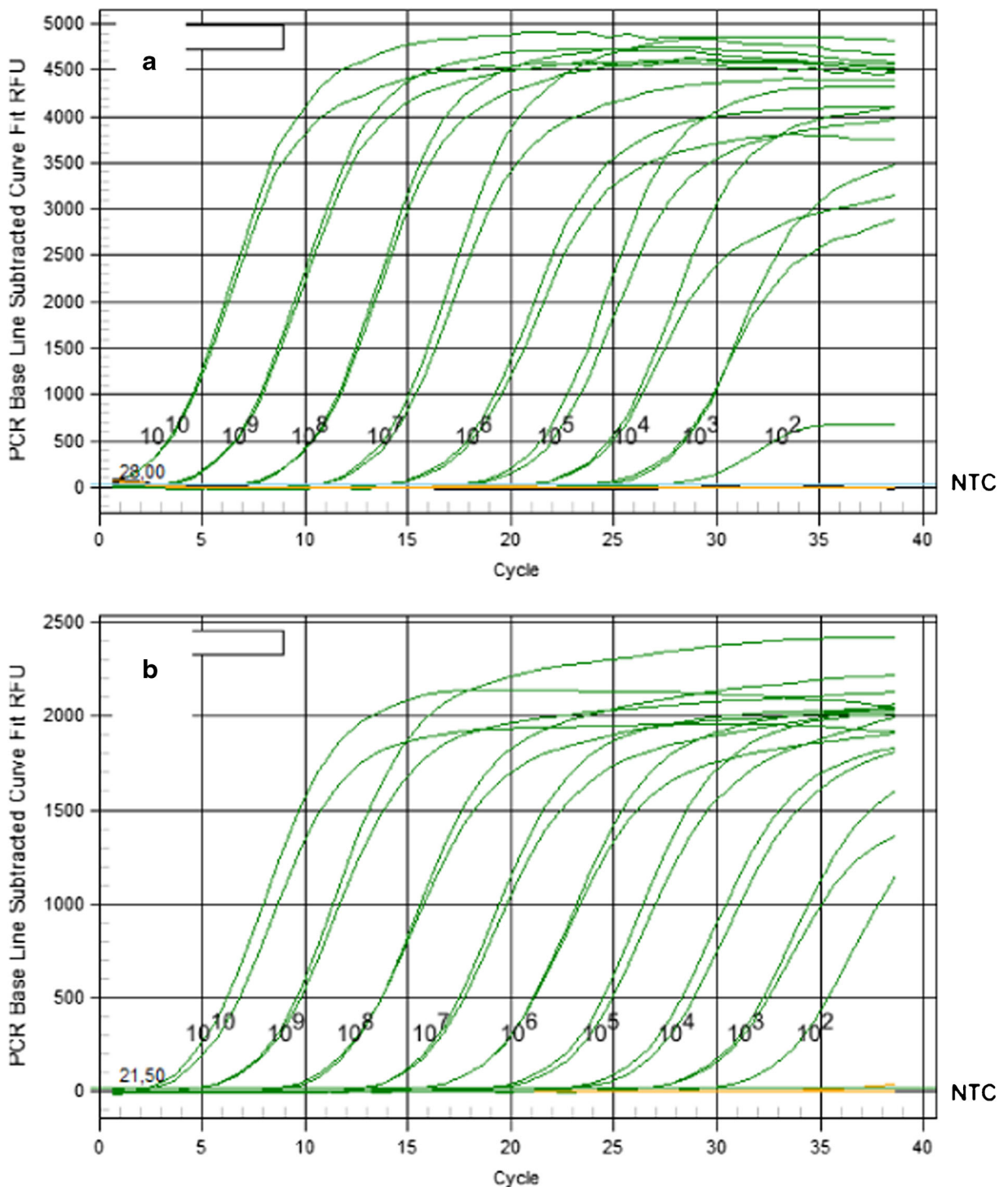
Fig. 4 Standard curves for the dilution series of in vitro RNA transcripts of PLRV genomic (**a**) and sub genomic (**b**) RNA. The dilution was tenfold from 10^{10} to 100 RNA copies. Non-template controls (NTC) did not cross the threshold line. Efficiency (**a**): 94.1 %, R squared (**a**): 0.999, slope (**a**): −3.472, y-intercept (**a**): 34.621. Efficiency (**b**): 94.1 %, R squared (**b**): 0.998, slope (**b**): −3.472, y-intercept (**b**): 36.757

Fig. 5 The absolute numbers of PLRV RNA copies presented on a logarithmic scale detected by real-time RT-qPCR in the potato cultivars 'Hermes'(**a**), 'Saturna' (**b**) and 'Desirée' (**c**). Up to 24 samples of each variant were analyzed. All values are presented as 95 % intervals, in which the *upper* and *lower line* of the *boxes* are the *upper* and *lower* confidence limits, respectively. The *line* in the *middle* of the *boxes* is the mean. Significant differences are indicated by different letters above the boxes. Names of the PLRV isolates are abbreviated (Jok = JokerMV10, Sym = SymlessLS10, ASL = ASL2000). PLRV RNA copies were detected using genomic (g) and sub genomic (sg) RNA specific primers
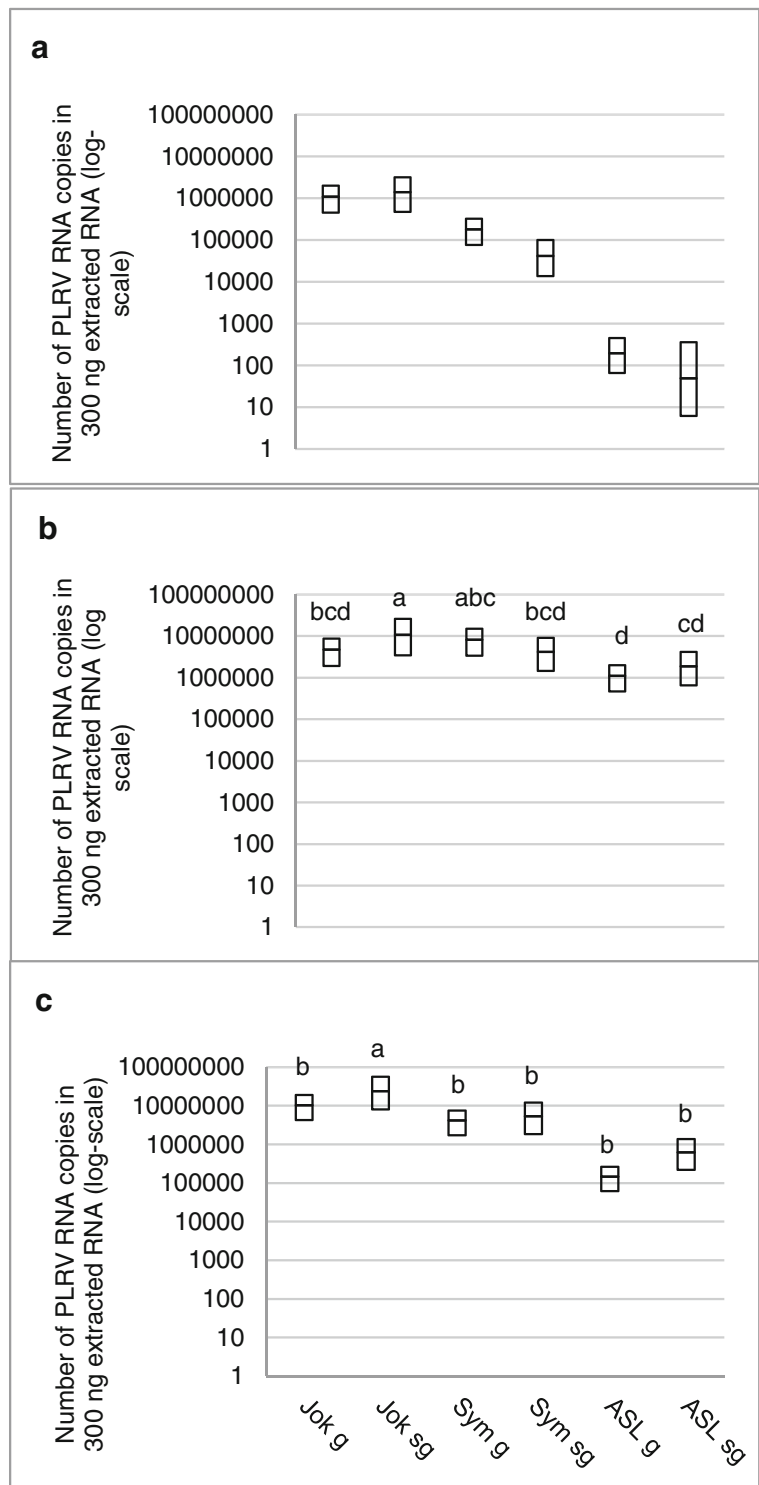
plants infected with isolate JokerMV10. Furthermore, JokerMV10 was the only isolate that generated higher numbers of sg than g RNA in all test plants.

The isolates ASL2000 and SymlessLS10 did not produce different amounts of the two viral RNAs after 25 dpi.

Sequence analysis of isolates of PLRV

Initially, the newly obtained sequences from the three isolates of PLRV (Tab. 1) proved to be very similar to published sequences. A phylogenetic analysis of the complete sequences and a Neighbour Joining analysis indicate the relationships presented in Fig. 6. There are two clusters: The Australian isolate (AUS) and isolate SymlessLS10 form the first and the remaining isolates form the second cluster. For the remaining isolates there are two sub-clusters. The newly sequenced isolates, ASL2000 and JokerMV10, cluster together with one of the three Czech, an Indian and a Peruvian isolate. The isolates from North America (CAN and US_CA) have the same internal node and are thus very closely related.

The phylogenetic analysis of ORF0 was done at the amino acid level, since it is reported that the clustering at this level is more stable (Zarghani et al. 2012). The phylogenetic tree of P0 clearly forms two main clusters (Fig. 7). In one of them the newly sequenced isolates cluster together with the Czech, some of the Tunisian and Polish, one Indian, the Peruvian and the 14.1 isolate from France. In the other main cluster the genetic distances of four of the five Australian isolates are a slightly bigger than those of the other isolates, but there are no separate clusters.

The newly sequenced isolates have no mutations within the F-box-like motifs 76-**LP**RHLHYECL E**WG**LLCGTH**P**-95 and G-139/W-140/G-141 and the F-220 residue located in the amino acid sequence of ORF0 (Zhuo et al. 2014) responsible for silencing suppression. Although all recently sequenced isolates are highly conserved within the F-box-like motifs, the Canadian isolate (CAN) has a P77S substitution.

To identify other genes with high variability their nucleotide sequences were compared (Tab. 3). Strain
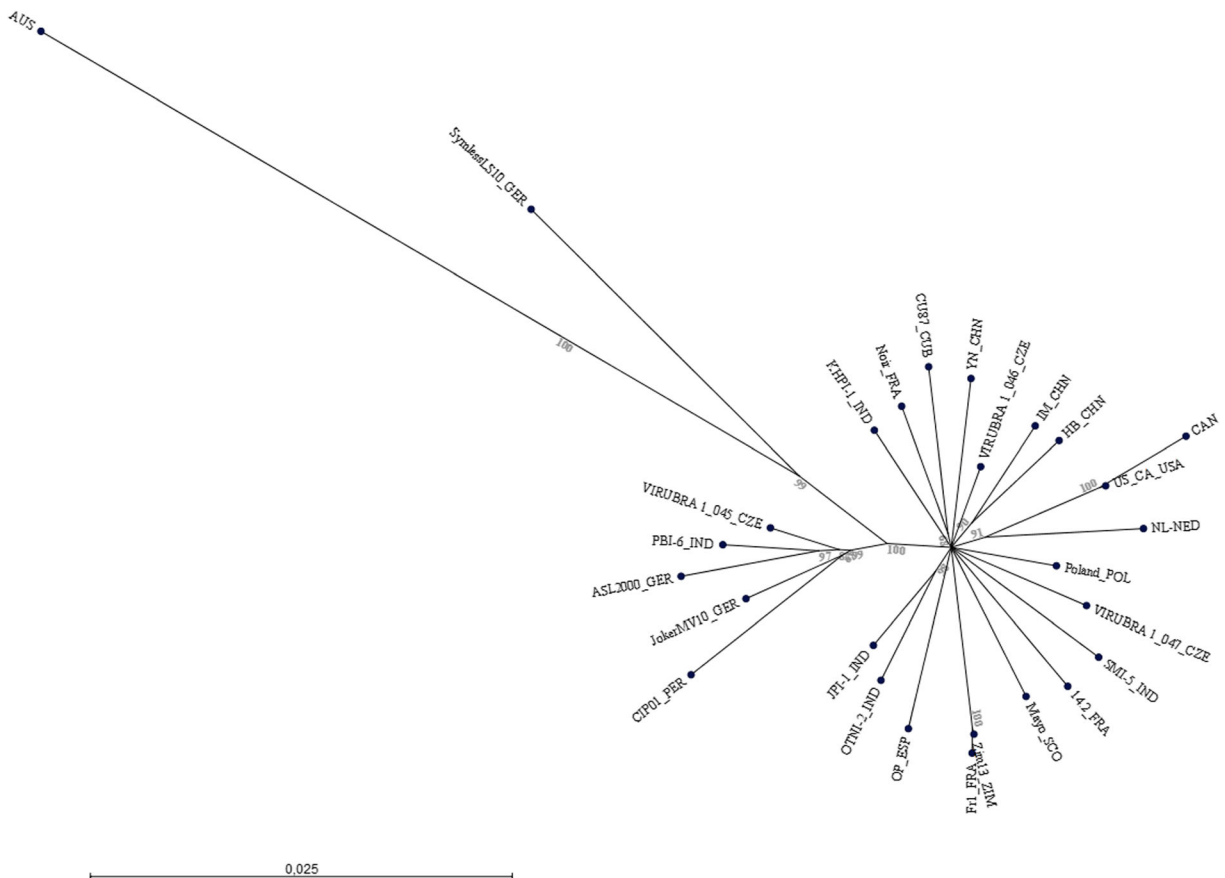
Fig. 6 Phylogenetic radial tree based on sequences of the complete genomes of PLRV isolates in GenBank. Sequences were trimmed at 5′ ends. Branches with bootstrap values less than 50 % were collapsed. The sequence CYDV-RPV (NC_004751) was used as an out group, but in the graph it is hidden. The *scale bar* represents the genetic distance. Recombinants were ignored by the software

Fig. 7 Phylogenetic radial tree based on previously published amino acid sequences of full-length ORF0. Branches with bootstrap values less than 70 % were collapsed. The sequence CYDV-RPV (NC_004751) was used as an out group, but is hidden in the graph. The *scale bar* represents the genetic distance. Recombinants were ignored by the software. For better visual clarity, some isolates with similar internal nodes and short genetic distances are hidden

SymlessLS10 differed in one striking way from all except Cu87 and the Australian isolate: P6 was interrupted due to a point mutation in ORF6 (TGG►TAG), which results in a shorter ORF6-protein, in which there are only 39 rather than 62 amino acids.

This is true of both sequenced clones. In the sg leader sequence of isolate SymlessLS10 there is a deletion. A similar deletion is recorded for the Australian isolate.

All complete PLRV sequences available at GenBank were used in a recombination analysis. The occurrence

Table 3 Sequence variations in ORF6 and the sub genomic (sg) leader sequence of selected isolates

	ORF6	sg leader sequence
JokerMV10	G_{5240}*ATCTATTGGAGAATTGGG	C_{3380}* TCACTAAAACTAG
SymlessLS10	G_{5239}*ATCTTT**TAG**AAAATTGGG	C_{3380}*TCA**C-A**AAACTAG
Australia (AUS)	G_{5256}*ATCTAT**TAG**AGAATTGGG	C_{3397}*TCAT**T-T**GACTAG
Cu87	G_{5240}*ATCTAT**TAG**AGAATTGGG	C_{3380}*TCACTA**T**AACTAG

*position in trimmed sequence

of putative recombination events was assessed using seven programs embedded in the RDP4 software (Martin et al. 2015). All three isolates from this study seem to be recombinants, with JokerMV10 consisting of one, ASL2000 of two and SymlessLS10 of three recombinants (Fig. 1b). Furthermore, SymlessLS10 is the minor parent of a recombination event in the Australian isolate.

Discussion

Since the middle of the 80's of the last century the importance of PLRV has decreased considerably. Unquestionably, this is due mainly to the use of rapid-acting systemic insecticides and the statutory schemes for the production of healthy seed potatoes (Zahn 2004; European European Union 2002). Valkonen (2007) assumes that the incidence of PLRV infections declined due to the use of PLRV resistant varieties. However, rating all the potato cultivars authorized by the German Federal Plant Variety Office over the last 30 years in terms of their susceptibility to PLRV has revealed that recently authorized cultivars are generally more susceptible to this virus than those authorized previously (Fig. 8, (Federal Plant Variety Office of Germany 1977–2014)). It is assumed, that due to the decrease in importance of PLRV breeders now focus less on PLRV resistance than 30 years ago.

Another reason for the decrease in the importance of PLRV could be a decline in the abundance of the main vector, *M. persicae*. The frequency of PLRV infections is correlated with the number of *M. persicae* caught early in the season (Basky 2002; Ioannou 1989). In North-West Germany the spectrum of aphid species has changed dramatically over the last 15 years. Previously, *M. persicae* made up more than two-thirds of all the species of aphids caught. Now, *Aphis* species dominate and *M. persicae* is caught only sporadically (Krüssel et al. 2011). However, this seems to be true only for central Europe since analyses of long-term suction-trap catches in the United Kingdom do not reveal a similar decrease in the abundance of *M. persicae* (Bell et al. 2015).

Although PLRV is now less important, the emergence of PLRV isolates causing no visible field symptoms in some potato cultivars may lead to a renewed interest in the significance of PLRV. There are reports in the past of strains of PLRV that cause mild symptoms in some potato cultivars and more severe symptoms in others and under different climatic conditions (Wright and MacCarthy 1963; MacCarthy 1963; Wright et al. 1967). Since very mild strains do not produce visible symptoms, infections are not apparent during the visual inspections of plants used for the production of seed potatoes. Infections are only revealed by ELISA tests, which could result in higher de-classification rates. Furthermore, regarding the breeding of potato cultivars and their PLRV susceptibility rating, the use of such strains during the assessment of a new variety can lead to false or varying susceptibility classifications.

Here we studied a "mild strain" found during growing-on tests in Lower Saxony. This was done by using *M. persicae* to transmit three PLRV strains to three different potato cultivars and recording the variation in the severity of their symptoms under controlled conditions. Whereas in the greenhouse and in other potato cultivars the three PLRV strains induced distinct symptoms, in our experiment we only recorded slight differences in visual symptoms (Fig. 3). Only isolate

Fig. 8 Mean susceptibility to PLRV of all the potato cultivars tested and authorized by the German Federal Plant Variety Office between 1977 and 2014
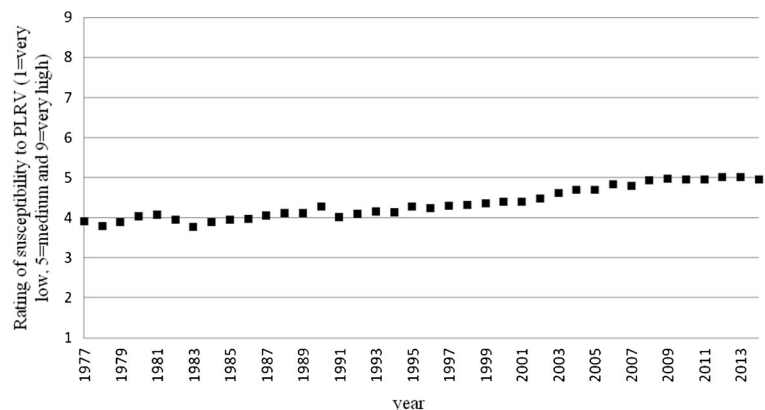
JokerMV10 produced slightly more severe symptoms in plants of all cultivars. All plants, independent of the PLRV strain and potato cultivar, showed typical symptoms, such as stunting and chlorotic and necrotic patches especially on older leaves.

We recorded the number of PLRV RNA copies in the infected plants in order to evaluate whether differences in the concentration of viral RNAs could account for the differences in symptom expression (Fig. 5). The lowest number of RNA copies was recorded in cultivar 'Hermes', which accords with its classification as less susceptible cultivar (Federal Plant Variety Office of Germany 1977–2014; Ummad-ud-Din et al. 2011; ECPD 2015). Although the susceptibility to PLRV is rated differentially for the two other cultivars, 'Saturna' (medium) and 'Desiree' (high), the detected RNA copy numbers recorded for these cultivars were nearly the same. Possibly 'Saturna' is more tolerant to the virus and thus rated as less susceptible. The differences in the RNA copy number recorded for the different isolates of PLRV are associated with the use of g or sg RNA specific primers. While in plants infected with isolate JokerMV10 the number of sg RNA copies was significantly higher than the number of g RNA copies, no differences between the amount of g RNA and sg RNA were recorded for the two other PLRV isolates. It seems that the isolates differ in the time to when they switch from the principal production of g RNA to the primary transcription of sg RNA. We speculate that JokerMV10 reproduces faster and can therefore switch earlier to primary sg RNA transcription. This is in accordance with the slightly more severe expression of symptoms by potato plants infected with this isolate. Comparisons of potato cultivars in terms of the quantifiable amount of PLRV RNA should be done using both, g RNA and sg RNA. By using only sg RNA (Mortimer-Jones et al. 2009) the number of PLRV RNA copies of some isolates could be overestimated, especially in the early stages of infection.

We searched for motifs responsible for differences in symptom expression by sequencing the whole genome of the three PLRV isolates used and comparing them with sequences from GenBank. Unfortunately, there is little information on the severity of symptoms induced by most isolates. In addition, it is difficult to compare results of different studies because of the different experimental conditions used. Since Guyader and Ducray (2002) included more isolates than other researchers in their study, we looked for sequences of

these isolates for the use in sequence analysis. A phylogenetic analysis of the whole PLRV genome did not reveal a correlation between sequence and symptom expression. All the above mentioned isolates and two of ours clustered together (Fig. 6). Djilani-Khouadja et al. (2005) described specific nucleotide changes at positions 556 and 557 within ORF0 that were correlated with the severity of symptoms. However, this seems to be true only for Tunisian isolates and isolate Cu87. Other isolates sequenced by Guyader and Ducray (2002) do not fit into this scheme. For example, the isolate Zim13 has A_{556}/A_{557} and should therefore be classified as very weak. In contrast, Guyader and Ducray (2002) categorized this isolate as severe. Nevertheless, based on the results of the phylogenetic analysis of ORF0, all three isolates in our study have A_{556}/C_{557} and form a clear cluster with other isolates all having the same nucleotide combination (Fig. 7). In addition, it is known that ORF0 is responsible for viral disease-like symptoms (van der Wilk et al. 1997) and silencing suppression. In this context an F-box-like motif plays an important role. Zhuo et al. (2014) report that the L_{76}/P_{77} residues are essential for silencing suppressor activity. Although the isolates used in our study and all recently sequenced isolates are highly conserved within the F-box-like motifs, the Canadian isolate (CAN) has a P77S substitution. However, it is not known, whether the silencing suppressor activity of this isolate (CAN) is reduced.

The role of protein P6 in the infection of plants by PLRV is still unknown. A +1 frame shift mutation in the ORF6 of *Barley yellow dwarf virus* (BYDV-PAV) results in an interruption in the corresponding amino acid sequence and the loss of infectivity (Young et al. 1991). A point mutation and deletion analyses (Mohan et al. 1995) revealed that knocking-out ORF6 prevents the accumulation of sg RNA2. Furthermore, they assume that the ORF6 protein may have a minor supporting role during replication. In vitro studies using BYDV-GAV reveal the RNA-silencing suppressor activity of P6 (Liu et al. (2012). Sequence analysis of the P6 protein of isolates SymlessLS10, Cu87 and the Australian isolate indicated that they are interrupted with the result that only 39 instead of 62 amino acids are produced. If P6 of PLRV acts as an RNA-silencing suppressor a defect in this protein should lead to a reduced virulence and weaker expression of symptoms similar to that of the silencing suppressor activity of P0 (Zhuo et al. 2014). Remarkably, it is reported that the isolates SymlessLS10

and Cu87 cause different symptoms to be expressed. Whereas Cu87 is characterized as very severe (Guyader and Ducray 2002) SymlessLS10 induced no symptoms in growing-on tests and medium to severe symptoms in a climate chamber. Therefore, we conclude, that the amino acids 40 to 62 of PLRV P6 are not involved in silencing suppression of RNA.

The sg leader sequence of PLRV is involved in regulating the translation of downstream located genes (Juszczuk et al. 2000; Juszczuk et al. 1997). Deletion analyses reveal that a lack of the sg leader sequence increases the translation efficiency fivefold (Juszczuk et al. 2000). There is a deletion of a single nucleotide in the isolate SymplessLS10, which is similar to the deletion recorded in the Australian isolate. Whether such a small deletion has an effect on the translation of sg RNA, this should clearly be a subject for further investigation.

The results of the recombination analysis revealed no reasons why isolate SymplessLS10 did not induce symptoms during growing-on tests. Either the recombination event was of unknown character or the classified event was not statistically supported by all embedded RDP programs.

Since we did not find any differences of the PLRV isolates at the molecular level and in the severity of symptoms under homogeneous environmental conditions, we assume that the discrepancy between the observations during the growing-on test in Lower Saxony and our experiments may be the result of dissimilar growth conditions and/or cultivar assortment. During the growing-on test plants were maintained in a glasshouse absorbing most of the UV radiation from sun light, especially considering that the test was accomplished in November without illumination. Our experiments were run in a climate chamber under EYE-Metal Halide lamps that emit a considerable portion of UV radiation, approximately identical to field conditions. Therefore, we propose, that the symptom development in plants infected with PLRV may be influenced by UV radiation. In the literature several examples can be found that describe the absence of symptoms in viral infected plants under low light conditions (Balachandran et al. 1994; Barba et al. 1989; Christov et al. 2007; Kumar and Poehling 2006; Osmond et al. 1990). Maybe the development of chlorotic viral disease symptoms is not merely a direct result from the inhibition of the chloroplast function by viral proteins (Banerjee et al. 1995) but rather a consequence from the reduced fitness of the

plant and the resulting increased destructive effect of unfavorable environmental conditions. Balachandran et al. (1997) conceived that "virus infections may have greater effects on fitness and competitive ability in low N [nitrogen], high light environments than in shaded, high nutrient conditions". In prior studies it was assumed that photo-damage to photosystem II (PSII) under sunlight is primarily associated with photosynthetically active light wavelengths. Now it was shown that the damage of PSII predominantly is caused by UV radiation (Takahashi et al. 2010) by increasing the production of reactive oxygen species (ROS) in the photosynthetic apparatus. In healthy plants ROS can be reduced to oxygen and water by the phytosystem I receiving electrons from the water-splitting PSII (Asada 1999). However, in some plants infected with viruses viral coat proteins accumulate within the PSII (Reinero and Beachy 1989) and the water-splitting activity and photosynthetic electron transport rates of PSII are reduced (Takahashi and Ehara 1992; Rahoutei et al. 2000). In high-light conditions this may lead to an overproduction of ROS resulting in photoinhibition. This, in turn, involves the damage to the D1 protein and other PSII reaction centre components leading to pigment photo-oxidation and chlorotic symptoms (Asada 1994).

The accumulation of carbohydrates in leaves of infected plants is another typical property of a PLRV infection causing growth deformation and stunting (Loebenstein 2001). However, the amount of soluble sugars in infected but symptomless plants was not measured and no upward rolling of leaves or an upright habit was observed during the growing-on test in Lower Saxony. The plants in the climate chamber, however, showed all these typical symptoms of a PLRV infection, indicating an indirect influence of UV radiation on these symptoms. Possibly, under low-light conditions fewer carbohydrates are produced whose accumulation does not lead to overt symptoms.

Since not all potato plants infected with PLRV were symptomless during the growing-on test in Lower Saxony, there has to be a considerable influence of the individual fitness of each plant on the severity of symptoms. Potato plants with a reduced fitness probably are not able to repair their PSII by de novo-synthesizing of D1 protein, sufficiently (Anderson et al. 1998). Altogether, there is need for further research investigating the influence of different environmental and plant fitness conditions on the development of PLRV symptoms.

In this study we compared the effects of the new PLRV isolate SymlessLS10 and other isolates, which did not induce any symptoms during growing-on tests, visually and at the molecular level. We did not find any differences in the severity of symptoms and the amount of PLRV RNA copies in potato plants. The sequences of the isolates used in this study are very similar. Several motifs, which could play a role in symptom expression, were analyzed, but none of them seem to be responsible for the absence of symptoms in potato plants infected with isolate SymlessLS10 during growing-on tests. Therefore, we discussed the variability of symptom expression in the context of stress physiology of virus-infected plants and concluded that the development of chlorotic viral disease symptoms may be not merely a direct result from the inhibition of the chloroplast function by viral proteins but rather a consequence from the reduced fitness of the plant and the resulting increased destructive effect of unfavorable environmental conditions.

References

Anderson, J. M., Park, Y. I., & Soon, W. S. (1998). Unifying model for the photoinactivation of photosystem II in vivo under steady-state photosynthesis. *Photosynthesis Research, 56*(1), 1–13. doi:10.1023/a:1005946808488.

Asada, K. (1994). Production and action of active oxygen species in photosynthetic tissues. In C. H. Foyer, & P. M. Mullineaux (Eds.), *Causes of photooxidative stress and amelioration of defense systems in plants.* (pp. 77–104).

Asada, K. (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology, 50*, 601–639. doi:10.1146/annurev.arplant.50.1.601.

Balachandran, S., Osmond, C. B., & Makino, A. (1994). Effects of 2 strains of tobacco mosaic-virus on photosynthetic characteristics and nitrogen partitioning in leaves of nicotiana-tabacum Cv Xanthi during photoacclimation under 2 nitrogen nutrition regimes. *Plant Physiology, 104*(3), 1043–1050.

Balachandran, S., Hurry, V. M., Kelley, S. E., Osmond, C. B., Robinson, S. A., Rohozinski, J., et al. (1997). Concepts of plant biotic stress. Some insights into the stress physiology of virus-infected plants, from the perspective of photosynthesis. *Physiologia Plantarum, 100*(2), 203–213. doi:10.1034/j.1399-3054.1997.1000201.x.

Banerjee, N., Wang, J. Y., & Zaitlin, M. (1995). A single nucleotide change in the coat protein gene of *Tobacco mosaic virus* is involved in the induction of severe chlorosis. *Virology, 207*(1), 234–239. doi:10.1006/viro.1995.1070.

Barba, M., Cupidi, A., & Faggioli, F. (1989). In vitro culture of grapevine infected by closterovirus type III*. *Journal of Phytopathology, 126*(3), 225–230. doi:10.1111/j.1439-0434.1989.tb01108.x.

Barker, H., & Woodford, J. A. T. (1987). Unusually mild symptoms of Potato leafroll virus in the progeny of late-infected mother plants. *Potato Research, 30*(2), 345–348.

Basky, Z. (2002). The relationship between aphid dynamics and two prominent potato viruses (PVY and PLRV) in seed potatoes in Hungary. *Crop Protection, 21*(9), 823–827. doi:10.1016/s0261-2194(02)00045-5.

Bell, J. R., Alderson, L., Izera, D., Kruger, T., Parker, S., Pickup, J., et al. (2015). Long-term phenological trends, species accumulation rates, aphid traits and climate: five decades of change in migrating aphids. *Journal of Animal Ecology, 84*(1 (Suppl. 4)), 21–34. doi:10.1111/1365-2656.12282.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., & Ziegler-Graff, V. (2007). The polerovirus F box protein PO targets ARGONAUTE1 to suppress RNA silencing. *Current Biology, 17*(18), 1615–1621. doi:10.1016/j.cub.2007.07.061.

Bradley, R. H. E. (1978). Search for leaf roll virus that does not cause diagnostic symptoms in potato foliage. *Canadian Plant Disease Survey, 58*(3), 56–60.

Brault, V., Vandenheuvel, J. F. J. M., Verbeek, M., Zieglergraff, V., Reutenauer, A., Herrbach, E., et al. (1995). Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. *EMBO Journal, 14*(4), 650–659.

Christov, I., Stefanov, D., Velinov, T., Goltsev, V., Georgieva, K., Abracheva, P., et al. (2007). The symptomless leaf infection with grapevine leafroll associated virus 3 in grown in vitro plants as a simple model system for investigation of viral effects on photosynthesis. *Journal of Plant Physiology, 164*(9), 1124–1133. doi:10.1016/j.jplph.2005.11.016.

Djilani-Khouadja, F., Rouzé-Jouan, J., Guyader, S., Marrakchi, M., & Fakhfakh, H. (2005). Biological and molecular characterization of Tunisian isolates of Potato leafroll virus. *Journal of Plant Pathology, 87*(2), 91–99.

Domier, L. L., & D'Arcy, C. J. (2008). Luteoviruses. In B. W. J. Mahy & M. H. V. Van Regenmortel (Eds.), *Ecyclopedia of virology* (3rd ed., Vol. 3, pp. 231–238). Amsterdam: Academic.

ECPD: The European Cultivated Potato Database (2015) http://www.europotato.org. Accessed 22nd June 2015.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*(5), 1792–1797. doi:10.1093/nar/gkh340.

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Federal Plant Variety Office of Germany (1977–2014). *Descriptive Variety Lists - Potato*. Hannover, Germany: Federal Plant Variety Office of Germany.

Guyader, S., & Ducray, D. G. (2002). Sequence analysis of potato leafroll virus isolates reveals genetic stability, major evolutionary events and differential selection pressure between overlapping reading frame products. *Journal of General Virology, 83*, 1799–1807.

Hwang, Y. T., Kalischuk, M., Fusaro, A. F., Waterhouse, P. M., & Kawchuk, L. (2013). Small RNA sequencing of potato leafroll virus-infected plants reveals an additional subgenomic RNA encoding a sequence-specific RNA-binding protein. *Virology, 438*(2), 61–69. doi:10.1016/j.virol. 2012.12.012.

Ioannou, N. (1989). The infection pressure of potato leafroll virus and potato virus Y in relation to aphid populations in Cyprus. *Potato Research, 32*(1), 33–47. doi:10.1007/bf02365815.

Jaag, H. M., Kawchuk, L., Rohde, W., Fischer, R., Emans, N., & Prüfer, D. (2003). An unusual internal ribosomal entry site of inverted symmetry directs expression of a potato leafroll polerovirus replication-associated protein. *Proceedings of the National Academy of Sciences of the United States of America, 100*(15), 8939–8944.

Juszczuk, M., Zagorski-Ostoja, W., & Hulanicka, D. M. (1997). Studies on the translation mechanism of subgenomic RNA of potato leafroll virus. *Acta Biochimica Polonica, 44*(1), 69–78.

Juszczuk, M., Paczkowska, E., Sadowy, E., Zagorski, W., & Hulanicka, D. M. (2000). Effect of genomic and subgenomic leader sequences of potato leafroll virus on gene expression. *FEBS Letters, 484*(1), 33–36. doi:10.1016/s0014-5793(00)02122-0.

Kamer, G., & Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Research, 12*(18), 7269–7282. doi:10.1093/nar/12.18.7269.

Krüssel, S. Langjährige Beobachtungen zum Auftreten von relevanten Blattlausarten in Kartoffeln in Niedersachsen. In *DPG-Arbeitskreis Integrierter Pflanzenschutz, Projektgruppe Kartoffel, Julius Kühn-Institut, Braunschweig, Germany, 2nd March 2011*.

Kumar, P., & Poehling, H. M. (2006). UV-blocking plastic films and nets influence vectors and virus transmission on greenhouse tomatoes in the humid tropics. *Environmental Entomology, 35*(4), 1069–1082. doi:10.1603/0046-225x-35.4.1069.

Liu, Y., Zhai, H., Zhao, K., Wu, B. L., & Wang, X. F. (2012). Two suppressors of RNA silencing encoded by cereal-infecting members of the family Luteoviridae. *Journal of General Virology, 93*, 1825–1830. doi:10.1099/vir.0.042135-0.

Loebenstein, G. (2001). Potato leafroll virus (PLRV; Genus Polerovirus; family Luteoviridae). In G. Loebenstein, P. H. Berger, A. A. Brunt, & R. H. Lawson (Eds.), *Virus and virus-like diseases of potatoes and production of seed-potatoes* (pp. 69–75). Dordrecht: Kluwer Academic Publishers.

MacCarthy, H. R. (1963). Instability of symptoms of potato leaf roll virus. *Phytopathology, 53*(10), 1161–1163.

Martin, D. P., Murrell, B., Golden, M., Khoosal, A., & Muhire, B. (2015). RDP4: detection and analysis of recombination patterns in virus genomes. *Virus Evolution, 1*(1), 1–5.

Mayo, M. A., Robinson, D. J., Jolly, C. A., & Hyman, L. (1989). Nucleotide-sequence of potato leafroll luteovirus RNA. *Journal of General Virology, 70*, 1037–1051.

Miller, J. S., & Mayo, M. A. (1991). The location of the 5′ end of the potato leafroll luteovirus subgenomic coat protein mRNA. *Journal of General Virology, 72*(11), 2633–2638. doi:10.1099/0022-1317-72-11-2633.

Mohan, B. R., Dinesh-Kumar, S. P., & Miller, W. A. (1995). Genes and cis-acting sequences involved in replication of barley yellow dwarf virus-PAV RNA. *Virology, 212*(1), 186–195. doi:10.1006/viro.1995.1467.

Mortimer-Jones, S. M., Jones, M. G. K., Jones, R. A. C., Thomson, G., & Dwyer, G. I. (2009). A single tube, quantitative real-time RT-PCR assay that detects four potato viruses simultaneously. *Journal of Virological Methods, 161*(2), 289–296. doi:10.1016/j.jviromet.2009.06.027.

Osmond, C. B., Berry, J. A., Balachandran, S., Buchenosmond, C., Daley, P. F., & Hodgson, R. A. J. (1990). Potential consequences of virus infection for shade-sun acclimation in leaves. *Botanica Acta, 103*(3), 226–229.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., et al. (2006). F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. *Proceedings of the National Academy of Sciences of the United States of America, 103*(6), 1994–1999. doi:10.1073/pnas.0510784103.

Peters, D. (1967). Purification of potato leafroll virus from its vector myzus persicae. *Virology, 31*(1), 46–54.

Prüfer, D., Kawchuk, L., Monecke, M., Nowok, S., Fischer, R., & Rohde, W. (1999). Immunological analysis of potato leafroll luteovirus (PLRV) P1 expression identifies a 25 kDa RNA-binding protein derived via P1 processing. *Nucleic Acids Research, 27*(2), 421–425.

Rahoutei, J., García-Luque, I., & Barón, M. (2000). Inhibition of photosynthesis by viral infection: effect on PSII structure and function. *Physiologia Plantarum, 110*(2), 286–292. doi:10.1034/j.1399-3054.2000.110220.x.

Reinero, A., & Beachy, R. N. (1989). Reduced photosystem II activity and accumulation of viral coat protein in chloroplasts of leaves infected with *Tobacco mosaic virus*. *Plant Physiology, 89*(1), 111–116. doi:10.1104/pp.89.1.111.

Robert, Y. (1999). Epidemiology of potato leafroll disease. In H. G. Smith & H. Barker (Eds.), *The Luteoviridae* (pp. 221–228). Wallingford: CABI Publishing.

Rohde, W., Gramstat, A., Schmitz, J., Tacke, E., & Prüfer, D. (1994). Plant viruses as model systems for the study of noncanonical translation mechanisms in higher plants. *Journal of General Virology, 75*, 2141–2149. doi:10.1099/0022-1317-75-9-2141.

Rowhani, A., & Stacesmith, R. (1979). Purification and characterization of potato leafroll virus. *Virology, 98*(1), 45–54.

Schenk, G. (1991). Dynamik des Virusbefalls von Kartoffeln in Mecklenburg-Vorpommern, Sachsen-Anhalt, Brandenburg, Thüringen und Sachsen. *Archives of Phytopathology and Plant Protection, 27*(6), 425–437. doi:10.1080/03235409109438085.

Smirnova, E., Firth, A. E., Miller, W. A., Scheidecker, D., Brault, V., Reinbold, C., et al. (2015). Discovery of a Small non-AUG-initiated ORF in poleroviruses and luteoviruses that is required for long-distance movement. *PLoS Pathogens, 11*(5), e1004868. doi:10.1371/journal.ppat.1004868.

Solomon-Blackburn, R. M., & Barker, H. (2001). Breeding virus resistant potatoes (Solanum tuberosum): a review of traditional and molecular approaches. *Heredity, 86*(Pt 1), 17–35.

Tacke, E., Prüfer, D., Salamini, F., & Rohde, W. (1990). Characterization of a potato leafroll luteovirus subgenomic RNA—differential expression by internal translation initiation and UAG suppression. *Journal of General Virology, 71*, 2265–2272. doi:10.1099/0022-1317-71-10-2265.

Takahashi, H., & Ehara, Y. (1992). Changes in the activity and the polypeptide composition of the oxygen-evolving complex in photosystem II of tobacco leaves infected with *Cucumber mosaic virus* strain. *Molecular Plant-Microbe Interactions, 5*(3), 269–272. doi:10.1094/mpmi-5-269.

Takahashi, S., Milward, S. E., Yamori, W., Evans, J. R., Hillier, W., & Badger, M. R. (2010). The solar action spectrum of photosystem II damage. *Plant Physiology, 153*(3), 988–993. doi:10.1104/pp.110.155747.

Taliansky, M., Mayo, M. A., & Barker, H. (2003). Potato leafroll virus: a classic pathogen shows some new tricks. *Molecular Plant Pathology, 4*(2), 81–89.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725–2729. doi:10.1093/molbev/mst197.

Tukey, J. (1949). Comparing individual means in the analysis of variance. *Biometrics, 5*(2), 99–114.

Ummad-ud-Din, U., Khan, M. A., Nazir, J., & Bushra, S. (2011). Evaluation of resistance against PLRV in potato cultivars. *Pakistan Journal of Phytopathology, 23*(1), 14–19.

European Union. (2002). Council directive 2002/56/EC of 13 June 2002 on the marketing of seed potatoes. *Official Journal of the European Communities, L 193*(45), 60–73.

Valkonen, J. P. (2007). Viruses: Economical losses and biotechnological potential. In D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D. K. L. McKarren, & M. A. Taylor (Eds.), *Potato biology and biotechnology—advances and perspectives* (pp. 619–641). Amsterdam: Elsevier.

van der Wilk, F., Houterman, P., Molthoff, J., Hans, F., Dekker, B., van den Heuvel, J., et al. (1997). Expression of the potato leafroll virus ORF0 induces viral-disease-like symptoms in transgenic potato plants. *Molecular Plant-Microbe Interactions, 10*(2), 153–159.

Wright, N. S., & MacCarthy, H. R. (1963). Expresson and detection of leaf roll virus strains in potato. *American Potato Journal, 40*, 154–162.

Wright, N. S., MacCarthy, H. R., & Cole, E. F. (1967). Detection and control of mild strains of potato leaf roll virus. [Article]. *American Potato Journal, 44*(7), 245–248. doi:10.1007/bf02862520.

Young, M. J., Kelly, L., Larkin, P. J., Waterhouse, P. M., & Gerlach, W. L. (1991). Infectious in vitro transcripts from a cloned cDNA of barley yellow dwarf virus. *Virology, 180*(1), 372–379. doi:10.1016/0042-6822(91)90042-A.

Zahn, V. (2004). Verändertes auftreten von viren. *Kartoffelbau, 55*(11), 394–397.

Zahn, V. (2014). Ergebnisse der Kartoffelvirustestung in der Saison 2013/2014. https://www.lwk-niedersachsen.de/index.cfm/portal/pflanze/nav/505/article/24333.html.

Zarghani, S., Shams-Bakhsh, M., Zand, N., Sokhandan-Bashir, N., & Pazhouhandeh, M. (2012). Genetic analysis of Iranian population of potato leafroll virus based on ORF0. *Virus Genes, 45*(3), 567–574. doi:10.1007/s11262-012-0804-z.

Zhuo, T., Li, Y.-Y., Xiang, H.-Y., Wu, Z.-Y., Wang, X.-B., Wang, Y., et al. (2014). Amino acid sequence motifs essential for P0-mediated suppression of rna silencing in an isolate of potato leafroll virus from inner mongolia. *Molecular Plant-Microbe Interactions, 27*(6), 515–527. doi:10.1094/mpmi-08-13-0231-r.

5.4 Evaluation of whether genetically modified potatoes are equivalent in their level of susceptibility to *Potato virus Y*

Evaluation of whether genetically modified potatoes are equivalent in their level of susceptibility to *Potato virus Y*

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Abstract

Genetically modified potatoes are evaluated according to their agronomic characteristics with the view to determining any unintended effects of potential safety concern. One of these effects could be an enhanced susceptibility to diseases. In this study several transgenic lines of the potato varieties 'Albatros' and 'Desirée' were compared with the non-transgenic counterparts and with a large assortment of commercial varieties in order to determine whether the transgenic lines are equivalent in terms of their level of susceptibility to *Potato virus Y* (PVY). In order to achieve this, a reverse transcription quantitative PCR (RT-qPCR) assay was developed to accurately quantify the number of PVY RNA copies in extracted plant RNA. The amount of virus RNA was used as an indicator of susceptibility to PVY. Transgenic 'Albatros' lines were assessed as equivalent to the commercial varieties. The equivalence of transgenic lines of variety 'Desirée', however, could not be evaluated, because the susceptibility of the non-transgenic counterpart was even higher than that of the commercial varieties used for the comparison. Therefore, we recommend the use of (extremely) virus resistant genotypes for genetic modification. Otherwise, it has to be reckoned that growers reject the cultivation of susceptible transgenic lines due to yield losses caused by virus infections.

Keywords

Real-time-RT-PCR, Genetic Engineering, Resistance, GMO, Risk assessment, *Potato virus Y*

Introduction

Potato (*Solanum tuberosum* L. subsp. *tuberosum*) is the world's fourth most important food crop behind maize, wheat and rice, with nearly 376 million metric tons produced in 2013 (FAOSTAT 2015). The demand for potato as a vegetable or processed product, especially in developing countries, is increasing. Projections predict an increase of the worldwide potato production in the next few decades of two to three percent annually (FAO 2008). This challenge is met by potato breeders by breeding new varieties that have several of the following traits: high yield, resistance to as many pathogens as possible, good taste, excellent processing properties, and tolerance to abiotic stresses, such as drought and high temperatures. However, potato breeding depends on vegetative propagation and is constrained by the autotetraploid nature of the potato genome and associated high degree of heterozygosity. Furthermore, it may take up to 50 years to develop a new variety using conventional potato breeding approaches (Rakosy-Tican et al. 2015). Breeding schemes using wild species to extend the genetic pool are often expensive and lengthy processes since various cycles of backcrosses are required to eliminate undesirable traits originating from the wild potato species. Genetic engineering may therefore offer potato breeders a broader genetic pool and reduce the costs and time needed to achieve their breeding goals. First transgenic potato plants were generated in 1986 (An et al. 1986; Ooms et al. 1986; Shahin and Simpson 1986). Genetic engineering is used to improve the resistance of potato to pests and pathogens (Chung et al. 2013; Dinh et al. 2014; Jahan et al. 2015; Zhang et al. 2015), herbicide and abiotic stress (Ahn et al. 2011; Kikuchi et al. 2015) and enhance the nutritive value of tubers (Li et al. 2014).

Due to the current non-acceptance by the consumers, genetic engineering of potato currently is almost exclusively done in the context of non-food use, e. g. "molecular pharming" to produce vaccines (Jose et al. 2014; Rukavtsova et al. 2015) or the utilization by the processing industry (Romano et al. 2005; Zhu et al. 2014). In contrast to transgenic disease resistance, which is at the risk being overcome by evolutionary changes in pathogens, this kind of transformation is stable and easily inherited because of its monogenetic character. "Molecular pharming" for the production of plant-derived vaccines in transgenic plants is an alternative of costly systems of recombinant immunogenic protein expression. Plant-derived vaccines can be produced cheaply in large quantities by using plants as "bio-factories". Furthermore, contamination by animal diseases or other adventitious agents can be excluded (WHO 2005). An example for the production of vaccines against animal diseases is the expression of the VP60 capsid protein of the *Rabbit hemorrhagic disease virus* (RHDV) in transgenic potato

plants. The lethal viral Rabbit Hemorrhagic Disease (RHD) often affects commercial and domestic rabbit populations. Rabbits immunized with this recombinant VP60 protein were fully protected against the hemorrhagic disease. Tuber-specific expression of VP60 is reported by Mikschofsky et al. (2011a) in potato cultivars 'Albatros' and 'Desiree'.

An example for the usage of genetically modified potatoes by the processing industry is the production of biodegradable polymers, such as polyaspartate, which can be obtained from the cyanobacterial storage compound cyanophycin (Simon 1976). The cyanophycin synthetase gene *cph*A from the cyanobacterium *Thermosynechococcus elongates* was successfully expressed in the tubers of the potato cultivar 'Albatros' (Hühns et al. 2009).

The OECD has released consensus documents considering the special components to be compared for the assessment of new (potato) varieties (OECD 2002). The document recommends, for instance, a comparison of "agronomic characteristics […], including yield, susceptibility and tolerance towards specific diseases". These traits can indicate "unintended effects of potential safety concerns that would require further investigations" (OECD 2002). Such an unintended effect of a genetic modification could be an impaired signaling pathway in response to a virus infection leading to an increase in the susceptibility of the plant.

In this study we examined transgenic potato lines for possible changes regarding their level of susceptibility to *Potato virus Y* (PVY), one of the most important potato viruses worldwide. The transgenic potatoes were either transformed with *cph*A and neomycin-phosphotransferase II (*npt*II) as a plant selection marker gene, VP60 and *npt*II or the *npt*II marker gene alone. We compared the transgenic lines with their laboratory-derived near isogenic varieties (niv) in order to determine differences in the level of PVY susceptibility, which we measured by means of the detectable number of PVY RNA copies within extracted plant RNA. The equivalence of PVY susceptibility was determined and discussed with regards to several commercial varieties with a broad range of PVY susceptibility.

Material and Methods

Potato lines and virus isolates used in the experiments

The transgenic potato lines of the varieties 'Albatros' and 'Desirée', expressing either the *cph*A and *npt*II or VP60 and *npt*II (Mikschofsky et al. 2011b), were compared with the niv and the control plants expressing only the kanamycin resistance marker gene *npt*II. In Table 1 all the potato lines analyzed are listed with information on the transgenes and the suppliers.

Tab. 1 Potato lines analyzed in this study

Genotype	Cultivar	Vector	Event	Transgenic product	Provided by
Alb niv[1,4]	Albatros	-	-	-	University of Rostock
Alb C12	Albatros	PsbY-cphA[3]	12	Cyanophycin, nptII	University of Rostock
Alb VP60	Albatros	35Svp60SEK[4]	204	VP60, nptII	University of Rostock
Alb NPTII	Albatros	35S (NPTII)[3]	205	nptII	University of Rostock
Des niv[1,4]	Desirée	-	-	-	University of Rostock
Des VP60	Desirée	35Svp60SEK[4]	6	VP60, nptII	University of Rostock
Des NPTII	Desirée	35S (NPTII)[3]	6	nptII	University of Rostock
Sat[2]	Saturna	-	-	-	University of Rostock
Her[2]	Hermes	-	-	-	Julius Kühn-Institut
Pri[2]	Princess	-	-	-	Julius Kühn-Institut
MaG[2]	Mayan Gold (*S. phureja*)	-	-	-	Purchased (garden centre "Naturwuchs", Bielefeld, Germany)

[1]Near isogenic variety
[2]Commercial varieties
[3]Hühns et al. (2009)
[4]Mikschofsky et al. (2011a)

To examine the equivalence of transgenic potato lines regarding PVY susceptibility, non-transgenic commercial varieties were used that had a wide range of susceptibility to PVY (Tab. 2). A scheme of the trial is presented in Fig. 1. With the aid of these commercial varieties an upper 95% equivalence limit can be set based upon estimates of the natural variation (Perry et al. 2009). Details of statistical calculations are described below.
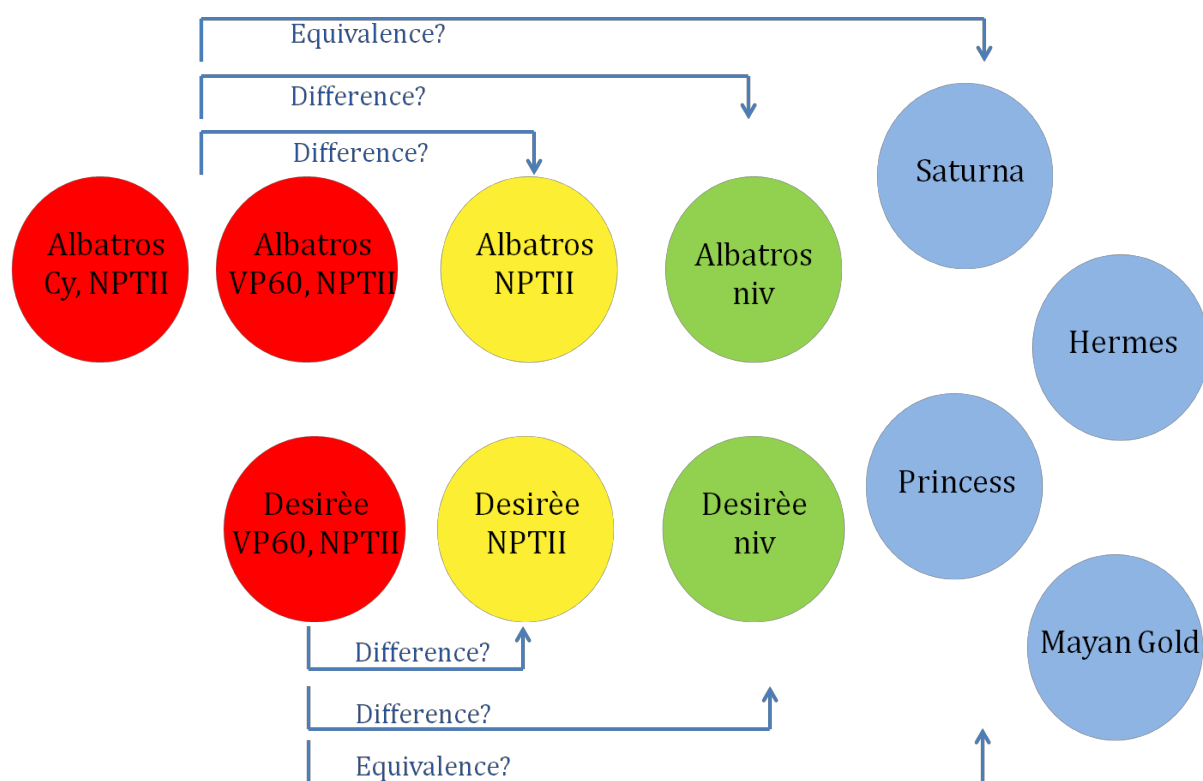

Fig. 1 Scheme of the trial

All experimental plants were infected either with PVY^{NTN}, isolate Gr99 (accession number AJ890343) or PVY^{N}Wilga, isolate 261-4 (accession number AM113988), since recombinant strains of PVY are of increasing importance in most of the potato growing regions and are highly virulent (Lindner and Billenkamp 2005; Karasev et al. 2011; Quintero-Ferrer and Karasev 2013). Furthermore, PVY^O could not be used because 'Desirée' harbors the *Ny* gene and as a result has a hypersensitive response (HR) to and resistant to PVY^O (Rowley et al. 2015).

Tab. 2 Susceptibility of potato varieties to PVY

Genotype	Susceptibility to PVY^{N-Wi} and PVY^{NTN}	
	According to ECPD[1] or BSA[2]	According to the results of this study
'Albatros'/ Alb niv[5]	1[2]	2[3]
Alb C12	-	2[3]
Alb VP60	-	1[3]
Alb NPTII	-	2[3]
'Desirée'/ Des niv[5]	1-3[1], 5[2]	5
Des VP60	-	5
Des NPTII	-	5
Sat[4]	1-4[1]	4
Her[4]	1-2[1]	5
Pri[4]	1-2[1]	5
MaG[4]	3[1]	1

The grades of susceptibility: 1=very low, 2=low, 3=medium, 4=high, 5=very high
[1]European Cultivated Potato Database (2015)
[2] Federal Plant Variety Office of Germany (2014)
[3]true for ≤ 20 °C, otherwise overcomes the HR, leading to high susceptibility and plant damage
[4]Commercial varieties
[5]near isogenic variety

Healthy potato plants were propagated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 20 g/l of sucrose and maintained *in vitro* in a climate chamber at 24 °C under a 16 h/8 h light/dark cycle. They were free from any virus and endogenous bacteria. The PVY isolates Gr99 and 261-4 were maintained on tobacco (*Nicotiana tabacum* 'Samsun NN') in a climate chamber under constant conditions (20 °C, 16h/8h light/dark).

Transfer of PVY infected potato varieties from in vivo to *in vitro*

To minimize the experimental error resulting from mechanical inoculation, the quantification of PVY RNA copies was foreseen to be done on PVY-infected *in vitro* plants grown under identical conditions for several cycles of propagation. For the transfer of PVY infected potato plants from *in vivo* to *in vitro* several plants of each potato line were potted and inoculated mechanically in a greenhouse. Four weeks after inoculation the success of PVY infection was validated by DAS-ELISA ("PVY monoclonal cocktail" purchased from Bioreba AG, Reinach, Switzerland). Plants that tested positive were dissected into stem segments

containing at least one internode. For sterilization, the segments were washed with 70% ethanol for two minutes and then rinsed in sterile distilled water. Subsequently, the plant pieces were treated with 1.3% sodium hypochlorite for ten minutes. Afterwards, the segments were washed four times with sterile distilled water. Then the plant pieces were trimmed with a sterile scalpel to remove damaged cell layers and cultured in single glass tubes on MS medium containing 20 g/l of sucrose. The glass tubes were maintained in a climate chamber at 22 °C under a 16 h/8 h light/dark cycle. After four weeks vigorous non-contaminated sprouts were further propagated *in vitro* and evaluated regarding their suitability for being analyzed by using reverse transcription real-time PCR (RT-qPCR).

Greenhouse experiments

As it will be explained in the results section, using PVY-infected *in vitro* plants for RT-qPCR was not successful. Therefore, healthy potato lines originating from *in vitro* culture were potted into a standard commercial substrate and at least 50 plants of each line were placed in a greenhouse cabin with a day and night temperature maximum of 24 °C and 16 °C, respectively. After one week of recovery under a plastic tunnel the plants of each line were separated into two groups for mechanical inoculation either with PVY isolate Gr 99 or 261_4. For the inoculation, 1 g of PVY-infected tobacco leaves (second leaf above inoculated tobacco leaf) was homogenized in 10 ml of phosphate-buffered saline and mixed with carborundum powder (SiC). 150 µl of the virus suspension was then rubbed on the upper surface of potato leaves with a glass spatula. After 10 min the inoculated leaves were rinsed with tap water. All variants were randomly distributed within the greenhouse cabin. After two further weeks samples were taken from all tests plants. By using the lid of a 1.5 ml centrifuge tube two leaf discs per plant were punched out from the upper fully developed leaves. Samples were immediately frozen in liquid nitrogen. The experiment was conducted twice: in October 2010 and in May 2011. During the first and second experiment, the average day temperatures in the greenhouse chamber were 19 °C and 23 °C, respectively

RNA extraction and quantitative reverse transcription real-time PCR (RT-qPCR)

Each sample was homogenized in a 1.5 ml microcentrifuge tube containing 500 µl of RNA isolation reagent (Concert™ Plant RNA Reagent, Life Technologies™) and three 2.5 mm stainless steel beads, using a Retsch® mixer mill (Retsch GmbH). Further purification of RNA was done following the manufacturer's protocol. The purified precipitated RNA was redissolved in diethylpyrocarbonate (DEPC)-treated distilled water to a concentration of 200

to 400 ng per µl. The total RNA amount was measured using ultraviolet absorbance in a NanoDrop 8000 instrument (Thermo Fisher Scientific). For the quantification of PVY RNA, using reverse transcription quantitative PCR (RT-qPCR), RNA standards were prepared consisting of a dilution series of a PVY RNA transcript. The sequence of the transcripts corresponded to PVY isolate Gr99 from position 8600, which is a highly conserved region of the coat protein, in the poly-A tail. Reverse transcription was performed using 2 µl purified RNA and 18 µl RT mixture [DEPC-treated water; 1 x RT reaction buffer (Promega) consisting of 50 mM Tris-HCl (pH 8.3), 7 5mM KCl, 3 mM $MgCl_2$ and 10 mM DTT; 200 µM of each dNTP; 0.4 µM reverse primer (PVYall3-9420, primer sequence: CGGAGAGACACTACATCAC); 80 U M-MLV RT (Promega) and 4 U RNase inhibitor (RiboLock™, Thermo Fisher Scientific)]. The RT reaction was done in duplicate without denaturation at 42 °C for 50 min following 10 min at 72 °C for enzyme inactivation. In a second step 2 µl of cDNA reaction mix was combined with 18 µl qPCR reaction mix [distilled water; 1x reaction buffer (Bioron GmbH) consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 and 0.1% Tween 20; 150 µM of each dNTP; 0.3 µM of forward and reverse primers (PVYall5-9194, primer sequence: AGGTCACATCAC GAACAC and PVYall3-9420); 2.5 mM $MgCl_2$; 2 U Taq DNA polymerase (Bioron GmbH) and 1 x Sybr®Green I (Life Technologies™)]. PCR reaction was performed in white 96-well plates (Biozym Scientific GmbH) sealed with adhesive clear seals (Thermo Scientific ABsolute™ QPCR Seal) in a MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the following protocol: 2 minutes (min) at 95 °C for initial denaturation followed by 40 cycles with 15 seconds (s) at 94 °C for denaturation , 15 s at 58 °C for annealing, 20 s at 72 °C for elongation and 15 s at 85 °C for melting of non specific products. Fluorescence was measured after each cycle. Following real-time PCR, the amplicons were melted at 95°C for 1 min and then fully reannealed at 55°C for 1 min. For determination of the melting point(s) of the PCR product(s) the temperature was then increased incrementally to 95°C at a rate of 0.5°C every 10 s (as fixed on the MyiQ™2). Melting of amplicons generated with PVY primers happened at 87.5 °C whereas that of the primer dimers at 82 °C.

Statistics

The cDNAs of the samples and the standards were applied in duplicates. Standards were applied only to the first 96-well plate. An interplate calibrator (IPC) consisting of the diluted PCR generated with the PVY amplification primers was used for normalization and variation compensation of all plates. To calculate absolute quantities from amplification data, the software GenEx Enterprise 5 (MultiD Analyses AB, Sweden) was used for reverse

calibration. Results and the qPCR efficiency were calculated based on a 95% confidence level. The estimated efficiency over all qPCR plates was corrected to 100% using GenEx software. For each sample the total RNA per µl was determined after RNA extraction. Therefore, the absolute quantities of PLRV RNA could be given as copies of PLRV RNA per 300 ng total RNA. By using the Microsoft® Office Excel® 2007 (Microsoft® Cooperation, Redmond, USA) graphs were generated that contain the confidence intervals and means of the detected number of PVY RNA copies of GM varieties and the upper confidence limit of detected PVY RNA copies of all the commercial potato cultivars tested. A test for significance was conducted by using the procedure mixed/adjust=simulate within the software SAS, version 9.4 (SAS Institute Inc., Cary, USA). Equivalence was confirmed, if the mean of detected PVY RNA copies in transgenic lines was smaller than the upper 95% confidence limit of detected PVY RNA copies of all tested commercial potato varieties (van der Voet et al. 2011).

Results

The use of in vitro propagated PVY-infected plants

The experimental error may increase considerably, if the potato plants are mechanically inoculated with PVY. First because, different batches of inoculum mixtures can contain varying amounts of virus because PVY is not evenly distributed within *N. tabacum* due to recovery (Nie and Molen 2015). Second because, it is not possible to determine whether negative results may due to the resistance of a potato variety to PVY or a failure of inoculation (Solomon-Blackburn and Barker 2001). Therefore, propagated PVY-infected *in vitro* plants should be used for the determination of the absolute number of PVY RNA copies in transgenic and non-transgenic potato lines. It was assumed that during *in vitro* cultivation the virus titer in transgenic lines would reach a distinct level that could be evaluated for its equivalence with commercial varieties.

However, after four weeks of *in vitro* cultivation the shoots of some potato lines started to became necrotic with complete necrosis after eight weeks of cultivation (Fig. 1). This was observed only on shoots of genotypes of the varieties 'Albatros' and 'Desirée' infected with PVY^{N-Wi}, isolate 261-4. It is assumed that the necrosis was due to a systemic hypersensitive response (HR) caused either by a so far unknown resistance gene or by the *Ny* gene that, because of environmental conditions, was also activated by PVY^{N-Wi}.
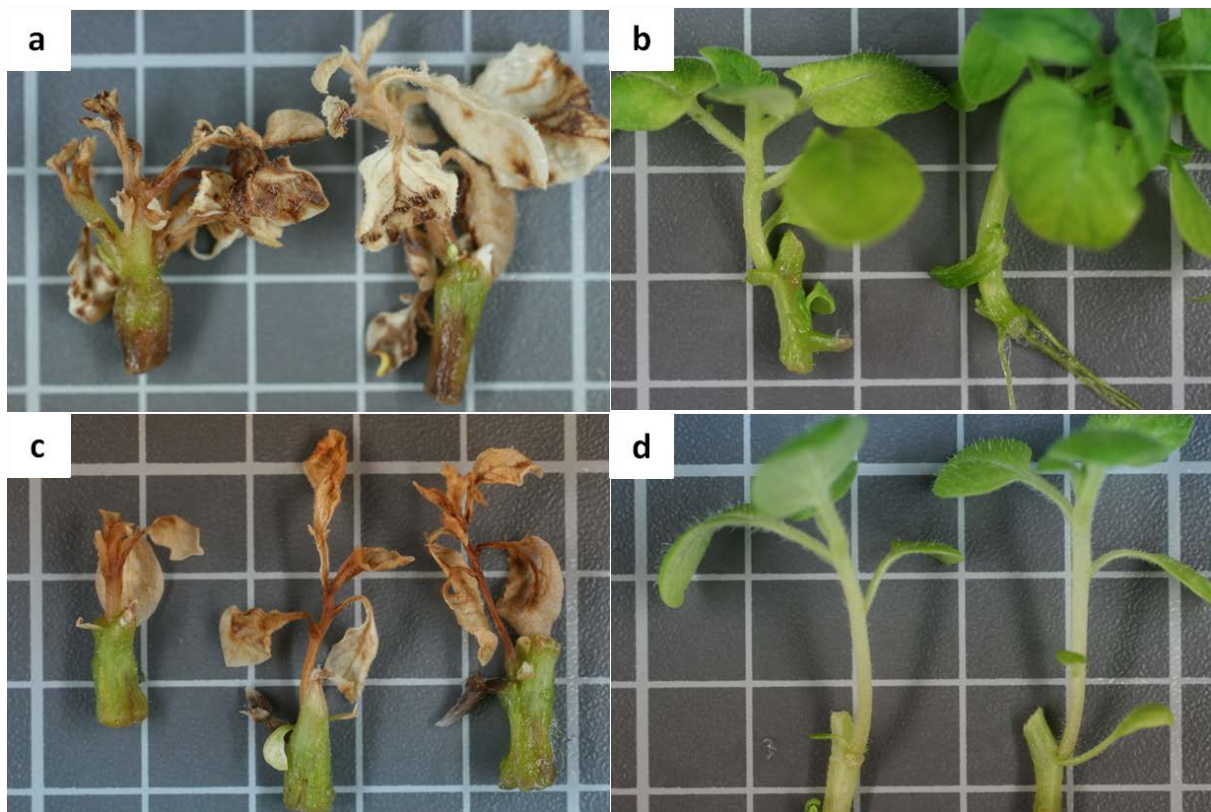
Fig. 1: Shoots from *in vitro* cultivated stem segments either infected with PVY^{N-Wi}, isolate 261-4 (**a,c**) or healthy (**b,d**). The picture was taken eight weeks after transferring the segments from *in vivo* to *in vitro*. The sprouts from niv varieties 'Albatros' (**a,b**) and 'Desirée' (**c,d**) show systemic necrosis (**a,c**), which indicates a systemic hypersensitive response (HR).

Because not all of the genotypes could be cultivated as *in vitro* plants, the experiments were conducted in a greenhouse with healthy *in vitro* plants that were mechanically inoculated but with special handling of the inoculum source. Only the second emerging leaf of PVY-infected *N. tabacum* 'Samsun NN' was used as the inoculum in order to obtain a consistent virus load. Furthermore, the inoculum was prepared in abundance and randomly rubbed onto the leaves of the different potato genotypes.

Comparison of the number of PVY RNA copies

The commercial varieties used in this study were 'Hermes', 'Saturna', 'Princess' and 'Mayan Gold'. The last mentioned variety is a *S. phureja* genotype expressing a Ry_{phu} gene for extreme resistance to PVY (Torrance et al. 2009; Al-Abedy et al. 2012). As expected, in plants of the variety 'Mayan Gold' no PVY was recorded in the isolate Gr99 or isolate 261-4, which is in contrast to the estimation of the European Cultivated Potato Database (ECPD) that classifies the resistance of this variety as "medium". If the number of PVY RNA copies detected is accepted as an indicator of susceptibility, the other three commercial varieties can

be classed as highly susceptible (Tab. 2). Regardless of which isolate was used for infection, the upper 95% confidence limit of detected PVY RNA copies of varieties 'Hermes', 'Saturna' and 'Princess' was very similar and ranged between 6.8E6 (Gr99) and 7.4E6 (261-4). Therefore, the commercial varieties were suitable for use in an evaluation of the equivalence of transgenic potato lines.

'Albatros'

The average number of PVY RNA copies in 'Albatros' genotypes was always lower than the upper 95% confidence limit of commercial varieties, independent of the PVY isolate (Fig. 2a). The near isogenic variety (niv) and the control expressing only the kanamycin resistance marker gene had similar virus titers, whereas the transgenic 'Albatros' lines expressing either the VP60 and *npt*II or the *cph*A and *npt*II genes showed lower and higher numbers of PVY RNA copies, respectively, than the control and the niv. Particularly, the transgenic line expressing the *cph*A and *npt*II genes seems to be more susceptible to isolate 261-4 than to isolate Gr99. In comparison to an infection with isolate Gr99, the virus titers increased by a factor of ten, when the control and the niv were infected with isolate 261-4. In the transgenic line expressing the *cph*A and *npt*II genes, the virus titer increased by a factor of 100 when infected with isolate 261-4. Nevertheless, the means of all 'Albatros' genotypes were smaller than the upper 95% confidence limit of commercial varieties indicating equivalence regarding the level of PVY susceptibility.

'Desirée'

In the 'Desirée' genotypes the virus titers increased only by approximately four times, when the plants were infected with isolate 261-4 (in comparison to an infection with isolate Gr99). However, since 'Desirée' is highly susceptible to infection with PVY, this factor was sufficient to cross the 95% confidence limit of the commercial varieties. Whereas the means of all 'Desirée' genotypes, independent of whether transgenic or non-transgenic, were smaller than the 95% confidence limit, when the plants were infected with isolate Gr99, however, the picture changed, when plants were infected with isolate 261-4 (Fig. 2b). All means of the 'Desirée' genotypes were higher than the 95% confidence limit. Even the niv completely exceeded the upper 95% confidence limit of commercial varieties. In contrast to the commercial varieties, which hardly differed in the number of PVY RNA copies detected
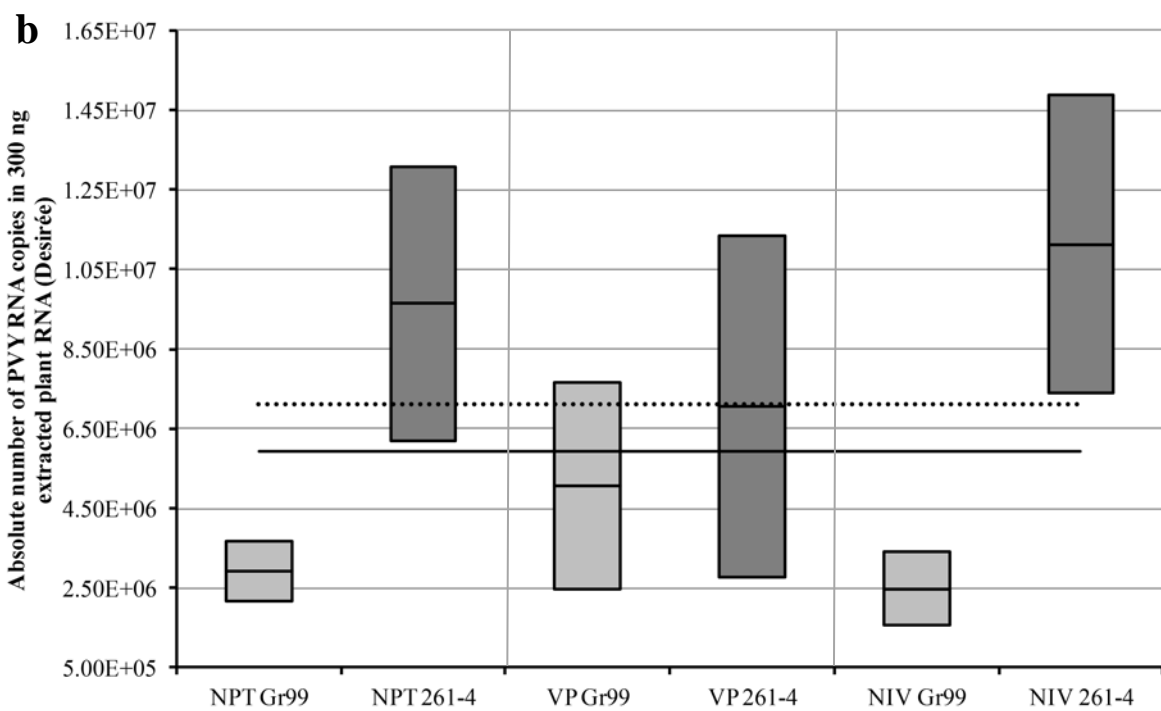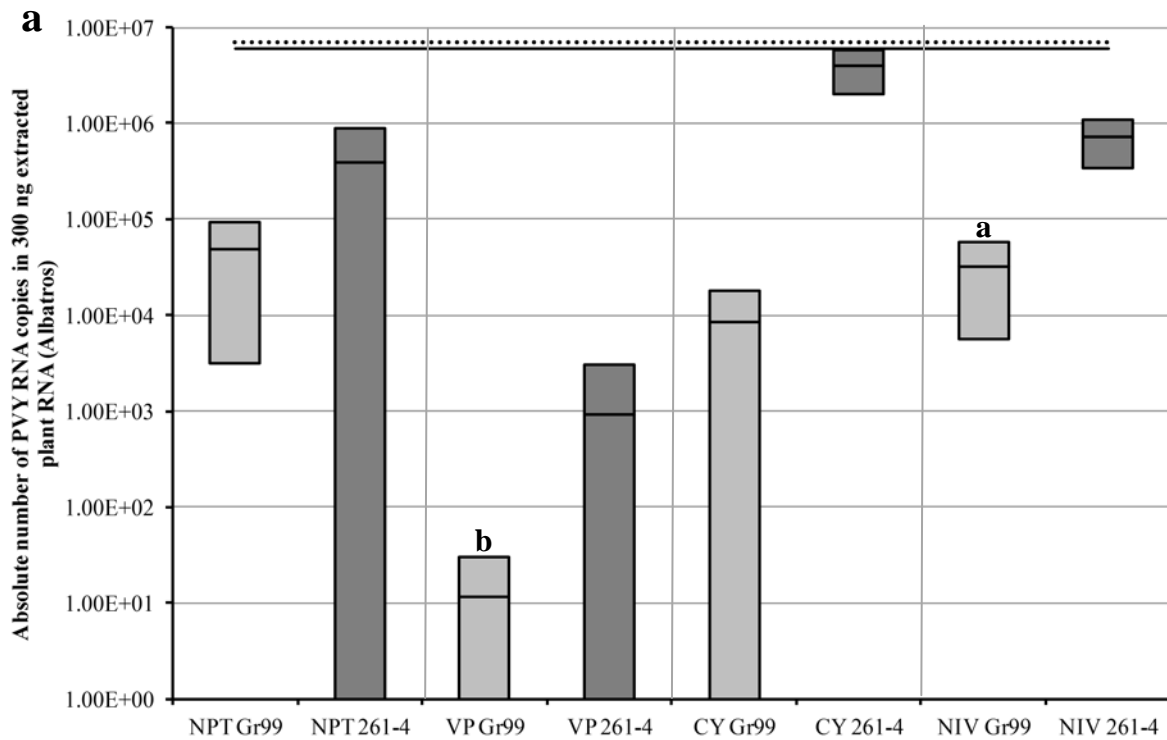
Fig. 2: The absolute number of PVY RNA copies in transgenic and non-transgenic lines of varieties 'Albatros' (**a**) and Desirée (**b**). NIV = near isogenic variety, NPT = *npt*II, CY = cyanophycin synthetase (*cph*A) and *npt*II, VP = VP60 and *npt*II. The solid and dotted horizontal lines represent the mean and upper 95% confidence limit, respectively, of all commercial varieties. All values of transgenic and non- transgenic lines are presented as 95% confidence intervals, in which the upper and lower lines of the boxes are the upper and lower 95% confidence limits, respectively. The line in the middle of the boxes is the mean. During the test of significance the niv was tested against the other lines at the strain level. Significant differences are indicated by different letters above the boxes. The y-axis in graph **a** is plotted on a logarithmic scale. n>45

for the different isolates, the 'Desirée' genotypes seem to be more susceptible to an infection with isolate 261-4. However, it is not correct to reject equivalence regarding the level of PVY susceptibility for transgenic 'Desirée' lines, since the niv was also not equivalent. In this case the assortment of commercial varieties and the choice of 'Desirée' for genetic engineering have to be discussed.

Influence of temperature on multiplication of PVY in the 'Albatros' lines

It is known, that 'Albatros' expresses the HR gene (*Ny*-1) in a temperature-dependent manner (Szajko et al. 2014). In this study we determined whether the HR gene of 'Albatros' is able to restrict the virus at 18 °C and 24 °C, respectively. Therefore, the niv and two transgenic lines of 'Albatros', expressing either the *npt*II or the *cph*A and *npt*II genes, were mechanically inoculated with PVY[N-Wi] (isolate 261-4) and PVY[NTN] (isolate Gr99), respectively. Subsequently, the plants were further cultivated in climate chambers, one at constant 18 °C and the other at 24 °C. Three weeks after inoculation, the plants were sampled and subjected to RT-qPCR as described in the materials and methods.

At the time of sampling, no PVY symptoms and only a few local lesions were visible on plants that were maintained at 18 °C (right plants in Fig. 3). In contrast, plants developed severe PVY symptoms and extensive necrotic lesions when maintained at 24 °C (left plants in Fig. 3). Plants infected with PVY[N-Wi] (isolate 261-4) and the transgenic lines expressing the *cph*A and *npt*II genes were severely necrotic.
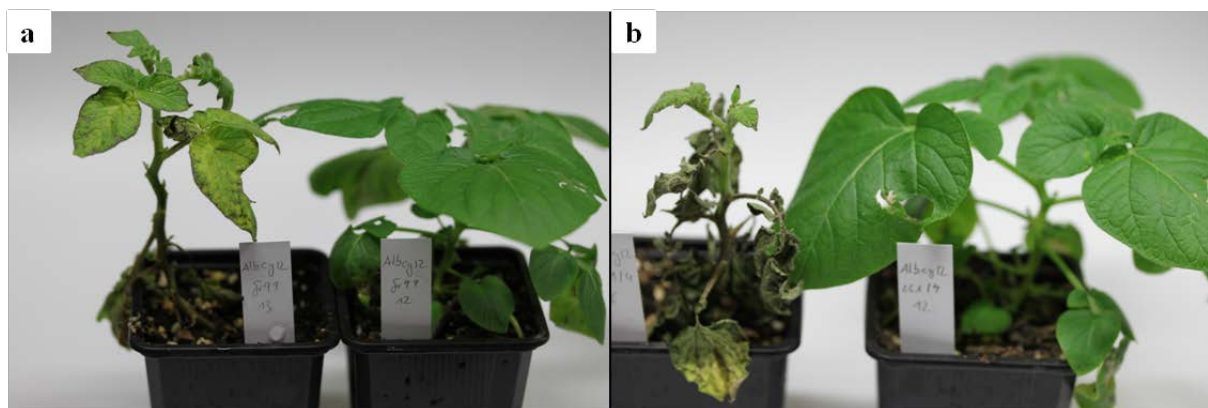
Fig. 3: Plants of the transgenic lines of 'Albatros' expressing the *cph*A and *npt*II genes infected either with isolate Gr99 (**a**) or 261-4 (**b**). The left plant in each picture was maintained at 24 °C, the right plant at 18 °C.
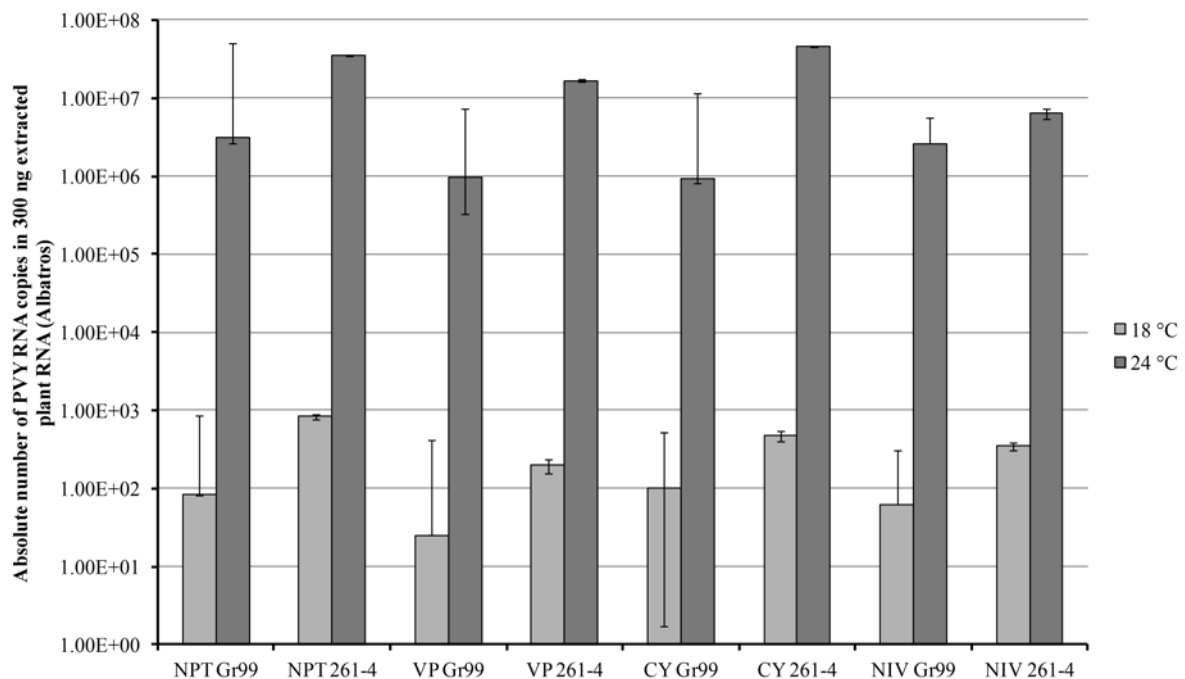
Fig. 4: Means of the absolute number of PVY RNA copies of isolate Gr99 and 261-4 in transgenic and non-transgenic lines of variety 'Albatros'. NPT = *npt*II, VP = VP60 and *npt*II, CY = cyanophycin synthetase (*cph*A) and *npt*II, NIV = near isogenic variety. The error bars illustrate the standard deviation. The y-axis is plotted on a logarithmic scale.

The visual assessment was confirmed by the evaluation of detected PVY RNA copies in the 'Albatros' genotypes (Fig. 4). In plants that were maintained at 18 °C and developed single necrotic lesions, the number of detected PVY RNA was low and ranged between 100 and 1,000 RNA copies in 300 ng of extracted plant RNA. In contrast, plants that were maintained at 24 °C and showed severe PVY symptoms and necrosis were heavily infected, with recorded PVY RNA copy numbers of between 1.0E6 and 1.0E8 detected in 300 ng extracted plant RNA. Although commercial varieties were not tested during this experiment, the means of PVY (261-4) RNA copies detected in transgenic 'Albatros' lines maintained at 24 °C probably are higher than the 95% confidence limit of commercial varieties, which was 7.4E6 PVY RNA copies in the other experiments. Therefore, the equivalence of transgenic 'Albatros' lines regarding the level of PVY susceptibility has to be evaluated in terms of the prevailing temperature and different PVY isolates.

Discussion

Unintended effects may appear in transgenic plants that respond differently in stress situations, such as unfavorable environmental conditions or infections by pathogens (Matthews et al. 2005). Although rarely documented, modifications in the genome could be responsible for unintended metabolic changes causing increased susceptibility to pathogens (Ting et al. 2008). To analyze equivalence non-transgenic counterparts and unintended effects, several molecular methods have been developed over time, such as DNA, protein or metabolite profiling (Vega and Marina 2014). However, the application of these techniques is costly and changes in the protein level may not *per se* have a negative effect on the performance of a plant, for instance, when stressed. Therefore, infection studies can reveal unintended effects by evaluating the susceptibility to a pathogen of transgenic lines. If the susceptibility of a transgenic line to a pathogen is non-equivalent to commercial varieties, then a further assessment is needed involving the use of several molecular techniques as previously mentioned.

In this study we examined transgenic potato lines either transformed with *cph*A and *npt*II, VP60 and *npt*II or the *npt*II marker gene alone for possible changes in their susceptibility to PVY, one of the most important potato viruses worldwide. We compared the transgenic lines with their near isogenic varieties (niv) in order to determine any differences in PVY susceptibility, which we measured by means of the total number of PVY RNA copies in extracted plant RNA.

Equivalence regarding the susceptibility to PVY was confirmed for transgenic lines, whose average number of detected PVY RNA copies did not cross the upper 95% confidence limit calculated for several commercial potato varieties. This is in accordance with van der Voet et al. (2011), who recommend that the differences and equivalences should be assessed in a comparable manner. In this study we selected commercial potato varieties, which have a broad range of susceptibility to PVY infection, according to the Eupopean Cultivated Potato Database (ECPD) and Plant Variety Office of Germany (BSA) (Tab. 2). However, by using the detectable amounts of PVY RNA copies as an indicator of PVY susceptibility, the results in this study differ from the classification of these two institutions. The resistance to PVY of the variety 'Mayan Gold' is classed as "medium" by the ECPD, which differs from our results. We could not detect any PVY RNA in the plants of this variety. 'Mayan Gold' is a *Solanum phureja* cultigen with has at least one gene for extreme resistance (Torrance et al. 2009; Al-Abedy et al. 2012). An infection with PVY is therefore extremely unlikely. Furthermore, in this study the detected amounts of PVY RNA copies in varieties 'Princess'

and 'Hermes' were in the same range as for the highly susceptible variety 'Desiree'(results not shown). However, 'Princess' and 'Hermes' are classed as lowly susceptible by the BSA (Tab. 2). This discrepancy is mainly due the use of field assessments by the BSA rather than mechanical inoculation, which results in a higher virus pressure. Furthermore, BSA uses the severity of symptoms as an indicator for PVY susceptibility. However, the severity of symptoms is not correlated with the detectable amounts of PVY RNA copies (Hühnlein et al. 2013; Lindner et al. 2015) and symptom tolerant potato varieties may serve as a source of infection in the field as they contain considerable amounts of virus. Therefore, the number of detectable PVY RNA copies is a more reliable indicator of PVY susceptibility.

'Albatros' is recorded as resistant to PVY infection due to its dominant HR gene (*Ny*-1). However, the HR gene is expressed in a temperature-dependent manner.
In experiments conducted by Szajko et al. (2014) plants of variety 'Albatros' were inoculated with PVY^{N-Wi} and maintained at 20 °C and 28 °C, respectively. At 20 °C the virus was localized due to the formation of local lesions. At 28 °C, the PVY-infection became systemic without any symptoms or signs of a HR. This differs from our results, in which plants of the 'Albatros' lines developed severe PVY symptoms and necrosis when maintained at 24 °C. It seems that two critical factors promoting the severe symptoms coincide (i): at 24 °C the virus spreads too fast to be restricted by programmed cell death and (ii): at the same temperature the HR gene is still expressed leading to a massive synthesis of reactive oxygen species (ROS) due to the systemic spread of the virus causing death of whole tissues.

The temperature-dependent resistance of 'Albatros' to PVY was the reason for the equivalence of transgenic 'Albatros' lines with commercial varieties. However, the 'Albatros' lines contained considerable amounts of PVY. This was due to the elevated temperatures in the greenhouse in May 2011 leading to a partial absence of HR in this lines. Therefore, experiments used to evaluate the equivalence of transgenic lines in terms of their susceptibility to a virus should always be done under the "worst case" conditions, which are most likely to facilitate the spread of the virus. This in turn requires a complete understanding of the mechanisms underlying the plant-virus interactions of each line that is tested. Surprisingly, the number of PVY RNA copies that were detected in the transgenic 'Albatros' line expressing the VP60 was considerably lower than in the other 'Albatros' lines, independent of the virus strain used for infection. One could speculate, that the coat protein of the RHDV may cause a kind of cross resistance to a PVY infection. However, this was not observed in 'Desirée' genotypes and, therefore, needs further investigation.

'Desirée' turned out to be an unfavorable variety for genetic modification because of its inherently high susceptibility to PVY. The cultivation of transgenic 'Desirée' lines would

require extensive measures to control vector aphids in the field, and yield losses due to a PVY infection probably would not be accepted by growers. Therefore, genetic modification should be applied to varieties that are highly resistant to PVY (and indeed also to other pathogens), preferably with extreme resistance, which is expressed independent of environmental conditions. For instance, progenies of somatic hybridization between *S. tuberosum* and *S. tarnii* are extremely resistant to all known PVY strains and in addition, highly resistant to infection with potato late blight caused by *Phytophthora infestans* (Thieme et al. 2008).

In this study the equivalence regarding the level of susceptibility to PVY was confirmed for transgenic 'Albatros' lines. However, equivalence cannot be assessed if the susceptibility of the niv is higher than the upper 95% confidence limit of commercial varieties, which was the case for transgenic 'Desirée' lines. Therefore, we recommend the use of extremely resistant rather than highly susceptible varieties for genetic modification.

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References

Ahn, S.-Y., Cho, K.-S., Seo, H.-W., Yi, J.-Y., Bae, S.-C., Cho, J.-H., et al. (2011). Evaluation of agronomic characteristics, nutritional contents, and insect response of the transgenic potato resistant to glufosinate ammonium. *Korean Journal of Horticultural Science & Technology, 29*(3), 247-254.

Al-Abedy, A., Cowan, G., McLean, K., Bryan, G., MacFarlane, S., & Torrance, L. (2012). Genetic analysis of Potyvirus resistance in *Solanum tuberosum* group *Phureja*. *The Dundee Conference. Crop Protection in Northern Britain, 2012, Dundee, UK, 28-29 February 2012*, 291-295.

An, G., Watson, B. D., & Chiang, C. C. (1986). Transformation of tobacco, tomato, potato, and *Arabidopsis thaliana* using a binary TI vector system. *Plant Physiology, 81*(1), 301-305, doi:10.1104/pp.81.1.301.

Chung, B. N., Yoon, J.-Y., & Palukaitis, P. (2013). Engineered resistance in potato against *Potato leafroll virus, Potato virus A* and *Potato virus Y*. *Virus Genes, 47*(1), 86-92, doi:10.1007/s11262-013-0904-4.

Dinh, P. T. Y., Zhang, L., Brown, C. R., & Elling, A. A. (2014). Plant-mediated RNA interference of effector gene Mc16D10L confers resistance against *Meloidogyne chitwoodi* in diverse genetic backgrounds of potato and reduces pathogenicity of nematode offspring. *Nematology, 16*, 669-682, doi:10.1163/15685411-00002796.

ECPD (2015). ECPD: The European Cultivated Potato Database. The European Cultivated Potato Database. http://www.europotato.org. Accessed 22nd June 2015.

FAO (2008). Food Outlook - Global Market Analysis, pp. 58-60.

FAOSTAT (2015). Worldwide potato production in 2013. Food and Agriculture Organization of the United Nations - Statistics. http://faostat3.fao.org/browse/Q/QC/E. Accessed 15th July 2015.

Federal Plant Variety Office of Germany (2014). *Descriptive Variety Lists - Potato*. Hannover, Germany: Federal Plant Variety Office of Germany.

Hühnlein, A., Drechsler, N., Steinbach, P., Thieme, T., & Schubert, J. (2013). Comparison of three methods for the detection of *Potato virus Y* in seed potato certification. *Journal of Plant Diseases and Protection, 120*(2), 57-69.

Hühns, M., Neumann, K., Hausmann, T., Klemke, F., Lockau, W., Kahmann, U., et al. (2009). Tuber-specific *cphA* expression to enhance cyanophycin production in potatoes. *Plant Biotechnology Journal, 7*(9), 883-898, doi:10.1111/j.1467-7652.2009.00451.x.

Jahan, S. N., Asman, A. K. M., Corcoran, P., Fogelqvist, J., Vetukuri, R. R., & Dixelius, C. (2015). Plant-mediated gene silencing restricts growth of the potato late blight pathogen *Phytophthora infestans*. *Journal of Experimental Botany, 66*(9), 2785-2794, doi:10.1093/jxb/erv094.

Jose, S., Ignacimuthu, S., Ramakrishnan, M., Srinivasan, K., Thomas, G., Kannan, P., et al. (2014). Expression of GroES TB antigen in tobacco and potato. *Plant Cell Tissue and Organ Culture, 119*(1), 157-169, doi:10.1007/s11240-014-0522-4.

Karasev, A. V., Hu, X., Brown, C. J., Kerlan, C., Nikolaeva, O. V., Crosslin, J. M., et al. (2011). Genetic diversity of the ordinary strain of *Potato virus Y* (PVY) and origin of recombinant PVY strains. *Phytopathology, 101*(7), 778-785, doi:10.1094/phyto-10-10-0284.

Kikuchi, A., Huu Duc, H., Endo, T., & Watanabe, K. (2015). Review of recent transgenic studies on abiotic stress tolerance and future molecular breeding in potato. *Breeding Science, 65*(1), 85-102, doi:10.1270/jsbbs.65.85.

Li, W., Wang, B., Wang, M., Chen, M., Yin, J.-M., Kaleri, G. M., et al. (2014). Cloning and characterization of a potato StAN11 gene involved in anthocyanin biosynthesis regulation. *Journal of Integrative Plant Biology, 56*(4), 364-372, doi:10.1111/jipb.12136.

Lindner, K., & Billenkamp, N. (2005). Veränderungen im Stammspektrum des Kartoffelvirus Y: Eine Ursache für die Zunahme der Virusanfälligkeit von Kartoffel- und Tabaksorten? *Journal für Kulturpflanzen, 57*(12), 245-353.

Lindner, K., Trautwein, F., Kellermann, A., & Bauch, G. (2015). *Potato virus Y* (PVY) in seed potato certification. *Journal of Plant Diseases and Protection, 122*(3), 109-119.

Matthews, D., Jones, H., Gans, P., Coates, S., & Smith, L. M. J. (2005). Toxic secondary metabolite production in genetically modified potatoes in response to stress. *Journal of Agricultural and Food Chemistry, 53*(20), 7766-7776, doi:10.1021/jf050589r.

Mikschofsky, H., Hartmann, A., Janczyk, P., Keil, G. M., Konig, P., Schirrmeier, H., et al. (2011a). Expression of the viral antigen VP60 in transgenic potatoes and its effect on the nutritional composition of tubers. *Food and Nutrition Sciences, 2*(2), 74-86, doi:10.4236/fns.2011.22010.

Mikschofsky, H., Heilmann, E., Schmidtke, J., Schmidt, K., Meyer, U., Leinweber, P., et al. (2011b). Greenhouse and field cultivations of antigen-expressing potatoes focusing on the variability in plant constituents and antigen expression. *Plant Molecular Biology, 76*(1-2), 131-144, doi:10.1007/s11103-011-9774-0.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497, doi:10.1111/j.1399-3054.1962.tb08052.x.

Nie, X., & Molen, T. A. (2015). Host recovery and reduced virus level in the upper leaves after *Potato virus Y* infection occur in tobacco and tomato but not in potato plants. *Viruses-Basel, 7*(2), 680-698, doi:10.3390/v7020680.

OECD (2002). Consensus document on compositional considerations for new varieties of potatoes: Key food and feed nutrients, anti-nutrients and toxins. (ENV/JM/MONO(2002)5). Series on the safety of novel foods and feeds, pp. 1-26.

Ooms, G., Bossen, M. E., Burrell, M. M., & Karp, A. (1986). Genetic manipulation in potato with *Agrobacterium rhizogenes. Potato Research, 29*(3), 367-379.

Perry, J. N., Ter Braak, C. J. F., Dixon, P. M., Duan, J. J., Hails, R. S., Huesken, A., et al. (2009). Statistical aspects of environmental risk assessment of GM plants for effects on non-target organisms. *Environmental Biosafety Research, 8*(2), 65–78, doi:10.1051/ebr/2009009.

Quintero-Ferrer, A., & Karasev, A. V. (2013). First report of recombinant *Potato virus Y* strains in potato in Jalisco, Mexico. *Plant Disease, 97*(3), 430-430, doi:10.1094/pdis-10-12-0919-pdn.

Rakosy-Tican, E., Thieme, R., Nachtigall, M., Molnar, I., & Denes, T.-E. (2015). The recipient potato cultivar influences the genetic makeup of the somatic hybrids between five potato cultivars and one cloned accession of sexually incompatible species *Solanum bulbocastanum* Dun. *Plant Cell Tissue and Organ Culture, 122*(2), 395-407, doi:10.1007/s11240-015-0777-4.

Romano, A., van der Plas, L. H. W., Witholt, B., Eggink, G., & Mooibroek, H. (2005). Expression of poly-3-(R)-hydroxyalkanoate (PHA) polymerase and acyl-CoA-transacylase in plastids of transgenic potato leads to the synthesis of a hydrophobic polymer, presumably medium-chain-length PHAs. *Planta, 220*(3), 455-464, doi:10.1007/s00425-004-1349-8.

Rowley, J., Gray, S., & Karasev, A. (2015). Screening potato cultivars for new sources of resistance to *Potato virus Y*. *American Journal of Potato Research, 92*(1), 38-48, doi:10.1007/s12230-014-9409-5.

Rukavtsova, E. B., Rudenko, N. V., Puchko, E. N., Zakharchenko, N. S., & Buryanov, Y. I. (2015). Study of the immunogenicity of hepatitis B surface antigen synthesized in transgenic potato plants with increased biosafety. *Journal of Biotechnology, 203*, 84-88, doi:10.1016/j.jbiotec.2015.03.019.

Shahin, E. A., & Simpson, R. B. (1986). Gene-transfer system for potato. *HortScience, 21*(5), 1199-1201.

Simon, R. D. (1976). Biosynthesis of multi-L-arginyl-poly(L-aspartic acid) in filamentous cyanobacterium *Anabaena cylindrica*. *Biochimica et Biophysica Acta, 422*(2), 407-418, doi:10.1016/0005-2744(76)90151-0.

Solomon-Blackburn, R. M., & Barker, H. (2001). Breeding virus resistant potatoes *(Solanum tuberosum)*: a review of traditional and molecular approaches. *Heredity, 86*, 17-35, doi:10.1046/j.1365-2540.2001.00799.x.

Szajko, K., Strzelczyk-Żyta, D., & Marczewski, W. (2014). *Ny*-1 and *Ny*-2 genes conferring hypersensitive response to *Potato virus Y* (PVY) in cultivated potatoes: mapping and marker-assisted selection validation for PVY resistance in potato breeding. *Molecular Breeding, 34*(1), 267-271, doi:10.1007/s11032-014-0024-4.

Thieme, R., Rakosy-Tican, E., Gavrilenko, T., Antonova, O., Schubert, J., Nachtigall, M., et al. (2008). Novel somatic hybrids (*Solanum tuberosum* L. (+) *Solanum tarnii*) and their fertile BC$_1$ progenies express extreme resistance to *Potato virus Y* and late blight. *Theoretical and Applied Genetics, 116*(5), 691-700.

Ting, M. Y., Shih, H. D., & Lin, C. Y. (2008). Increased susceptibility of rice following insertion of amylopullulanase gene, to brown spot caused by *Bipolaris oryzae*. *Journal of Phytopathology, 156*(9), 530-533, doi:10.1111/j.1439-0434.2008.01407.x.

Torrance, L., Liu, H., Cowan, G., Bradshaw, J., & MacFarlane, S. (2009). Extreme resistance to potyviruses in *Solanum tuberosum* group *phureja*. *Aspects of Applied Biology*(94), 1-4.

Vega, E. D., & Marina, M. L. (2014). Characterization and study of transgenic cultivars by capillary and microchip electrophoresis. *International Journal of Molecular Sciences, 15*(12), 23851-23877, doi:10.3390/ijms151223851.

van der Voet, H., Perry, J. N., Amzal, B., & Paoletti, C. (2011). A statistical assessment of differences and equivalences between genetically modified and reference plant varieties. *BMC Biotechnology, 11*, doi:10.1186/1472-6750-11-15.

WHO (2005). WHO informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants, World Health Organization, Geneva, Switzerland, p. 16.

Zhang, J., Khan, S. A., Hasse, C., Ruf, S., Heckel, D. G., & Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science, 347*(6225), 991-994, doi:10.1126/science.1261680.

Zhu, X., Richael, C., Chamberlain, P., Busse, J. S., Bussan, A. J., Jiang, J., et al. (2014). Vacuolar invertase gene silencing in potato (*Solanum tuberosum* L.) improves processing quality by decreasing the frequency of sugar-end defects. *PLoS ONE, 9*(4), doi:10.1371/journal.pone.0093381.

6 Discussion and outlook

In this thesis the development and applications of RT-qPCR detection assays were presented, that are able to quantify the worldwide most important potato viruses PVY and PLRV and thus can contribute to several areas of potato research. But why is research on potato so essential, especially concerning virus infections?

6.1 High infection rates of potatoes with PVY and PLRV in developing countries

Within the industrialized nations agriculture is highly technologized. Substantial measures in pest management, fertilization and irrigation lead to high commercial crop yields that are close to the respective potential yield, which is the maximum yield of a given cultivar in a certain region under optimal environmental conditions (Pereira et al. 2008; Licker et al. 2010). The potential yield of potato varies considerably depending on the geographic region. The highest yield potential with up to 69 tones/ha can be found in regions, such as Southeastern Europe, at the foot of the Pamir Mountains, in the Shanxi province of China and along the Andes Mountains (Licker et al. 2010; Foley et al. 2011). In developing countries potato is a valuable crop especially where it contributes as a staple to hunger reduction and increasing the employment rate and income (Thiele et al. 2010). The good nutritive value and cooking versatility have led to a tripling of potato consumption in developing countries in the last 50 years (Lutaladio and Castaidi 2009). However, while the size of arable land utilized for potato production increased in the last decades in developing countries, the yield remained static (Pandey 2007). On the one hand, developing countries lack an efficient system for regular multiplication and distribution of certified seed tubers. On the other hand, pests and diseases considerably constrain potato production because of an insufficient rapid deployment of new, improved varieties and due to poor crop management measures (Lutaladio et al. 2010). In developing countries the yield per hectare reaches only less than the half of the yield in industrialized nations. Even in India and China, the most important producers of potatoes worldwide, the yields per hectare are relatively low with only 22.9 and 17.0 tones/hectare, respectively (FAOSTAT 2014) and the high potential yield is not reached.

The major cause of this low productivity is considered to be seed degeneration over multiple generations due to seed borne diseases, such as bacterial wilt (*Ralstonia solanacearum*), virus infections (PVY and PLRV), soft rot (*Erwinia chrysanthemi*) and *Fusarium* wilt and dry rot (*Fusarium solani*) (Fuglie 2007; Gildemacher et al. 2009). In contrast, industrialized countries have effective seed certification systems, where the seed tubers are replaced by certified seeds, annually. This "certified seed replacement paradigm" led seed degeneration to become only a background noise in Western Europe and North America (Thomas-Sharma et al. 2016).

However, in several developing countries, particularly in Western and Central Africa, suboptimal production areas are prevalent with a warm and humid climate leading to a high disease pressure. There, formal seed certification systems economically make less sense, because the costs for producing healthy seeds would exceed the gain from their cultivation. For such countries an alternative may be an integrated seed health strategy that has a stronger focus on on-farm management practices, such as roguing of diseased plants or vector management, and the use of resistant varieties (Thomas-Sharma et al. 2016). A study conducted 2006 in Kenya revealed an average incidence of PVY and PLRV infections of seed potatoes purchased from rural markets of 78% and 74%, respectively (Gildemacher et al. 2009). According to Nolte et al. (2004) a 1% increase of the incidence of PVY infections results in yield reduction of 0.17-0.18 tones/ha, independent from the cultivar used. Thus, a 78% incidence of PVY infections, as mentioned above, would result in an average yield loss of 12.95 tones/ha, which is more than the half of the potential yield calculated for Kenyan potato growing regions (Licker et al. 2010; Foley et al. 2011). Therefore, developing countries, especially those with suboptimal growing conditions, need potato cultivars that harbor the so-called "pro-poor" traits, such as a broad range of disease resistance and a high adaptability to regional climatic conditions (Thiele et al. 2010). With the quantitative detection of PVY and PLRV, presented in this thesis, it is possible to evaluate the resistance type of a potato variety and to record slight differences in the accumulation of virus within a plant under various climatic conditions (Hühnlein et al. 2016c). The authors demonstrated that in potatoes harboring genes for extreme resistance (ER) against an infection with PVY small amounts of virus RNA can be detected by RT-qPCR but not with ELISA. Furthermore, the sensitive method was able to detect differences in the number of virus particles between wild potato species regarded as extremely resistant. Whereas in *S. pinnatisectum*, no PVY RNA copies were recorded independently from the PVY strain used for inoculation, low amounts of virus RNA were detected in *S. etuberosum.* In the plants of this genotype 240 and 1,400 PVY RNA copies were recorded on average in 300 ng of total plant RNA for infections with PVY[N-Wi] (isolate 261-4) and PVY[NTN] (isolate Gr99), respectively. From this it can be inferred that the mechanism and the efficiency of resistance genes differ between wild potato species. This should be expected as they are located on different chromosomes. The finding that the mechanisms of resistance differ was further supported by the fact that the recorded virus titer differed not only between the genotypes but also between the PVY strains used for inoculation. Whereas in plants of *S. cardiophyllum* PVY was detected only after an infection with PVY[N-Wi], in plants of *S. stoloniferum* successful infections (500 PVY RNA copies in 300 ng extracted plant RNA) were only recorded for PVY[NTN]. It seems that the virus strains harbor different virulence genes. These findings and the developed RT-qPCR assay can be useful to support potato breeding research. However, for practical purposes it does not make a

difference whether a plant accumulates 100 or 1,000 virus RNA copies by remaining symptomless. Therefore, the ELISA test constitutes a kind of threshold, up to which resistance is able to restrict the virus sufficiently enough to prevent yield reduction and transmission of the virus by vectors. For RT-qPCR such a threshold would have to be defined, which is complicated because the threshold will vary between PVY strains and between different environmental conditions. However, since *S. stoloniferum* is a well studied representative of ER, a threshold could be generated by using cultivars, whose ancestry contains a *S. stoloniferum* accession, such as 'Assia' or 'Fanal' (Flis et al. 2005b). A threshold could then be calculated from the average number of detected PVY RNA copies of different PVY strains. Genotypes that later on will be compared with this threshold could be classed partly resistant, if they have significantly higher amounts of virus RNA than the threshold value. Genotypes with lower virus amounts or for which the difference is not significant could be classed as resistant. However, non-significant differences have to be verified by a power analysis, because the "absence of evidence is not evidence of absence" (Altman and Bland 1995). A power analysis could be conducted, for instance, by using the code described in Littell (2006, p. 480) and the software SAS, version 9.4 (SAS Institute Inc., Cary, USA).

6.2   Climate change may increase the prevalence of PVY and PLRV infections

A further important challenge in potato research is the climate change. During the last 130 years the global surface temperature shows an average increase of 0.85 K and the global temperature is expected to increase further by at least 2 K and maximal 4 K, dependent on the amount of future anthropogenic CO_2 emissions (IPCC 2014). An increase of the global temperature will also increase the number of periods of heat and drought, which can have a drastic negative effect on potato yield, especially in tropical, subtropical and continental climates (Hijmans 2003). However, a temperature increase has also several direct and indirect impacts on the significance of viral diseases on potato. Direct impacts are known to result from a reduced performance of temperature-dependent resistance genes, such as the dominant *N* genes triggering hypersensitive response (HR) upon a virus infection (Canto and Palukaitis 2002; Szajko et al. 2014; Hühnlein et al. 2016c). At temperatures higher than 28 °C, the HR becomes inactive leading to a systemic infection of the host plant. In this thesis it was demonstrated that transgenic and non-transgenic 'Albatros' lines grown at constant 24 °C were dramatically affected by a PVY infection. Whereas at 28 °C the infection becomes systemic and symptomless (Szajko et al. 2014), at 24 °C the *Ny-1* gene was still active but was not able to restrict the virus resulting in systemic HR and severe necroses leading to plant death (Hühnlein et al. 2016a). Potato varieties harboring *N* genes for virus resistance may therefore be unsuitable for cultivation in warmer climates. In contrast, resistance mechanisms

that are based on RNA silencing seem to be positively affected by increased temperatures, resulting in an increase of virus-derived siRNAs and recovery at elevated temperatures (Szittya et al. 2003; Sun et al. 2008; Wu et al. 2008). In other studies, however, no correlation was found between the temperature and the efficiency of RNA silencing (Sos-Hegedus et al. 2005; Ali et al. 2013; Romon et al. 2013) indicating different mechanisms for different hosts and targeted genes. Nevertheless, genetic engineered potato lines harboring resistance traits based on RNA interference may be promising for the use in warmer climates, especially through possible combinations with other resistance or tuber traits. However, such plants will be regulated by the governmental GMO legislation implicating an assessment of possible risks for humans and the environment as well as an evaluation of these transgenic lines regarding their substantial equivalence. In this thesis a method was presented, with which the equivalence of transgenic potato lines regarding their susceptibility to PVY can be assessed. Since its high sensitivity, this method enables also the detection of virus RNA in highly resistant varieties. Moreover, significant differences of the detectable amounts of virus RNA, although on a very low level, can be recognized, which offers an opportunity to register possible unintended effects of the new traits within transgenic potato plants (Hühnlein et al. 2016a). At an international workshop on transgenic potatoes for the benefit of resource-poor farmers in developing countries (Collins et al. 2000) it came out that the fourth most needed trait, for which transgenic potatoes could provide a solution in developing countries, is resistance to PVY and PLRV. The International Potato Center (CIP) in Peru encourages public research on potato resulting in adapted and resistant varieties that can be used by poor farmers in developing countries (CIP 2016b). Recently, late blight resistant potato varieties were presented during field trials in Uganda (CIP 2016a). Transgenic genotypes were generated from the potato variety 'Desirée'. However, in this thesis, 'Desirée' turned out to be an unsuitable variety for genetic modification due to its inherent high susceptibility to the recombinant PVY strains PVY^{N-Wi} and PVY^{NTN} (Hühnlein et al. 2016a). The number of detected PVY^{N-Wi} RNA copies in the transgenic and non-transgenic genotypes of this variety was even higher than the upper 95% confidence limit of several commercial potato varieties showing a wide range of PVY susceptibility. Therefore, successful cultivation of transgenic potato lines requires stacked resistance traits to at least the most important pathogens of the potato crop, such as late blight, PVY and, especially in developing countries: PLRV, potato tuber moth (*Phthorimaea operculella*) and bacterial wilt (*R. solanacearum*) (Collins et al. 2000).

An indirect impact of global warming on the significance of virus diseases in potato results from an increased abundance of aphid vectors that is associated with several factors, such as an increased number of insect generations, an extended length of the aphid flying season or

higher overwintering rates (Zhou et al. 1996; Finlay and Luck 2011). In addition, aphids may shift their distribution areas and become present in geographic regions where they were not native previously (Toth et al. 2008). For instance, in the Tyrnävä-Liminka area of Finland, one of the five European High Grade Seed Potato Production Zones (HG zones), *Aphis gossypii* was previously found only indoors or in greenhouses (Albrecht 2010). However, during the last 10 years this aphid species, which is an efficient vector for PVY, was also found in seed potato fields of the Tyrnävä-Liminka area (Kirchner et al. 2013). This raised new concerns about the future reliability of the Finish HG zone in the course of increased vector and thus virus pressure. Therefore, in seed certification systems, everywhere in the world, there is need for very sensitive methods that detect potato viruses and particularly PVY independent from strain or isolate-specific sequence polymorphisms. In this thesis, primers were presented that detect more than 70 different isolates of PVY with an equal efficiency, which lowers the risk that the detection of single isolates fails due to mismatches between primer and virus sequences. In addition, by using immuno-capture (IC) instead of costly RNA extraction procedures, the advantages of ELISA (ease and low costs) can be combined with the high sensitivity of RT-qPCR. Furthermore, by using direct tuber testing, no greenhouse is needed since the tuber flesh itself is tested for virus infestation and not the leaves emerging from tuber sprouts. Direct tuber testing detects PVY with the same efficacy, if non-dormant tubers are sampled (Hühnlein et al. 2013).

6.3   New more virulent PVY and PLRV strains may evolve

In this thesis it is assumed that quantitative real-time PCR allows studying virus virulence and evolution. According to the trade-off hypothesis, virulence (here used as a synonym for symptom severity) is an unavoidable consequence of virus transmission (Anderson and May 1982). The higher the virulence of a strain is, the shorter the lifetime of the host becomes, which will decrease the number of transmissions by vectors. However, the transmission capacity is positively correlated with virus accumulation in the host (Escriu et al. 2000), which in turn was recorded to be positively correlated with virulence (Fraser et al. 2007; Pagan et al. 2007; Longdon et al. 2015; Patterson et al. 2015; Souto et al. 2015; Girerd-Genessay et al. 2016). Therefore, to obtain a maximum of transmission, viruses have evolved to harm their hosts until a finite optimal level of virulence (Anderson and May 1982; Frank 1996). On the contrary, plants have evolved different mechanisms to avoid (extreme resistance), repress (quantitative resistance) or tolerate (tolerance) infections by viruses. According to Miller et al. (2006) the last mechanism comprises different types of tolerances leading either to higher or lower viral accumulation rates within the host plant. If the host completely tolerates viral accumulation, the virus will evolve towards higher accumulation

rates in order to achieve a higher transmission capacity. On the other hand, if the tolerance of a host plant is saturated on a specific level, it will select for viruses with reduced replication. Since viruses and their host plants have coevolved, host-virus interactions depend on both, the host's and the parasite's genotypes (Pagan et al. 2007). Therefore, virus multiplication and thus virulence or symptom severity depends on various interactions between the genotypes of hosts and viruses. This is often the case for uncorrelated data originating from studies that aim to find a relationship between the virus isolate or strain and symptom severity (Rodriguezcerezo et al. 1991; Shi et al. 2002; Carrillo-Tripp et al. 2007; Doumayrou et al. 2013; Lindner et al. 2015). Either one virus isolate was used to infect several accessions of plants or several virus isolates were inoculated to one plant genotype. However, to reveal isolate x accession interactions it is necessary to infect each plant genotype within a variety of different accessions with one of several virus isolates (Pagan et al. 2007; Froissart et al. 2010).

In the publication of this thesis that compares three different methods for the detection of *Potato virus Y* in seed potato certification (Hühnlein et al. 2013), no relationship was found between the high virulence and the number of detectable virus RNA copies of PVY^{N-Wi} isolate 261-4 in potato plants (see Fig. 7 within the mentioned publication). The impact of the potato variety on virus accumulation was greater than the impact of the virus isolate, particularly since the small number of replicates probably have weakened the conclusion. In a further publication presented in this thesis each of three potato varieties were infected with either one of three different isolates of PLRV assuming that they induce diverse symptom severities. However, the plants maintained under controlled conditions did not differ in the severity of symptoms although the number of detectable PLRV RNA copies indeed varied between the cultivars. All plants showed clear symptoms of a PLRV infection. However, the use of *in vitro* plants instead of emerging sprouts from tubers diminished the estimation of symptoms since *in vitro* plants *per se* have a distinct habitus. Nevertheless, even if clear differences in the severity of symptoms would have been observed, this would not necessarily result in a correlation of symptom severity and virus accumulation, because viruses are unevenly distributed not only in different plant organs but also during different stages of infection (Wang and Maule 1995). Furthermore, viruses induce several perturbations in the signaling pathways of their host plants (Whitham et al. 2006), which occur in infected as well as in uninfected tissues of the plant. Symptom severity therefore results not merely from a competition between virus and host for host resources, but is rather a consequence of the interaction and interference between virus and host components that affect host physiology (Culver and Padmanabhan 2007). Moreover, a virus can induce a change of the expression of more than 4,000 host genes with a considerable variation among the virus species used for the

infection (Whitham et al. 2003; Senthil et al. 2005; Pallas and Garcia 2011). Alterations in the expression pattern of host genes effects several biological processes, such as photosynthesis, pigment metabolism and plant-pathogen interactions (Lu et al. 2012), which in turn are involved in symptom severity. Moreover, it is assumed, that the number of genes altered in their expression is positively correlated with the severity of symptoms (Dardick 2007; Mochizuki et al. 2014; Yang et al. 2014; Kogovsek et al. 2016). Therefore, expression profiling may deliver better quantitative indicators for the virulence of a given virus isolate than the quantitative determination of the virus concentration in a plant.

Nevertheless, the investigation of virus virulence is an important challenge in potato research, because multiple historic reports exist documenting the occurrence of new virus strains with increased virulence. Ross (1959) reported on the establishment of a necrotic strain of PVY during the 1950s in Germany and other European countries. The new strain, meanwhile named as PVY^N, mostly displaced the common PVY^O strain and caused severe epidemics in tobacco and potato fields. The yield of infected potato plants was remarkably reduced while the leaves almost remained symptomless. This led to a failed control through seed certification and a replacement of many susceptible potato cultivars.

A dramatic shift in virus strains towards isolates causing increased yield reduction was recorded for PVY (see chapter 1.2.1.2). Thirty years have passed since the first reports were published about recombinant strains of PVY that induced symptoms of veinal necrosis on tobacco, but reacted with PVY^O- specific antibodies (PVY^{N-Wi}, PVY$^{N:O}$) (Chrzanowska 1987; Singh et al. 2003) or that caused mosaic symptoms on tobacco and reacted with PVY^N- specific antibodies (PVY^{NTN}). The last mentioned strain furthermore induces the potato tuber necrotic ring spot disease (PTNRD) that causes severe symptoms and yield losses (Beczner et al. 1984). Since recombination between PVY strains that infect non-potato hosts and potato-infecting PVY strains are scarce, Visser et al. (2012) suggested that PVY$^{N-Wi/N:O}$ and PVY^{NTN} strains have evolved as specialists of potato cultivars due to the increasing international trade since the 20th century. Indeed, within a few decades recombinant PVY strains have become the most prevalent strains worldwide with reports from Germany and France (Lindner and Billenkamp 2005; Rolland et al. 2008), Russia (Volkov et al. 2009), Syria, Jordan and Saudi Arabia (Chikh Ali et al. 2007; Anfoka et al. 2014; Chikh-Ali et al. 2016a), Vietnam (Schubert et al. 2015), China (Hu et al. 2009a), Japan (Ogawa et al. 2008), Australia (Kehoe and Jones 2011), Indonesia (Chikh-Ali et al. 2016b), New Zealand, although only PVY^{NTN} and not PVY$^{N:O}$ (Fletcher and Lister 2004; Fomitcheva et al. 2009), USA and Canada (Karasev and Gray 2013b), Argentina and Brazil (Colavita et al. 2007; Sawazaki et al. 2009) as well as Tunisia, Kenya and South Africa (Larbi et al. 2012; Were et al. 2013; Visser and Bellstedt 2015). Even in Greenland PVY is now present, since farmers grow imported tubers from

Europe instead of own certified seeds (Neergaard et al. 2014). The authors did not examine the PVY strain, but if recombinant PVY strains are not already there, it will only be a question of time before they will be detected.

In tolerant potato varieties recombinant PVY strains often remain symptomless (Chrzanowska 1991; McDonald and Singh 1996a; Glais et al. 2005). This led to a rapid dispersal of recombinant PVY strains particularly in the USA where seed certification relies mainly on visual field inspections and where thousands of hectares are grown with tolerant potato cultivars, such as 'Russet Norkotha', 'Shepody' or 'Silverton Russet' (Groves 2009; Karasev and Gray 2013a). In these tolerant potato cultivars isolates are selected with high viral accumulation, since their transmission capacity is increased. This poses a danger to non-tolerant varieties, if they are exposed to virus isolates that have evolved in response to tolerance. According to Miller et al. (2006) "intolerant populations may suffer catastrophic levels of mortality". A recent report about highly virulent recombinant PVY strains that cause severe symptoms on potato may result from such a scenario (Schubert et al. 2015). Although it could not be validated in this thesis (Hühnlein et al. 2013), studies exist that describe higher virus multiplication rates of recombinant PVY isolates than of non-recombinant isolates (Kerlan 2004; Kogovsek et al. 2010). Besides from the fact that isolates with higher replication have a higher transmission capacity, such isolates have also a fitness advantage over low-replicating isolates within the host plant (Levin and Bull 1994). In mathematic models it was demonstrated that if two (or more) virus strains are related and use the same intracellular resources, then the faster growing strain will win and exclude the slower growing strain (Phan and Wodarz 2015). Taken together, these could be reasons why recombinant strains of PVY may have been becoming the most prevalent strains worldwide. According to the author's estimation, no studies exist determining the viral accumulation of different PVY strains co-infecting one host plant over time. Results from such investigations may deliver more insights into the dynamic process that accompanies multiple infections with more than one PVY strain and could examine the hypothesis that recombinant strains repress non-recombinant strains within one host plant due to a fitness advantage (Kamangar et al. 2014; Karasev 2014). Such experiments need RT-qPCR assays that are able to discriminate and quantify different PVY strains with equal efficiencies. Unfortunately, such assays could not be developed during this project due to the lack of probe efficiencies (see chapter 4.6.2). To achieve such a successful discrimination and quantification, recently developed digital PCR (dPCR) approaches could be utilized.

6.4   Digital PCR as an alternative to qPCR for absolute quantification?

dPCR has two decisive advantages over qPCR: considerable less vulnerability to decreased PCR efficiencies and a greater tolerance to inhibitors (Whale et al. 2012; Manoj 2016). qPCR is still one of the most widely used molecular techniques to detect and quantify nucleic acids (Bustin 2004a). However, it is constrained by its susceptibility to errors resulting from a high dependency on various efficiencies, such as equal nucleic acid extraction efficiencies of the samples, equal amplification efficiencies of samples and standard or constant qPCR efficiencies during all amplification cycles. Furthermore, the experimental error can be immensely high, if the efficiency of a qPCR run has a wide confidence interval (CI). Wide CIs originate from too few replicates especially within the standard serial dilutions. Since post-qPCR statistic calculations are mostly based on the mean of C_q-values or reverse calibrated absolute quantities, high experimental errors are disguised. Even in the MIQE guidelines the importance to specify the CIs for qPCR efficiencies is assessed as non-essential, although at least desirable (Bustin et al. 2009). For instance, if the mean of qPCR efficiency is 97% and the lower and upper confidence limits are 94% and 100%, respectively, then the lower and upper confidence limits of a sample containing an average sequence copy number of 10,000 are 4,200 and 24,000 copies, respectively. From an absolute point of view, this is a high error margin, although the CI of the efficiency is comparatively small [e.g. 85%-90% in Isolani et al. (2012), which is in fact a praised paper cited in Bustin et al. (2013)]. Therefore, even the compliance with the MIQE guidelines does not *per se* imply a high reliability of the data presented. Conceivably, this might be a reason why the majority of publications with results based on qPCR reveal a lack of transparent reporting of essential technical information (Bustin et al. 2013). Some results may become erratic or even meaningless, if the variability of efficiencies would be included in the calculations. Therefore, especially for absolute quantification, dPCR could be an alternative since it does not require normalization or a calibration curve. The random nature of the distribution of DNA molecules across the partitions can be calculated and predicted by the Poisson distribution making the measurement very precise and reproducible (see chapter 2.2 and Huggett et al. 2015). However, as is the case with qPCR, dPCR is also highly susceptibility to upstream errors during sampling or extraction of nucleic acids. Whereas this problem can be most widely solved by the usage of a sufficient number of replicates and calibrated lab equipment, another aspect constrains dPCR particularly for its application in quantification of nucleic acids with highly varying incidences, for instance virus RNA copies in plant tissue. The dynamic range of a dPCR assay is dependent on the number of partitions, in which the PCR reaction mix is divided. If the number of partitions is small, highly abundant nucleic acids cannot be quantified because all partitions will show positive results. Samples with high amounts of target sequences would have to be diluted further. If the number of partitions is high (e. g.

100,000 or even a million) the assay features a high dynamic range making different dilution strategies unnecessary (Huggett et al. 2015). However, as it was clearly shown by Jacobs et al. (2014), the CI increases considerably for lowly abundant sequences when only few partitions show a positive signal. Consequently, it is at least questionable to use a technique for absolute quantification that itself needs a pre-estimation of the number of targeted molecules. Nevertheless, its strengths (high sensitivity and reproducibility) are superior and for applications with a known range of target nucleic acids, such as the evaluation of the virus resistance type in potato varieties or the assessment of an altered susceptibility to viruses of transgenic potatoes, dPCR probably will generate more reliable data than qPCR. In this thesis the MIQE guidelines were applied to the methodical documentation of RT-qPCR results and the CIs of qPCR efficiencies were small ranging from 89%-99% (Hühnlein et al. 2013), 95%-102% (Hühnlein et al. 2016a; Hühnlein et al. 2016c) and 92%-107% (Hühnlein et al. 2016b). However, the results of all presented publications were weakened by differences among the tested treatments that could not by validated by significance. This was due to the high variation of the detected number of virus RNA copies in the plants. The use of vegetative clones from *in vitro* culture and the meticulous sampling procedure using always the same developmental stage of leaves were not sufficient to mitigate high standard deviations. Therefore, the determination of virus RNA copies by using quantitative detection methods requires an extremely high number of fully-randomized replicates. However, this applies to both, qPCR as well as dPCR. Perhaps, the standard deviation could have been decreased by using relative quantification of the virus titer as percentage difference from reference genes. Then the increase or decrease of the percentage difference from reference genes could be subjected to a statistical test of significance. However, a parallel quantification of target and reference genes in one well of a qPCR plate is not possible with Sybr® Green and requires the use of different fluorophore-labeled probes. Furthermore, target and reference genes have to be amplified with equal efficiencies, which is difficult to obtain. In this thesis the use of TaqMan® probes was not successful resulting in reduced efficiencies in comparison to Sybr® Green. A parallel quantification of target and reference genes in different qPCR wells consumes too much lab equipment and reagents. Therefore, the author of this thesis decided for the use of absolute quantification, especially since the aim of most applications was the determination of the absolute quantity of virus RNA.

7　References

Aaziz, R., & Tepfer, M. (1999). Recombination in RNA viruses and in virus-resistant transgenic plants. *Journal of General Virology, 80*, 1339-1346.

Abdul-Razzak, A., Guiraud, T., Peypelut, M., Walter, J., Houvenaghel, M.-C., Candresse, T., et al. (2009). Involvement of the cylindrical inclusion (CI) protein in the overcoming of an eIF4E-mediated resistance against Lettuce mosaic potyvirus. *Molecular Plant Pathology, 10*(1), 109-113, doi:10.1111/j.1364-3703.2008.00513.x.

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., et al. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science, 232*(4751), 738-743, doi:10.1126/science.3457472.

Adelphi, PRC, & EURAC (2015). Vulnerabilität Deutschlands gegenüber dem Klimawandel. Climate Change 24/2015, Umweltbundesamt, Dessau-Roßlau, p. 690.

Ai, T., Zhang, L., Gao, Z., Zhu, C. X., & Guo, X. (2011). Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. *Plant Biology, 13*(2), 304-316, doi:10.1111/j.1438-8677.2010.00374.x.

Al-Abedy, A., Cowan, G., McLean, K., Bryan, G., MacFarlane, S., & Torrance, L. (2012). Genetic analysis of Potyvirus resistance in *Solanum tuberosum* group *Phureja*. *The Dundee Conference. Crop Protection in Northern Britain, 2012, Dundee, UK, 28-29 February 2012*, 291-295.

Ala-Poikela, M., Goytia, E., Haikonen, T., Rajamaki, M.-L., & Valkonen, J. P. T. (2011). Helper Component Proteinase of the Genus Potyvirus Is an Interaction Partner of Translation Initiation Factors eIF(iso)4E and eIF4E and Contains a 4E Binding Motif. *Journal of Virology, 85*(13), 6784-6794, doi:10.1128/jvi.00485-11.

Albrecht, A. (2010). Atlas of the aphids of finland. LUOMUS, Finnish Museum of Natural History. http://www.luomus.fi/elaintiede/hyonteiset/tutkimus/kirvat/atlas.htm. Accessed 4th March 2016.

Ali, E. M., Kobayashi, K., Yamaoka, N., Ishikawa, M., & Nishiguchi, M. (2013). Graft Transmission of RNA Silencing to Non-Transgenic Scions for Conferring Virus Resistance in Tobacco. *PLoS ONE, 8*(5), doi:10.1371/journal.pone.0063257.

Alidjinou, E. K., Bocket, L., & Hober, D. (2015). Quantification of viral DNA during HIV-1 infection: A review of relevant clinical uses and laboratory methods. *Pathologie Biologie, 63*(1), 53-59, doi:10.1016/j.patbio.2014.07.007.

Allefs, J. J. H. M., Muskens, M. W. M., & van der Vossen, E. A. G. (2005). Breeding for foliage late blight resistance in the genomics era. In A. J. Haverkort, & P. C. Struik (Eds.), *Potato in progress: science meets practice* (pp. 255-266). Wageningen, The Netherlands: Wageningen Academic Publishers.

Almasi, R., Miller, W. A., & Ziegler-Graff, V. (2015). Mild and severe cereal yellow dwarf viruses differ in silencing suppressor efficiency of the P0 protein. *Virus Research, 208*, 199-206, doi:10.1016/j.virusres.2015.06.020.

Altman, D. G., & Bland, J. M. (1995). Statistics notes - Absence of evidence is not evidence of absence. *British Medical Journal, 311*(7003), 485-485.

Ames, M., & Spooner, D. M. (2008). DNA from herbarium specimens settles a controversy about origins of the European potato. *American Journal of Botany, 95*(2), 252-257, doi:10.3732/ajb.95.2.252.

An, L., Tang, W., Ranalli, T. A., Kim, H.-J., Wytiaz, J., & Kong, H. (2005). Characterization of a Thermostable UvrD Helicase and Its Participation in Helicase-dependent Amplification. *Journal of Biological Chemistry, 280*(32), 28952-28958, doi:10.1074/jbc.M503096200.

Anandalakshmi, R., Marathe, R., Ge, X., Herr, J. M., Mau, C., Mallory, A., et al. (2000). A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science, 290*(5489), 142-144, doi:10.1126/science.290.5489.142.

Andersen, K., & Johansen, I. E. (1998). A single conserved amino acid in the coat protein gene of pea seed-borne mosaic potyvirus modulates the ability of the virus to move systemically in Chenopodium quinoa. *Virology, 241*(2), 304-311, doi:10.1006/viro.1997.8967.

Anderson, R. M., & May, R. M. (1982). Coevolution of hosts and parasites. *Parasitology, 85*(OCT), 411-426.

Andrivon, D. (1996). The origin of Phytophthora infestans populations present in Europe in the 1840s: a critical review of historical and scientific evidence. *Plant Pathology, 45*(6), 1027-1035, doi:10.1046/j.1365-3059.1996.d01-196.x.

Anfoka, G., Ahmad, F. H., Altaleb, M., Abadi, M., Abubaker, S., Levy, D., et al. (2014). First report of recombinant *Potato virus Y* strains infecting potato in Jordan. *Plant Disease, 98*(7), 1017-1017, doi:10.1094/PDIS-03-14-0236-PDN.

Anindya, R., Chittori, S., & Savithri, H. S. (2005). Tyrosine 66 of Pepper vein banding virus genome-linked protein is uridylylated by RNA-dependent RNA polymerase. *Virology, 336*(2), 154-162, doi:10.1016/j.virol.2005.03.024.

Anindya, R., & Savithri, H. S. (2004). Potyviral NIa proteinase, a proteinase with novel deoxyribonuclease activity. *Journal of Biological Chemistry, 279*(31), 32159-32169, doi:10.1074/jbc.M404135200.

Appel, O., & Schlumberger, O. (1911). *Die Blattrollkrankheit und unsere Kartoffelernten* (Vol. 190, Arbeiten der Deutschen Landwirtschafts-Gesellschaft). Berlin: P. Parey.

Arif, M., Azhar, U., Arshad, M., Zafar, Y., Mansoor, S., & Asad, S. (2012). Engineering broad-spectrum resistance against RNA viruses in potato. *Transgenic Research, 21*(2), 303-311, doi:10.1007/s11248-011-9533-7.

Ashoub, A., Rohde, W., & Prüfer, D. (1998). In planta transcription of a second subgenomic RNA increases the complexity of the subgroup 2 luteovirus genome. *Nucleic Acids Research, 26*(2), 420-426.

Atreya, C. D., & Pirone, T. P. (1993). Mutational analysis of the helper component-proteinase gene of a potyvirus - Effects of amino-acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility. *Proceedings of the National Academy of Sciences of the United States of America, 90*(24), 11919-11923, doi:10.1073/pnas.90.24.11919.

Audy, P., Palukaitis, P., Slack, S. A., & Zaitlin, M. (1994). Replicase-mediated resistance to *Potato virus Y* in transgenic tobacco plants. *Molecular Plant-Microbe Interactions, 7*(1), 15-22, doi:10.1094/mpmi-7-0015.

Ayadi, M., Bouaziz, D., Nouri-Ellouz, O., Rouis, S., Drira, N., & Gargouri-Bouzid, R. (2012). Efficient resistance to *Potato virus Y* infection conferred by cytosolic expression of anti-viral protease single-chain variable fragment antibody in transgenic potato plants. *Journal of Plant Pathology, 94*(3), 561-569.

Bahner, I., Lamb, J., Mayo, M. A., & Hay, R. T. (1990). Expression of the genome of *Potato leafroll virus* - Readthrough of the coat protein termination codon *in vivo*. *Journal of General Virology, 71*, 2251-2256, doi:10.1099/0022-1317-71-10-2251.

Baker, M. (2012). Digital PCR hits its stride. *Nat Meth, 9*(6), 541-544, doi:10.1038/nmeth.2027.

Baldauf, P. M., Gray, S. M., & Perry, K. L. (2006). Biological and serological properties of *Potato virus Y* isolates in northeastern United States potato. *Plant Disease, 90*(5), 559-566, doi:10.1094/pd-90-0559.

Barker, H., & Woodford, J. A. T. (1987). Unusually mild symptoms of *Potato leafroll virus* in the progeny of late-infected mother plants. *Potato Research, 30*(2), 345-348.

Basky, Z. (2002). The relationship between aphid dynamics and two prominent potato viruses (PVY and PLRV) in seed potatoes in Hungary. *Crop Protection, 21*(9), 823-827, doi:10.1016/s0261-2194(02)00045-5.

Bauhin, C. (1596). *Phytopinax: seu enumeratio plantarum*. Basel: digitized by the Bavarian State Library.

Baumberger, N., Tsai, C.-H., Lie, M., Havecker, E., & Baulcombe, David C. (2007). The Polerovirus Silencing Suppressor P0 Targets ARGONAUTE Proteins for Degradation. *Current Biology, 17*(18), 1609-1614, doi:10.1016/j.cub.2007.08.039.

Baunoch, D., Das, P., & Hari, V. (1988). Intracellular-localization of TEV capsid and inclusion proteins by immunogold labeling. *Journal of Ultrastructure and Molecular Structure Research, 99*(3), 203-212, doi:10.1016/0889-1605(88)90064-x.

Beauchemin, C., & Laliberte, J.-F. (2007). The poly(A) binding protein is internalized in virus-induced vesicles or redistributed to the nucleolus during turnip mosaic virus infection. *Journal of Virology, 81*(20), 10905-10913, doi:10.1128/jvi.01243-07.

Beczner, L., Horvath, J., Romhanyi, I., & Forster, H. (1984). Studies on the etiology of tuber necrotic ringspot disease in potato. *Potato Research, 27*(3), 339-352.

Bendahmane, M., Chen, J., Asurmendi, S., Bazzini, A. A., Szecsi, J., & Beachy, R. N. (2007). Coat protein-mediated resistance to TMV infection of *Nicotiana tabacum* involves multiple modes of interference by coat protein. *Virology, 366*(1), 107-116, doi:10.1016/j.virol.2007.03.052.

Benedict, C. A., McMoran, D. W., Inglis, D. A., & Karasev, A. V. (2015). Tuber symptoms associated with recombinant strains of *Potato virus Y* in specialty potatoes under western Washington growing conditions. *American Journal of Potato Research, 92*(5), 593-602, doi:10.1007/s12230-015-9472-6.

Besong-Ndika, J., Ivanov, K. I., Hafren, A., Michon, T., & Makinen, K. (2015). Cotranslational coat protein-mediated inhibition of Potyviral RNA translation. *Journal of Virology, 89*(8), 4237-4248, doi:10.1128/jvi.02915-14.

Bio-Rad (2016). What is Real-Time PCR (qPCR)? http://www.bio-rad.com/de-de/applications-technologies/what-real-time-pcr-qpcr. Accessed 22nd January 2016.

Bizouarn, F. (2014). Introduction to digital PCR. In R. Biassoni, & A. Raso (Eds.), *Quantitative real-time PCR: Methods and protocols* (pp. 27-41). New York, Heidelberg, Dordrecht, London: Humana Press.

Blanc, S., Ammar, E. D., Garcia-Lampasona, S., Dolja, V. V., Llave, C., Baker, J., et al. (1998). Mutations in the potyvirus helper component protein: effects on interactions with virions and aphid stylets. *Journal of General Virology, 79*, 3119-3122.

Blanco-Urgoiti, B., Tribodet, M., Leclere, S., Ponz, F., de San Roman, C. P., Legorburu, F. J., et al. (1998). Characterization of potato potyvirus Y (PVY) isolates from seed potato batches. Situation of the NTN, Wilga and Z isolates. *European Journal of Plant Pathology, 104*(8), 811-819, doi:10.1023/a:1008698719645.

Boland, G. J., Melzer, M. S., Hopkin, A., Higgins, V., & Nassuth, A. (2004). Climate change and plant diseases in Ontario. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 26*(3), 335-350.

Bömer, A., & Mattis, H. (1924). Der Solaningehalt der Kartoffeln. *Zeitschrift für Untersuchung der Nahrungs- und Genussmittel, 47*, 97-127.

Boonham, N., Walsh, K., Hims, M., Preston, S., North, J., & Barker, I. (2002). Biological and sequence comparisons of *Potato virus Y* isolates associated with potato tuber necrotic ringspot disease. *Plant Pathology, 51*(2), 117-126.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., & Ziegler-Graff, V. (2007). The polerovirus F box protein PO targets ARGONAUTE1 to suppress RNA silencing. *Current Biology, 17*(18), 1615-1621, doi:10.1016/j.cub.2007.07.061.

Boukhris-Bouhachem, S., Djilani-Khouadja, F., Fakhfakh, H., Glais, L., Tribodet, M., & Kerlan, C. (2010). Incidence and Characterization of *Potato virus Y* in Seed Potatoes in Tunisia. *Potato Research, 53*(3), 151-166.

Bowers, R. M., & Dhar, A. K. (2011). Effect of template on generating a standard curve for absolute quantification of an RNA virus by real-time reverse transcriptase-polymerase chain reaction. *Molecular and Cellular Probes, 25*(1), 60-64, doi:10.1016/j.mcp.2010.12.002.

Bradley, R. H. E. (1978). Search for Leaf roll virus that does not cause diagnostic symptoms in potato foliage. *Canadian Plant Disease Survey, 58*(3), 56-60.

Brantley, J. D., & Hunt, A. G. (1993). The N-terminal protein of the polyprotein encoded by the Potyvirus *Tobacco vein mottling virus* is an RNA-binding protein. *Journal of General Virology, 74*, 1157-1162, doi:10.1099/0022-1317-74-6-1157.

Brault, V., Bergdoll, M., Mutterer, J., Prasad, V., Pfeffer, S., Erdinger, M., et al. (2003). Effects of point mutations in the major capsid protein of *Beet western yellows virus* on capsid formation, virus accumulation, and aphid transmission. *Journal of Virology, 77*(5), 3247-3256, doi:10.1128/jvi.77.5.3247.3256.2003.

Brault, V., Mutterer, J., Scheidecker, D., Simonis, M. T., Herrbach, E., Richards, K., et al. (2000). Effects of point mutations in the readthrough domain of the *Beet western yellows virus* minor capsid protein on virus accumulation in planta and on transmission by aphids. *Journal of Virology, 74*(3), 1140-1148, doi:10.1128/jvi.74.3.1140-1148.2000.

Brault, V., Vandenheuvel, J. F. J. M., Verbeek, M., Zieglergraff, V., Reutenauer, A., Herrbach, E., et al. (1995). Aphid Transmission of Beet western yellows luteovirus requires the minor capsid read-through protein P74. *EMBO Journal, 14*(4), 650-659.

Brown, C. R., & Adiwilaga, K. D. (1991). Use of rescue pollination to make a complex interspecific cross in potato. *American Potato Journal, 68*(12), 813-820, doi:10.1007/bf02853855.

Brown, C. R., & Corsini, D. (2001). Genetics and breeding of virus resistance: Traditional methods. In G. Loebenstein, P. H. Berger, A. A. Brunt, & R. H. Lawson (Eds.), *Virus and virus-like diseases of potatoes and production of seed potatoes* (pp. 323-340). Dordrecht, Boston, London: Kluwer Academic Publishers.

Brown, C. R., Corsini, D., Pavek, J., & Thomas, P. E. (1997). Heritility of field resistance to *Potato leafroll virus* in cultivated potato. *Plant Breeding, 116*(6), 585-588, doi:10.1111/j.1439-0523.1997.tb02194.x.

Brown, C. R., & Thomas, P. E. (1993). Resistance to *Potato leafroll virus* derived from *Solanum chacoense*: characterization and inheritance. *Euphytica, 74*(1-2), 51-57, doi:10.1007/BF00033767.

Bucher, G. L., Tarina, C., Heinlein, M., Di Serio, F., Meins, F., & Iglesias, V. A. (2001). Local expression of enzymatically active class I beta-1,3-glucanase enhances symptoms of TMV infection in tobacco. *Plant Journal, 28*(3), 361-369, doi:10.1046/j.1365-313X.2001.01181.x.

Burton, W. G. (1989). *The potato* (3ed., The potato.). Harlow, Essex; UK: Longman Group Limited.

Bustin, S. A. (2004a). Quantification of nucleic acids by PCR. In S. A. Bustin (Ed.), *A-Z of quantitative PCR* (pp. 5-46). California, USA: International University Line.

Bustin, S. A. (2004b). *A-Z of quantitative PCR*. California, USA: International University Line.

Bustin, S. A., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., et al. (2013). The need for transparency and good practices in the qPCR literature. *Nature Methods, 10*(11), 1063-1067, doi:10.1038/nmeth.2697.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry, 55*(4), 611-622, doi:10.1373/clinchem.2008.112797.

Bustin, S. A., & Nolan, T. (2004). Data analysis and interpretation. In S. A. Bustin (Ed.), *A-Z of quantitative PCR* (pp. 441-492). Ja Lolla, USA: International University Line.

Butler, N. M., Atkins, P. A., Voytas, D. F., & Douches, D. S. (2015). Generation and inheritance of targeted mutations in potato *(Solanum tuberosum* L.) using the CRISPR/Cas system. *PLoS ONE, 10*(12), doi:10.1371/journal.pone.0144591.

Canto, T., & Palukaitis, P. (2002). Novel N gene-associated, temperature-independent resistance to the movement of *Tobacco mosaic virus* vectors neutralized by a *Cucumber mosaic virus* RNA1 transgene. *Journal of Virology, 76*(24), 12908-12916, doi:10.1128/jvi.76.24.12908-12916.2002.

Carputo, D., Frusciante, L., & Peloquin, S. J. (2003). The role of 2n gametes and endosperm balance number in the origin and evolution of polyploids in the tuber-bearing Solanums. *Genetics, 163*(1), 287-294.

Carrillo-Tripp, J., Lozoya-Gloria, E., & Rivera-Bustamante, R. F. (2007). Symptom remission and specific resistance of pepper plants after infection by *Pepper golden mosaic virus*. *Phytopathology, 97*(1), 51-59, doi:10.1094/PHYTO-97-0051.

Carrington, J. C., & Dougherty, W. G. (1987). Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. *Journal of Virology, 61*(8), 2540-2548.

Carrington, J. C., Freed, D. D., & Leinicke, A. J. (1991). Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. *Plant Cell, 3*(9), 953-962.

Carrington, J. C., Haldeman, R., Dolja, V. V., & Restrepohartwig, M. A. (1993). Internal cleavage and trans-proteolytic activities of the VPg-proteinase (NIa) of tobacco etch Potyvirus in-vivo. *Journal of Virology, 67*(12), 6995-7000.

Carrington, J. C., Jensen, P. E., & Schaad, M. C. (1998). Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. *Plant Journal, 14*(4), 393-400, doi:10.1046/j.1365-313X.1998.00120.x.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K., & Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell, 8*(10), 1669-1681.

Cascardo, R. S., Arantes, I. L. G., Silva, T. F., Sachetto-Martins, G., Vaslin, M. F. S., & Correa, R. L. (2015). Function and diversity of P0 proteins among cotton leafroll dwarf virus isolates. *Virology journal, 12*, doi:10.1186/s12985-015-0356-7.

de Cedron, M. G., Osaba, L., Lopez, L., & Garcia, J. A. (2006). Genetic analysis of the function of the plum pox virus CI RNA helicase in virus movement. *Virus Research, 116*(1-2), 136-145, doi:10.1016/j.virusres.2005.09.009.

Chavez, J. D., Cilia, M., Weisbrod, C. R., Ju, H.-J., Eng, J. K., Gray, S. M., et al. (2012). Cross-linking measurements of the *Potato leafroll virus* reveal protein interaction topologies required for virion stability, aphid transmission, and virus-plant interactions. *Journal of Proteome Research, 11*(5), 2968-2981, doi:10.1021/pr300041t.

Chen, X., Liu, J., Xu, L., Jiang, F., Xie, X., Zhu, C., et al. (2010). Inhibiting virus infection by RNA interference of the eight functional genes of the *Potato Virus Y* genome. *Journal of Phytopathology, 158*(11-12), 776-784, doi:10.1111/j.1439-0434.2010.01701.x.

Chikh-Ali, M., Alruwaili, H., Pol, D. V., & Karasev, A. V. (2016a). Molecular characterization of recombinant strains of *Potato virus Y* from Saudi Arabia. *Plant Disease, 100*(2), 292-297.

Chikh-Ali, M., Bosque-Pérez, N. A., Pol, D. V., Sembel, D., & Karasev, A. V. (2016b). Occurrence and molecular characterization of recombinant *Potato virus Y*[NTN] isolates from Sulawesi, Indonesia. *Plant Disease, 100*(2), 269-275.

Chikh-Ali, M., Gray, S. M., & Karasev, A. V. (2013). An improved multiplex IC-RT-PCR assay distinguishes nine strains of *Potato virus Y*. *Plant Disease, 97*, 1370-1374.

Chikh Ali, M., Maoka, T., & Natsuaki, K. T. (2007). The occurrence and characterization of new recombinant isolates of PVY displaying shared properties of PVY[NW] and PVY[NTN]. *Journal of Phytopathology, 155*(7-8), 409-415, doi:10.1111/j.1439-0434.2007.01251.x.

Chikh Ali, M., Maoka, T., Natsuaki, T., & Natsuaki, K. T. (2010). PVY[NTN-NW], a novel recombinant strain of *Potato virus Y* predominating in potato fields in Syria. *Plant Pathology, 59*(1), 31-41, doi:10.1111/j.1365-3059.2009.02174.x.

Choi, I. R., Horken, K. M., Stenger, D. C., & French, R. (2005). An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication. *Journal of General Virology, 86*, 2605-2614, doi:10.1099/vir.0.81081-0.

Chowdappa, P., Kumar, B. J. N., Madhura, S., Kumar, S. P. M., Myers, K. L., Fry, W. E., et al. (2015). Severe outbreaks of late blight on potato and tomato in South India caused by recent changes in the *Phytophthora infestans* population. *Plant Pathology, 64*(1), 191-199, doi:10.1111/ppa.12228.

Christie, R. G., & Edwardson, J. R. (1977). *Light and electron microscopy of plant virus inclusions* (Florida Agricultural Experiment Stations Monograph Series, Vol. 9).

Chrzanowska, M. (1987). Nowe izolaty wirusa Y zagrazajace ziemniakom w Polsce. *Hodowla Roslin I Nasienn*, 5-6.

Chrzanowska, M. (1991). New isolates of the necrotic strain of *Potato virus Y* (PVY[N]) found recently in Poland. *Potato Research, 34*, 179-182.

Chung, B. Y.-W., Miller, W. A., Atkins, J. F., & Firth, A. E. (2008). An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences, 105*(15), 5897-5902, doi:10.1073/pnas.0800468105.

CIP (2016a). First field observation in Uganda shows extreme resistance to late blight by GM potato. http://cipotato.org/press-room/blogs/first-field-observation-in-uganda-shows-extreme-resistance-to-late-blight-by-gm-potato/. Accessed 15th March 2016.

CIP (2016b). Vision, Mission, Values. http://cipotato.org/about-cip/vision-mission-values/. Accessed 15th March 2016.

Clasen, B. M., Stoddard, T. J., Luo, S., Demorest, Z. L., Li, J., Cedrone, F., et al. (2015). Improving cold storage and processing traits in potato through targeted gene knockout. [Article in Press]. *Plant Biotechnology Journal*, doi:10.1111/pbi.12370.

Cockerham, G. (1943). The reactions of potato varieties to viruses X, A, B and C. *Ann Appl Biol, 30*((4)), 338-344, doi:10.1111/j.1744-7348.1943.tb06712.x.

Cockerham, G. (1970). Genetical studies on resistance to potato viruses X and Y. *Heredity, 25*(3), 309-348.

Colavita, M., Massa, G., & Feingold, S. (2007). In the search of recombinant PVY strains. *13th European Association for Potato Research (EAPR) Virology Section Meeting*. Scotland, United Kingdom.

Collins, W., Witcombe, J., Lenne, J., & Eden-Green, S. Introduction and outcomes. In C. Lizárraga, & A. Hollister (Eds.), *Workshop on transgenic potatoes for the benefit of resource-poor farmers in developing countries, Manchester, United Kingdom, 5th-9th June 2000 2000* (pp. 5-8): International Potato Center (CIP)

Cooke, D. E. L., Cano, L. M., Raffaele, S., Bain, R. A., Cooke, L. R., Etherington, G. J., et al. (2012). Genome Analyses of an Aggressive and Invasive Lineage of the Irish Potato Famine Pathogen. *PLoS Pathogens, 8*(10), doi:10.1371/journal.ppat.1002940.

Costa, J.-M., Ernault, P., Olivi, M., Gaillon, T., & Arar, K. (2004). Chimeric LNA/DNA probes as a detection system for real-time PCR. *Clinical Biochemistry, 37*(10), 930-932, doi:10.1016/j.clinbiochem.2004.05.020.

Crosslin, J. M., Hamm, P. B., Hane, D. C., Jaeger, J., Brown, C. R., Shiel, P. J., et al. (2006). The occurrence of PVY^O, PVY^N, and PVY$^{N:O}$ strains of *Potato virus Y* in certified potato seed lot trials in Washington and Oregon. *Plant Disease, 90*(8), 1102-1105, doi:10.1094/pd-90-1102.

Cui, X., Wei, T., Chowda-Reddy, R. V., Sun, G., & Wang, A. (2010). The *Tobacco etch virus* P3 protein forms mobile inclusions via the early secretory pathway and traffics along actin microfilaments. *Virology, 397*(1), 56-63, doi:10.1016/j.virol.2009.11.015.

Culver, J. N., & Padmanabhan, M. S. (2007). Virus-induced disease: Altering host physiology one interaction at a time. In *Annual Review of Phytopathology* (Vol. 45, pp. 221-243, Annual Review of Phytopathology).

Dardick, C. (2007). Comparative expression profiling of *Nicotiana benthamiana* leaves systemically infected with three fruit tree viruses. *Molecular Plant-Microbe Interactions, 20*(8), 1004-1017, doi:10.1094/mpmi-20-8-1004.

Daros, J. A., Schaad, M. C., & Carrington, J. C. (1999). Functional analysis of the interaction between VPg-proteinase (NIa) and RNA polymerase (NIb) of tobacco etch potyvirus, using conditional and suppressor mutants. *Journal of Virology, 73*(10), 8732-8740.

De Bokx, J. A., & Huttinga, H. (1981). *Potato virus Y*. http://www.dpvweb.net/dpv/showdpv.php?dpvno=242. Accessed 2011-25-01 2011.

De Koeyer, D., Chen, H., & Gustafson, V. (2011). Molecular breeding for potato improvement. In J. M. Bradeen, & C. Kole (Eds.), *Genetics, genomics and breeding of potato* (pp. 41-67). Enfield, Hampshire, USA: science Publishers.

De Storme, N., & Geelen, D. (2014). Callose homeostasis at plasmodesmata: molecular regulators and developmental relevance. *Frontiers in Plant Science, 5*, doi:10.3389/fpls.2014.00138.

Delfosse, V. C., Agrofoglio, Y. C., Casse, M. F., Bonacic Kresic, I., Esteban Hopp, H., Ziegler-Graff, V., et al. (2014). The P0 protein encoded by *Cotton leafroll dwarf virus* (CLRDV) inhibits local but not systemic RNA silencing. *Virus Research, 180*, 70-75, doi:10.1016/j.virusres.2013.12.018.

van den Heuvel, J. F. J. M., Hogenhout, S. A., & van der Wilk, F. (1999). Recognition and receptors in virus transmission by arthropods. *Trends in Microbiology, 7*(2), 71-76, doi:10.1016/S0966-842X(98)01434-6.

Deng, P., Wu, Z., & Wang, A. (2015). The multifunctional protein CI of potyviruses plays interlinked and distinct roles in viral genome replication and intercellular movement. *Virology journal, 12*, doi:10.1186/s12985-015-0369-2.

Derrien, B., Baumberger, N., Schepetilnikov, M., Viotti, C., De Cillia, J., Ziegler-Graff, V., et al. (2012). Degradation of the antiviral component ARGONAUTE1 by the autophagy pathway. *Proceedings of the National Academy of Sciences of the United States of America, 109*(39), 15942-15946, doi:10.1073/pnas.1209487109.

Dietrich, C., Miller, J., Mckenzie, G., Palkovics, L., Balázs, E., Palukaitis, P., et al. (2007). No recombination detected in artificial potyvirus mixed infections and between potyvirus derived transgenes and heterologous challenging potyviruses. *Environmental Biosafety Research, 6*(3), 207-218.

Djilani-Khouadja, F., Rouzé-Jouan, J., Guyader, S., Marrakchi, M., & Fakhfakh, H. (2005). Biological and molecular characterization of Tunisian isolates of *Potato leafroll virus*. *Journal of Plant Pathology, 87*(2), 91-99.

Dodds, K. S., & Paxman, G. J. (1962). Genetic system of cultivated diploid potatoes. *Evolution, 16*(2), 154-&, doi:10.2307/2406194.

Dolja, V. V., Haldeman-Cahill, R., Montgomery, A. E., Vandenbosch, K. A., & Carrington, J. C. (1995). Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. *Virology (New York), 206*(2), 1007-1016, doi:10.1006/viro.1995.1023.

Domier, L. L., Shaw, J. G., & Rhoads, R. E. (1987). Potyviral proteins share amino-acid sequence homology with picornaviral, comoviral, and caulimoviral proteins. *Virology, 158*(1), 20-27, doi:10.1016/0042-6822(87)90233-9.

Douglas, D. R., & Pavek, J. J. (1972). Net necrosis of potato tubers associated with primary, secondary, and tertiary infection of leafroll. *American Potato Journal, 49*(9), 330-333.

Doumayrou, J., Avellan, A., Froissart, R., & Michalakis, Y. (2013). An experimental test of the transmission-virulence trade-off hypothesis in a plant virus. *Evolution, 67*(2), 477-486, doi:10.1111/j.1558-5646.2012.01780.x.

Dreo, T., Pirc, M., Ramsak, Z., Pavsic, J., Milavec, M., Zel, J., et al. (2014). Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot. *Analytical and Bioanalytical Chemistry, 406*(26), 6513-6528, doi:10.1007/s00216-014-8084-1.

Dufresne, P. J., Thivierge, K., Cotton, S., Beauchemin, C., Ide, C., Ubalijoro, E., et al. (2008). Heat shock 70 protein interaction with *Turnip mosaic virus* RNA-dependent RNA polymerase within virus-induced membrane vesicles. *Virology, 374*(1), 217-227, doi:10.1016/j.virol.2007.12.014.

Eagles, R. M., Balmorimelian, E., Beck, D. L., Gardner, R. C., & Forster, R. L. S. (1994). Characterization of NTPase, RNA-binding and RNA-helicase activities of the cytoplasmic inclusion protein of Tamarillo mosaic Potyvirus. *European Journal of Biochemistry, 224*(2), 677-684, doi:10.1111/j.1432-1033.1994.t01-1-00677.x.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*(5), 1792-1797, doi:10.1093/nar/gkh340.

Edwardson, J. R. (1974). Host ranges of viruses in the *Potato virus Y* group. *Florida Agricultural Experiment Stations Monograph Series*(5), 1-225.

Edwardson, J. R., & Christie, R. G. (1991). A monograph on the potyvirus group. *Monograph/Agricultural Experiment Station*.

EFSA (2015). Neonicotinoids: foliar spray uses confirmed as a risk to bees. http://www.efsa.europa.eu/en/press/news/150826. Accessed 3rd Febraury 2016.

EFSA (2016). Pesticides and bees: EFSA to update neonicotinoid assessments. http://www.efsa.europa.eu/en/press/news/160111. Accessed 3rd February 2016.

Eiamtanasate, S., Juricek, M., & Yap, Y.-K. (2007). C-terminal hydrophobic region leads PRSV P3 protein to endoplasmic reticulum. *Virus Genes, 35*(3), 611-617, doi:10.1007/s11262-007-0114-z.

El-Mohsen, A., Nashwa, M. A., El-Din, A. S. G., Sohair, I. E. A., Sadik, A. S., & Abdelmaksoud, H. M. (2003). Characterization of potato virus Y strain "N-Egypt". *Annals of Agricultural Science (Cairo), 48*(2), 485-504.

EPPO (2014). EPPO A1 and A2 lists of pests recommended for regulation as quarantine pest. (Vol. PM 1/2(23), pp. 16). Paris, France.

Escriu, F., Perry, K. L., & Garcia-Arenal, F. (2000). Transmissibility of *Cucumber mosaic virus* by *Aphis gossypii* correlates with viral accumulation and is affected by the presence of its satellite RNA. *Phytopathology, 90*(10), 1068-1072, doi:10.1094/phyto.2000.90.10.1068.

European Commission. Bees & Pesticides: Commission to proceed with plan to better protect bees. Press release from 29th April 2013.

European Commission (2015). Legislation of new plant breeding techniques. http://ec.europa.eu/food/plant/gmo/legislation/plant_breeding/index_en.htm. Accessed 20th January 2016.

European Community (2002). Council Directive 2002/56/EC of 13 June 2002 on the marketing of seed potatoes. *Official Journal of the European Communities, L193*, 60-73.

European Food Safety Authority (2012a). Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal, 10*(2), 2561-2594, doi:10.2903/j.efsa.2012.2561.

European Food Safety Authority (2012b). Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. *EFSA Journal, 10*(10), 2943-2974, doi:10.2903/j.efsa.2012.2943.

European Union (2001). Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of the European Union, 44*, 1-38.

European Union (2002). Council Directive 2002/56/EC of 13 June 2002 on the marketing of seed potatoes. *Official Journal of the European Communities, L 193, 45*, 60-73.

European Union (2003). L268-Regulation (EC) No 1829/2003 of the European Parliament and of the Coucil of 22 September 2003 on genetically modified food and feed. *Official Journal of the European Union, 46*, 1-23.

European Union (2013). Commission Implementing Directive 2013/63/EU of 17 December 2013 amending Annexes I and II to Council Directive 2002/56/EC as regards minimum conditions to be satisfied by seed potatoes and lots of seed potatoes Text with EEA relevance. *Official Journal of the European Union, L341*, 52–55.

Fanigliulo, A., Comes, S., Pacella, R., Harrach, B., Martin, D. P., & Crescenzi, A. (2005). Characterisation of *Potato virus Y* nnp strain inducing veinal necrosis in pepper: a naturally occurring recombinant strain of PVY. *Arch Virol, 150*(4), 709-720, doi:10.1007/s00705-004-0449-x.

FAOSTAT (2014). Potato production in China and India in 2014. FAO. http://faostat3.fao.org/download/Q/QC/E. Accessed 3rd March 2016.

FAOSTAT (2015). Worldwide potato production in 2013. Food and Agriculture Organization of the United Nations - Statistics. http://faostat3.fao.org/browse/Q/QC/E. Accessed 15th July 2015.

Federal Plant Variety Office of Germany (2014). *Descriptive Variety Lists - Potato*. Hannover, Germany: Federal Plant Variety Office of Germany.

Fellers, J., Wan, J. R., Hong, Y. L., Collins, G. B., & Hunt, A. G. (1998). In vitro interactions between a potyvirus-encoded, genome-linked protein and RNA-dependent RNA polymerase. *Journal of General Virology, 79*, 2043-2049.

Fellers, J. P., Tremblay, D., Handest, M. F., & Lommel, S. A. (2002). The *Potato virus Y* M(S)N(R) NIb-replicase is the elicitor of a veinal necrosis-hypersensitive response in root knot nematode resistant tobacco. *Mol Plant Pathol, 3*(3), 145-152, doi:10.1046/j.1364-3703.2002.00106.x.

Fernandez, A., Guo, H. S., Saenz, P., SimonBuela, L., deCedron, M. G., & Garcia, J. A. (1997). The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. *Nucleic Acids Research, 25*(22), 4474-4480, doi:10.1093/nar/25.22.4474.

Fernandez, A., Lain, S., & Garcia, J. A. (1995). RNA helicase activity of the plum pox Potyvirus CI protein expressed in *Escherichia coli* - Mapping of an RNA-binding domain. *Nucleic Acids Research, 23*(8), 1327-1332, doi:10.1093/nar/23.8.1327.

Finlay, K. J., & Luck, J. E. (2011). Response of the bird cherry-oat aphid (*Rhopalosiphum padi*) to climate change in relation to its pest status, vectoring potential and function in a crop-vector-virus pathosystem. *Agriculture Ecosystems & Environment, 144*(1), 405-421, doi:10.1016/j.agee.2011.08.011.

Fish, N., Karp, A., & Jones, M. G. K. (1988). Production of somatic hybrids by electrofusion in *Solanum. Theoretical and Applied Genetics, 76*(2), 260-266.

Flatken, S. (2006). *Auftreten, Variabilität und Rekombination von Kartoffelviren unter besonderer Berücksichtigung des Kartoffelvirus Y (Potato virus Y) in transgenen und nichttransgenen Kartoffeln*. Dissertation, Univ. Hannover,

Fletcher, J. D., & Lister, R. A. (2004). *Poster - PVY NTN in New Zealand potato crops* (EAPR Virology 2004, Abstracts of the 12th European Association for Potato Research Virology Section Meeting, Rennes, France, 13-19 June 2004).

Flis, B., Hennig, J., Strzelczyk-Zyta, D., Gebhardt, C., & Marczewski, W. (2005a). The *Ry-f(sto)* gene from *Solanum stoloniferum* for extreme resistant to *Potato virus Y* maps to potato chromosome XII and is diagnosed by PCR marker GP122(718) in PVY resistant potato cultivars. *Molecular Breeding, 15*(1), 95-101, doi:DOI 10.1007/s11032-004-2736-3.

Flis, B., Hermig, J., Strzelczyk-Zyta, D., Gebhardt, C., & Marezewski, W. (2005b). The *Ry-f(sto)* gene from *Solanum stoloniferum* for extreme resistance to *Potato virus Y* maps to potato chromosome XII and is diagnosed by PCR marker GP122(718) in PVY resistant potato cultivars. *Molecular Breeding, 15*(1), 95-101.

Foley, J. A., Ramankutty, N., Brauman, K. A., Cassidy, E. S., Gerber, J. S., Johnston, M., et al. (2011). Solutions for a cultivated planet. *Nature, 478*(7369), 337-342, doi:10.1038/nature10452.

Fomitcheva, V. W., Fletcher, J. D., & Schubert, J. (2009). *Potato virus Y* strain spectrum in New Zealand - Absence of recombinant N:O strains. *Journal of Phytopathology, 157*(7-8), 507-510, doi:10.1111/j.1439-0434.2008.01510.x.

Frank, S. A. (1996). Models of parasite virulence. *Quarterly Review of Biology, 71*(1), 37-78, doi:10.1086/419267.

Fraser, C., Hollingsworth, T. D., Chapman, R., de Wolf, F., & Hanage, W. P. (2007). Variation in HIV-1 set-point viral load: Epidemiological analysis and an evolutionary hypothesis. *Proceedings of the National Academy of Sciences of the United States of America, 104*(44), 17441-17446, doi:10.1073/pnas.0708559104.

Froissart, R., Doumayrou, J., Vuillaume, F., Alizon, S., & Michalakis, Y. (2010). The virulence–transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*(1548), 1907-1918, doi:10.1098/rstb.2010.0068.

Fry, W. E., McGrath, M. T., Seaman, A., Zitter, T. A., McLeod, A., Danies, G., et al. (2013). The 2009 Late Blight Pandemic in the Eastern United States - Causes and Results. *Plant Disease, 97*(3), 296-306, doi:10.1094/pdis-08-12-0791-fe.

Fuglie, K. O. (2007). Priorities for potato research in developing countries: Results of a survey. *American Journal of Potato Research, 84*(5), 353-365.

Gadkar, V. J., & Filion, M. (2014). New Developments in Quantitative Real-time Polymerase Chain Reaction Technology. *Current Issues in Molecular Biology, 16*, 1-5.

Gallie, D. R., & Walbot, V. (1992). Identification of the motifs within the *Tobacco mosaic virus* 5' leader responsible for enhancing translation. *Nucleic Acids Research, 20*(17), 4631-4638, doi:10.1093/nar/20.17.4631.

Galvez, L. C., Banerjee, J., Pinar, H., & Mitra, A. (2014). Engineered plant virus resistance. *Plant Science, 228*, 11-25, doi:10.1016/j.plantsci.2014.07.006.

Galvino-Costa, S. B. F., Figueira, A. d. R., Camargos, V. V., Geraldino, P. S., Hu, X.-J., Nikolaeva, O. V., et al. (2012). A novel type of *Potato virus Y* recombinant genome, determined for the genetic strain PVY[E]. *Plant Pathology, 61*, 388-398.

Gao, F., Chang, F., Shen, J., Shi, F., Xie, L., & Zhan, J. (2014). Complete genome analysis of a novel recombinant isolate of *Potato virus Y* from China. *Archives of Virology, 159*(12), 3439-3442, doi:10.1007/s00705-014-2184-2.

Garcia-Bustos, J., Heitman, J., & Hall, M. N. (1991). Nuclear protein localization. *Biochimica et Biophysica Acta, 1071*(1), 83-101, doi:10.1016/0304-4157(91)90013-m.

Garcia, J. A., Lain, S., Cervera, M. T., Riechmann, J. L., & Martin, M. T. (1990). Mutational analysis of plum pox potyvirus polyprotein processing by the NIa protease in *Escherichia coli*. *Journal of General Virology, 71*, 2773-2779, doi:10.1099/0022-1317-71-12-2773.

Gargouri-Bouzid, R., Jaoua, L., Rouis, S., Saidi, M. N., Bouaziz, D., & Ellouz, R. (2006). PVY-resistant transgenic potato plants expressing an anti-NIa protein scFv antibody. *Molecular Biotechnology, 33*(2), 133-140, doi:10.1385/mb:33:2:133.

Gebhardt, C. (2013). Bridging the gap between genome analysis and precision breeding in potato. *Trends in Genetics, 29*(4), 248-256, doi:10.1016/j.tig.2012.11.006.

Gibson, U. E. M., Heid, C. A., & Williams, P. M. (1996). A novel method for real time quantitative RT PCR. *Genome Research, 6*(10), 995-1001, doi:10.1101/gr.6.10.995.

Gildemacher, P. R., Demo, P., Barker, I., Kaguongo, W., Woldegiorgis, G., Wagoire, W. W., et al. (2009). A Description of Seed Potato Systems in Kenya, Uganda and Ethiopia. *American Journal of Potato Research, 86*(5), 373-382, doi:10.1007/s12230-009-9092-0.

Girerd-Genessay, I., Baratin, D., Ferry, T., Chidiac, C., Ronin, V., & Vanhems, P. (2016). Higher HIV RNA viral load in recent patients with symptomatic acute HIV infection in Lyon University hospitals. *PLoS ONE, 11*(1), doi:10.1371/journal.pone.0146978.

Glais, L., Faurez, F., Tribodet, M., Boulard, F., & Jacquot, E. (2015). The amino acid 419 in HC-Pro is involved in the ability of PVY isolate N605 to induce necrotic symptoms on potato tubers. *Virus Research, 208*, 110-119, doi:10.1016/j.virusres.2015.05.024.

Glais, L., Tribodet, M., & Kerlan, C. (2002). Genomic variability in Potato potyvirus Y (PVY): evidence that PVY^{N}W and PVY^{NTN} variants are single to multiple recombinants between PVY^{O}) and PVY^{N} isolates. *Archives of Virology, 147*(2), 363-378.

Glais, L., Tribodet, M., & Kerlan, C. (2005). Specific detection of the PVY^{N})-W variant of *Potato virus Y*. *Journal of Virological Methods, 125*(2), 131-136.

Goldmeyer, J., Kong, H., & Tang, W. (2007). Development of a novel one-tube isothermal Reverse Transcription thermophilic Helicase-Dependent Amplification platform for rapid RNA detection. *The Journal of molecular diagnostics : JMD, 9*(5), 639-644, doi:10.2353/jmoldx.2007.070012.

Gray, S., De Boer, S., Lorenzen, J., Karasev, A., Whitworth, J., Nolte, P., et al. (2010). *Potato virus Y*: An evolving concern for potato crops in the United States and Canada. *Plant Disease, 94*(12), 1384-1397, doi:10.1094/pdis-02-10-0124.

Greene, A. E., & Allison, R. F. (1994). Recombination between viral-RNA and transgenic plant transcripts. *Science, 263*(5152), 1423-1425, doi:DOI 10.1126/science.8128222.

Greer, S., Honeywell, R., Geletu, M., Arulanandam, R., & Raptis, L. (2010). Housekeeping genes; expression levels may change with density of cultured cells. *Journal of Immunological Methods, 355*(1–2), 76-79, doi:http://dx.doi.org/10.1016/j.jim.2010.02.006.

Groves, R. L. (2009). *Potato virus Y: a continuing risk to seed potato production; an emerging threat to commercial potato storagethreat storage.* Paper presented at the conference "Wisconsin Potato and Vegetable Growers AssociationWisconsin Association Grower Education ConferenceGrower Conference", Wisconsin, USA, 3rd February 2009.

Guntner, C., Gonzalez, A., DosReis, R., Gonzalez, G., Vazquez, A., Ferreira, F., et al. (1997). Effect of *Solanum* glycoalkaloids on potato aphid, Macrosiphum euphorbiae. *Journal of Chemical Ecology, 23*(6), 1651-1659, doi:10.1023/b:joec.0000006429.14373.91.

Guo, D. Y., Rajamaki, M. L., Saarma, M., & Valkonen, J. P. T. (2001). Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *Journal of General Virology, 82*, 935-939.

Guyader, S., & Ducray, D. G. (2002). Sequence analysis of *Potato leafroll virus* isolates reveals genetic stability, major evolutionary events and differential selection pressure between overlapping reading frame products. *Journal of General Virology, 83*, 1799-1807.

Hafren, A., Hofius, D., Ronnholm, G., Sonnewald, U., & Makinen, K. (2010). HSP70 and its cochaperone CPIP promote Potyvirus infection in *Nicotiana benthamiana* by regulating viral coat protein functions. *Plant Cell, 22*(2), 523-535, doi:10.1105/tpc.109.072413.

Hajimorad, M. R., Ding, X. S., Flasinski, S., Mahajan, S., Graff, E., HaldemanCahill, R., et al. (1996). Nla and Nlb of peanut stripe potyvirus are present in the nucleus of infected cells, but do not form inclusions. *Virology, 224*(2), 368-379, doi:10.1006/viro.1996.0544.

Hämäläinen, J. H., Sorri, V. A., Watanabe, K. N., Gebhardt, C., & Valkonen, J. P. T. (1998). Molecular examination of a chromosome region that controls resistance to potato Y and A potyviruses in potato. *Theoretical and Applied Genetics, 96*(8), 1036-1043, doi:10.1007/s001220050836.

Hampson, M. C. (1993). History, biology, and control of potato wart disease in Canada. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 15*(4), 223-244.

Han, Y. H., Xiang, H. Y., Wang, Q. A., Li, Y. Y., Wu, W. Q., Han, C. G., et al. (2010). Ring structure amino acids affect the suppressor activity of melon aphid-borne yellows virus P0 protein. *Virology, 406*(1), 21-27, doi:10.1016/j.virol.2010.06.045.

Hari, V. (1981). The RNA of *Tobacco etch virus* - Further characterization and detection of protein linked to RNA. *Virology, 112*(2), 391-399, doi:10.1016/0042-6822(81)90286-5.

Harrison, B. D. (1999). Steps in the development of Luteovirology. In H. G. Smith, & H. Barker (Eds.), *The Luteoviridae* (pp. 1-14). Wallingford, UK; New York, USA: CABI Publishing.

Hartung, F., & Schiemann, J. (2014). Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. *Plant Journal, 78*(5), 742-752, doi:10.1111/tpj.12413.

Hataya, T., Inoue, A. K., Ohshima, K., & Shikata, E. (1994). Characterization and strain identification of a *Potato virus Y* isolate non-reactive with monoclonal antibodies specific to the ordinary and necrotic strains. *Intervirology, 37*(1), 12-19.

Hawkes, J. G. (1990). *The potato: evolution, biodiversity and genetic resources*.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*(10), 986-994, doi:10.1101/gr.6.10.986.

Helgeson, J. P., Haberlach, G. T., Pohlman, J., & Austin, S. (1988). Somatic fusion of *Solanum* species. *Plant Cell Tissue and Organ Culture, 12*(2), 185-187, doi:10.1007/bf00040083.

Herbers, K., Tacke, E., Hazirezaei, M., Krause, K.-P., Melzer, M., Rohde, W., et al. (1997). Expression of a luteoviral movement protein in transgenic plants leads to carbohydrate accumulation and reduced photosynthetic capacity in source leaves. *The Plant Journal, 12*(5), 1045-1056, doi:10.1046/j.1365-313X.1997.12051045.x.

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Bio-Technology, 11*(9), 1026-1030, doi:10.1038/nbt0993-1026.

Hijmans, R. J. (2003). The effect of climate change on global potato production. *American Journal of Potato Research, 80*(4), 271-279.

Hoekema, A., Huisman, M. J., Molendijk, L., Vandenelzen, P. J. M., & Cornelissen, B. J. C. (1989). The genetic engineering of 2 commercial potato cultivars for resistance to *Potato virus X. Bio-Technology, 7*(3), 273-278, doi:10.1038/nbt0389-273.

Hofius, D., Herbers, K., Melzer, M., Omid, A., Tacke, E., Wolf, S., et al. (2001). Evidence for expression level-dependent modulation of carbohydrate status and viral resistance by the potato leafroll virus movement protein in transgenic tobacco plants. *Plant Journal, 28*(5), 529-543.

Hogenhout, S. A., vanderWilk, F., Verbeek, M., Goldbach, R. W., & Vandenheuvel, J. F. J. M. (1998). *Potato leafroll virus* binds to the equatorial domain of the aphid endosymbiotic GroEL homolog. *Journal of Virology, 72*(1), 358-365.

Hong, X. Y., Chen, J., Shi, Y. H., & Chen, J. P. (2007). The '6K1' protein of a strain of *Soybean mosaic virus* localizes to the cell periphery. *Archives of Virology, 152*(8), 1547-1551, doi:10.1007/s00705-007-0972-7.

Hong, Y. L., & Hunt, A. G. (1996). RNA polymerase activity catalyzed by a potyvirus-encoded RNA-dependent RNA polymerase. *Virology, 226*(1), 146-151, doi:10.1006/viro.1996.0639.

Hu, X., He, C., Xiao, Y., Xiong, X., & Nie, X. (2009a). Molecular characterization and detection of recombinant isolates of *Potato virus Y* from China. *Archives of Virology, 154*(8), 1303-1312, doi:10.1007/s00705-009-0448-z.

Hu, X., Karasev, A. V., Brown, C. J., & Lorenzen, J. H. (2009b). Sequence characteristics of *Potato virus Y* recombinants. *Journal of General Virology, 90*, 3033-3041, doi:10.1099/vir.0.014142-0.

Hu, X., Meacham, T., Ewing, L., Gray, S., & Karasev, A. V. (2009c). A novel recombinant strain of *Potato virus Y* suggests a new viral genetic determinant of vein necrosis in tobacco. *Virus Research, 143*(1), 68-76, doi: C:\Dokumente und Einstellungen\Anja.Huehnlein\Eigene Dateien\Literatur\PVY

Huaman, Z., & Spooner, D. M. (2002). Reclassification of landrace populations of cultivated potatoes (*Solanum* sect. Petota). *American Journal of Botany, 89*(6), 947-965, doi:10.3732/ajb.89.6.947.

Huggett, J. F., Cowen, S., & Foy, C. A. (2015). Considerations for digital PCR as an accurate molecular diagnostic tool. *Clinical Chemistry, 61*(1), 79-88, doi:10.1373/clinchem.2014.221366.

Hühnlein, A., Drechsler, N., Steinbach, P., Thieme, T., & Schubert, J. (2013). Comparison of three methods for the detection of *Potato virus Y* in seed potato certification. *Journal of Plant Diseases and Protection, 120*(2), 57-69.

Hühnlein, A., Schubert, J., & Thieme, T. (2016a). Evaluation of whether genetically modified potatoes are equivalent in their level of susceptibility to *Potato virus Y. unpublished*.

Hühnlein, A., Schubert, J., Zahn, V., & Thieme, T. (2016b). Examination of an isolate of *Potato leaf roll virus* that does not induce visible symptoms in the greenhouse. *European Journal of Plant Pathology*, doi:10.1007/s10658-016-0872-3.

Hühnlein, A., Thieme, R., Schubert, J., & Thieme, T. (2016c). The use of RT-qPCR to evaluate *Solanum* species and progenies of somatic hybrids regarding their resistance to *Potato virus Y. unpublished*.

Hwang, Y. T., Kalischuk, M., Fusaro, A. F., Waterhouse, P. M., & Kawchuk, L. (2013). Small RNA sequencing of *Potato leafroll virus*-infected plants reveals an additional subgenomic RNA encoding a sequence-specific RNA-binding protein. *Virology, 438*(2), 61-69, doi:10.1016/j.virol.2012.12.012.

Iglesias, V. A., & Meins, F. (2000). Movement of plant viruses is delayed in a beta-1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition. *Plant Journal, 21*(2), 157-166, doi:10.1046/j.1365-313x.2000.00658.x.

Inglis, D. A., & Gundersen, B. Impact of *Potato virus Y* on the quality of specialty potato tubers. In B. V. Pelt (Ed.), *Washington-Oregon Potato Conference, Kennewick, Washington, 2015* (pp. 7-14)

Ioannou, N. (1989). The infection pressure of *Potato leafroll virus* and *Potato virus Y* in relation to aphid populations in Cyprus. *Potato Research, 32*(1), 33-47, doi:10.1007/bf02365815.

IPCC (2014). Climate Change 2014 Synthesis Report: Summary for Policymakers, Intergovernmental Panel on Climate Change (IPCC), Geneva, Switzerland, p. 32.

ISAAA (2015). ISAAA's GM Approval Database. International Service for the Aquisistion of Agri-Biotech Applications. http://www.isaaa.org/gmapprovaldatabase/commercialtrait/default.asp?TraitTypeID=3&TraitType=Disease%20Resistance. Accessed 15th January 2016.

Isolani, M. E., Pietra, D., Balestrini, L., Borghini, A., Deri, P., Imbriani, M., et al. (2012). The in vivo effect of chelidonine on the stem cell system of planarians. *European Journal of Pharmacology, 686*(1–3), 1-7, doi:http://dx.doi.org/10.1016/j.ejphar.2012.03.036.

Ivanov, K. I., Eskelin, K., Lõhmus, A., & Mäkinen, K. (2014). Molecular and cellular mechanisms underlying potyvirus infection. *Journal of General Virology, 95*, 1415-1429, doi:10.1099/vir.0.064220-0.

Ivanov, K. I., Puustinen, P., Merits, A., Saarma, M., & Makinen, K. (2001). Phosphorylation down-regulates the RNA binding function of the coat protein of potato virus A. *Journal of Biological Chemistry, 276*(17), 13530-13540.

Jaag, H. M., Kawchuk, L., Rohde, W., Fischer, R., Emans, N., & Prüfer, D. (2003). An unusual internal ribosomal entry site of inverted symmetry directs expression of a potato leafroll polerovirus replication-associated protein. *Proceedings of the National Academy of Sciences of the United States of America, 100*(15), 8939–8944.

Jacobs, B., Goetghebeur, E., & Clement, L. (2014). Impact of variance components on reliability of absolute quantification using digital PCR. *BMC Bioinformatics, 15*(283), 1-13.

Jacobsen, E., & Schouten, H. J. (2009). Cisgenesis: an important sub-invention for traditional plant breeding companies. *Euphytica, 170*(1), 235-247, doi:10.1007/s10681-009-0037-y.

Jameson, P. E., & Clarke, S. F. (2002). Hormone-virus interactions in plants. *Critical Reviews in Plant Sciences, 21*(3), 205-228, doi:10.1080/0735-260291044241.

Jansky, S. (2006). Overcoming hybridization barriers in potato. *Plant Breeding, 125*(1), 1-12, doi:10.1111/j.1439-0523.2006.01178.x.

Jenner, C. E., Tomimura, K., Ohshima, K., Hughes, S. L., & Walsh, J. A. (2002). Mutations in *Turnip mosaic virus* P3 and cylindrical inclusion proteins are separately required to overcome two *Brassica napus* resistance genes. *Virology, 300*(1), 50-59, doi:10.1006/viro.2002.1519.

Jenner, C. E., Wang, X. W., Tomimura, K., Ohshima, K., Ponz, F., & Walsh, J. A. (2003). The dual role of the potyvirus P3 protein of *Turnip mosaic virus* as a symptom and avirulence determinant in brassicas. *Molecular Plant-Microbe Interactions, 16*(9), 777-784, doi:10.1094/mpmi.2003.16.9.777.

Jeschke, P., Nauen, R., Schindler, M., & Elbert, A. (2011). Overview of the Status and Global Strategy for Neonicotinoids. *Journal of Agricultural and Food Chemistry, 59*(7), 2897-2908, doi:10.1021/jf101303g.

Jiang, F., Song, Y., Han, Q., Zhu, C., & Wen, F. (2011). The choice of target site is crucial in artificial miRNA-mediated virus resistance in transgenic *Nicotiana tabacum*. *Physiological and Molecular Plant Pathology, 76*(1), 2-8, doi:http://dx.doi.org/10.1016/j.pmpp.2011.07.002.

Jiang, J., & Laliberté, J.-F. (2011). The genome-linked protein VPg of plant viruses — a protein with many partners. *Current Opinion in Virology, 1*(5), 347-354, doi:10.1016/j.coviro.2011.09.010.

Jin, Y., Ma, D., Dong, J., Jin, J., Li, D., Deng, C., et al. (2007). HC-Pro protein of *Potato virus Y* can interact with three *Arabidopsis* 20S proteasome subunits in planta. *Journal of Virology, 81*(23), 12881-12888, doi:10.1128/jvi.00913-07.

Johnston, S. A., & Hanneman, R. E. (1980). Support of the endosperm balance number hypothesis utilizing some tuber-bearing *Solanum* species. *American Potato Journal, 57*(1), 7-14, doi:10.1007/bf02852750.

Jonasson, T., & Olsson, K. (1994). The influence of glycoalkaloids, chlorogenic acid and sugars on the susceptibility of potato-tubers to wireworm. *Potato Research, 37*(2), 205-216, doi:10.1007/bf02360510.

Jones, D. R., & Baker, R. H. A. (2007). Introductions of non-native plant pathogens into Great Britain, 1970-2004. *Plant Pathology, 56*(5), 891-910, doi:10.1111/j.1365-3059.2007.01619.x.

Jones, R. A. C. (1990). Strain group specific and virus specific hypersensitive reactions to infection with potyviruses in potato cultivars. *Annals of Applied Biology, 117*(1), 93-105, doi:10.1111/j.1744-7348.1990.tb04198.x.

Jones, R. A. C. (2014). Virus disease problems facing potato industries worldwide: viruses found, climate change implications, rationalizing virus strain nomenclature, and addressing the *Potato virus Y* issue. In R. Navarre, & M. J. Pavek (Eds.), *The potato: botany, production and uses* (pp. 202-224).

Juszczuk, M., Paczkowska, E., Sadowy, E., Zagorski, W., & Hulanicka, D. M. (2000). Effect of genomic and subgenomic leader sequences of *Potato leafroll virus* on gene expression. *FEBS Letters, 484*(1), 33-36, doi:10.1016/s0014-5793(00)02122-0.

Juszczuk, M., Zagorski-Ostoja, W., & Hulanicka, D. M. (1997). Studies on the translation mechanism of subgenomic RNA of *Potato leafroll virus*. *Acta Biochimica Polonica, 44*(1), 69-78.

Kamangar, S. B., Smagghe, G., Maes, M., & De Jonghe, K. (2014). *Potato virus Y* (PVY) strains in Belgian seed potatoes and first molecular detection of the N-Wi strain. *Journal of Plant Diseases and Protection, 121*(1), 10-19.

Kamer, G., & Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Research, 12*(18), 7269-7282, doi:10.1093/nar/12.18.7269.

Kaniewski, W., Lawson, C., Sammons, B., Haley, L., Hart, J., Delannay, X., et al. (1990). Field-resistance of transgenic Russet Burbank potato to effects of infection by *Potato virus X* and *Potato virus Y*. *Nature Biotechnology, 8*(8), 750-754, doi:10.1038/nbt0890-750.

Karasev, A. V. (2014). Recombinant strains of *Potato virus Y* - a new problem in potato. *Phytopathology, 104*(11), 182-182.

Karasev, A. V., & Gray, S. M. (2013a). Continuous and emerging challenges of *Potato virus Y* in potato. *Annual Review of Phytopathology, 51*(1), 571-586, doi:10.1146/annurev-phyto-082712-102332.

Karasev, A. V., & Gray, S. M. (2013b). Genetic diversity of *Potato virus Y* complex. *American Journal of Potato Research, 90*(1), 7-13, doi:10.1007/s12230-012-9287-7.

Karasev, A. V., Hu, X., Brown, C. J., Kerlan, C., Nikolaeva, O. V., Crosslin, J. M., et al. (2011). Genetic diversity of the ordinary strain of *Potato virus Y* (PVY) and origin of recombinant PVY strains. *Phytopathology, 101*(7), 778-785, doi:10.1094/phyto-10-10-0284.

Kasai, K., Morikawa, Y., Sorri, V. A., Valkonen, J. P. T., Gebhardt, C., & Watanabe, K. N. (2000). Development of SCAR markers to the PVY resistance gene *Ry(adg)* based on

a common feature of plant disease resistance genes. *Genome, 43*(1), 1-8, doi:DOI 10.1139/gen-43-1-1.

Kasschau, K. D., & Carrington, J. C. (2001). Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology, 285*(1), 71-81, doi:10.1006/viro.2001.0901.

Kawchuk, L. M., Martin, R. R., & McPherson, J. (1991). Sense and antisense RNA-mediated resistance to *Potato leafroll virus* in Russet Burbank potato plants. *Molecular Plant-Microbe Interactions, 4*(3), 247-253, doi:10.1094/mpmi-4-247.

Keese, P., Martin, R. R., Kawchuk, L. M., Waterhouse, P. M., & Gerlach, W. L. (1990). Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll Luteovirus and their relationships with 2 European isolates. *Journal of General Virology, 71*, 719-724, doi:10.1099/0022-1317-71-3-719.

Kehoe, M. A., & Jones, R. A. C. (2011). A proposal to help resolve the disagreement between naming of *Potato virus Y* strain groups defined by resistance phenotypes and those defined by sequencing. *Archives of Virology, 156*(12), 2273-2278, doi:10.1007/s00705-011-1095-8.

Kehoe, M. A., & Jones, R. A. C. (2015). Improving *Potato virus Y* strain nomenclature: lessons from comparing isolates obtained over a 73-year period. *Plant Pathology*, n/a-n/a, doi:10.1111/ppa.12404.

Kerlan, C. (2004). Evolution in *Potato virus* Y: from recombination in the genome to emergence and spreading of variants. In D. Giblot-Ducray (Ed.), *EAPR Virology 2004, Abstracts of the 12th European Association for Potato Research Virology Section Meeting, Rennes, France, 13-19 June 2004* (pp. 23-27). Wageningen, Netherlands: European Association for Potato Research.

Kerlan, C. (2006). *Potato virus Y*. Description of plant viruses. http://www.dpvweb.net/dpv/showdpv.php?dpvno=414. Accessed 9th October 2015.

Kerlan, C., Nikolaeva, O. V., Hu, X., Meacham, T., Gray, S. M., & Karasev, A. V. (2011). Identification of the molecular make-up of the *Potato virus Y* Strain PVY^Z: Genetic Typing of PVY^{Z-NTN}. *Phytopathology, 101*(9), 1052-1060, doi:10.1094/phyto-11-10-0317.

Kerlan, C., Tribodet, M., Glais, L., & Guillet, M. (1999). Variability of *Potato Virus Y* in potato crops in France. *Journal of Phytopathology, 147*(11-12), 643-651, doi:10.1046/j.1439-0434.1999.00441.x.

Khan, M. A. (2002). *Correlation of environmental conditions with major potato viruses and their vectors* (Integrated plant disease management. Proceedings of 3rd National Conference of Plant Pathology, NARC, Islamabad, 1-3 Oct. 2001).

Killick, R. J. (1979). Effect on infection with *Potato leaf roll virus* (PLRV) on yield and some of its components in a variety of potato (*Solanum tuberosum*). *Annals of Applied Biology, 91*(1), 67-74, doi:10.1111/j.1744-7348.1979.tb07414.x.

Kirchner, S. M., Hiltunen, L., Döring, T. F., Virtanen, E., Palohuhta, J. P., & Valkonen, J. P. T. (2013). Seasonal Phenology and Species Composition of the Aphid Fauna in a Northern Crop Production Area. *PLoS ONE, 8*(8), e71030, doi:10.1371/journal.pone.0071030.

Kloosterman, B., Visser, R. G. F., & Bachem, C. W. B. (2006). Isolation and characterization of a novel potato Auxin/Indole-3-Acetic Acid family member (StIAA2) that is involved in petiole hyponasty and shoot morphogenesis. *Plant Physiology and Biochemistry, 44*(11-12), 766-775, doi:10.1016/j.plaphy.2006.10.026.

Knuhtsen, H., Hiebert, E., & Purciful.De (1974). Partial-purification and some properties of *Tobacco etch virus*-induces intranuclear inclusions. *Virology, 61*(1), 200-209, doi:10.1016/0042-6822(74)90254-2.

Kogovsek, P., Pompe-Novak, M., Baebler, S., Rotter, A., Gow, L., Gruden, K., et al. (2010). Aggressive and mild *Potato virus Y* isolates trigger different specific responses in susceptible potato plants. *Plant Pathology, 59*(6), 1121-1132, doi:10.1111/j.1365-3059.2010.02340.x.

Kogovsek, P., Pompe-Novak, M., Petek, M., Fragner, L., Weckwerth, W., & Gruden, K. (2016). Primary metabolism, phenylpropanoids and antioxidant pathways are regulated in potato as a response to *Potato virus Y* infection. *PLoS ONE, 11*(1), doi:10.1371/journal.pone.0146135.

Kontanis, E. J., & Reed, F. A. (2006). Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. *Journal of Forensic Sciences, 51*(4), 795-804, doi:10.1111/j.1556-4029.2006.00182.x.

Koonin, E. V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-stranded RNA viruses. *Journal of General Virology, 72*, 2197-2206, doi:10.1099/0022-1317-72-9-2197.

Kopek, B. G., Perkins, G., Miller, D. J., Ellisman, M. H., & Ahlquist, P. (2007). Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. *PLoS Biology, 5*(9), 2022-2034, doi:10.1371/journal.pbio.0050220.

Koshkin, A. A., Nielsen, P., Meldgaard, M., Rajwanshi, V. K., Singh, S. K., & Wengel, J. (1998). LNA (Locked Nucleic Acid): An RNA Mimic Forming Exceedingly Stable LNA:LNA Duplexes. *Journal of the American Chemical Society, 120*(50), 13252-13253, doi:10.1021/ja9822862.

Kozlowska-Makulska, A., Guilley, H., Szyndel, M. S., Beuve, M., Lemaire, O., Herrbach, E., et al. (2010). P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. *Journal of General Virology, 91*, 1082-1091, doi:10.1099/vir.0.016360-0.

Kuhl, J. C., Novy, R. G., Whitworth, J. L., Dibble, M. S., Schneider, B., & Hall, D. (2016). Development of molecular markers closely Linked to the *Potato leafroll virus* resistance gene, *Rlr*$_{etb}$, for use in marker-assisted selection. *American Journal of Potato Research, 10.1007/s12230-016-9496-6*.

Kühn, J. (1859). *Die Krankheiten der Kulturgewächse, ihre Ursachen und ihre Verhütung* (Vol. 2). Berlin: Gustav Bosselmann, landwirthschaftliche Verlagsbuchhandlung.

Kuiper-Goodman, T., & Nawrot, P. S. Solanine and chaconine. http://www.inchem.org/documents/jecfa/jecmono/v30je19.htm. Accessed 24th September 2015.

Lachman, J., & Hamouz, K. (2005). Red and purple coloured potatoes as a significant antioxidant source in human nutrition – a review. *Plant Soil Environment, 51*(11), 477–482.

Lain, S., Riechmann, J. L., & Garcia, J. A. (1990). RNA helicase - a novel activity associated with a protein encoded by a positive strand RNA virus. *Nucleic Acids Research, 18*(23), 7003-7006, doi:10.1093/nar/18.23.7003.

Lain, S., Riechmann, J. L., Mendez, E., & Garcia, J. A. (1988). Nucleotide sequence of the 3' terminal region of Plum pox Potyvirus RNA. *Virus Research, 10*(4), 325-342, doi:10.1016/0168-1702(88)90074-3.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., Liu, Y.-P., et al. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO Journal, 25*(12), 2768-2780, doi:10.1038/sj.emboj.7601164.

Larbi, I., Djilani-Khouadja, F., Khamassy, N., & Fakhfakh, H. (2012). Potato virus surveys and wide spread of recombinant PVY[NTN] variant in Central Tunisia. *African Journal of Microbiology Research, 6*(9), 2109-2115, doi:10.5897/ajmr11.1385.

Lawson, E. C., Weiss, J. D., Thomas, P. E., & Kaniewski, W. K. (2001). NewLeaf Plus (R) Russet Burbank potatoes: replicase-mediated resistance to *Potato leafroll virus*. *Molecular Breeding, 7*(1), 1-12, doi:10.1023/a:1009637325028.

Le Romancer, M., Kerlan, C., & Nedellec, M. (1994). Biological characterization of various geographical isolates of *Potato virus Y* inducing superficial necrosis on potato tubers. *Plant Pathology, 43*(1), 138-144.

Lee, L., Kaplan, I. B., Ripoll, D. R., Liang, D., Palukaitis, P., & Gray, S. M. (2005). A surface loop of the *Potato leafroll virus* coat protein is involved in virion assembly, systemic movement, and aphid transmission. *Journal of Virology, 79*(2), 1207-1214, doi:10.1128/jvi.79.2.1207-1214.2005.

Lee, T. Y., & Fry, W. E. (1997). Genetic analysis of metalaxyl resistance in *Phytophthora infestans*. *Phytopathology, 87*(6 SUPPL.), S57-S57.

Leonard, S., Viel, C., Beauchemin, C., Daigneault, N., Fortin, M. G., & Laliberte, J. F. (2004). Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein in planta. *Journal of General Virology, 85*, 1055-1063, doi:10.1099/vir.0.19706-0.

Levin, B. R., & Bull, J. J. (1994). Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends in Microbiology, 2*(3), 76-81, doi:http://dx.doi.org/10.1016/0966-842X(94)90538-X.

Levis, C., & Astiermanifacier, S. (1993). The 5' untranslated region of PVY RNA, even located in an internal position, enables initiation of translation. *Virus Genes, 7*(4), 367-379, doi:10.1007/bf01703392.

Li, B., Su, H., Wu, C., Zhou, M., & An, Y. (2010). Development of IC-RT real-time PCR method for detection of *Cowpea severe mosaic virus*. *Journal of Zhejiang University (Agriculture and Life Sciences), 36*(5), 491-496.

Li, H., Ma, D., Jin, Y., Tu, Y., Liu, L., Leng, C., et al. (2015). Helper component-proteinase enhances the activity of 1-deoxy-(D)-xylulose-5-phosphate synthase and promotes the biosynthesis of plastidic isoprenoids in *Potato virus Y*-infected tobacco. *Plant Cell and Environment, 38*(10), 2023-2034, doi:10.1111/pce.12526.

Li, X. H., Valdez, P., Olvera, R. E., & Carrington, J. C. (1997). Functions of the tobacco etch virus RNA polymerase (NIb): Subcellular transport and protein-protein interaction with VPg/proteinase (NIa). *Journal of Virology, 71*(2), 1598-1607.

Li, X. J., Halpin, C., & Ryan, M. D. (2007). A novel cleavage site within the *Potato leafroll virus* P1 polyprotein. *Journal of General Virology, 88*, 1620-1623, doi:10.1099/vir.0.82627-0.

Licker, R., Johnston, M., Foley, J. A., Barford, C., Kucharik, C. J., Monfreda, C., et al. (2010). Mind the gap: how do climate and agricultural management explain the 'yield gap' of croplands around the world? *Global Ecology and Biogeography, 19*(6), 769-782, doi:10.1111/j.1466-8238.2010.00563.x.

Lin, L., Luo, Z., Yan, F., Lu, Y., Zheng, H., & Chen, J. (2011). Interaction between potyvirus P3 and ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) of host plants. *Virus Genes, 43*(1), 90-92, doi:10.1007/s11262-011-0596-6.

Lindner, K., & Billenkamp, N. (2005). Veränderungen im Stammspektrum des Kartoffelvirus Y: Eine Ursache für die Zunahme der Virusanfälligkeit von Kartoffel- und Tabaksorten? *Journal für Kulturpflanzen, 57*(12), 245-353.

Lindner, K., Trautwein, F., Kellermann, A., & Bauch, G. (2015). *Potato virus Y* (PVY) in seed potato certification. *Journal of Plant Diseases and Protection, 122*(3), 109-119.

Link, K., Vogel, F., & Sonnewald, U. (2011). PD trafficking of *Potato leaf roll virus* movement protein in *Arabidopsis* depends on site-specific protein phosphorylation. *Frontiers in Plant Science, 2*, doi:10.3389/fpls.2011.00018.

Linnet, K., & Kondratovich, M. (2004). Partly nonparametric approach for determining the limit of detection. *Clinical Chemistry, 50*(4), 732-740, doi:10.1373/clinchem.2003.029983.

Littell, R. C. (2006). Power calculations for mixed models. In R. C. Littell, W. W. Stroup, G. A. Milliken, R. D. Wolfinger, & O. Schabenberger (Eds.), *SAS for Mixed Models, Second Edition* (pp. 479-496). Cary, USA: SAS Institute.

Liu, Y., Zhai, H., Zhao, K., Wu, B. L., & Wang, X. F. (2012). Two suppressors of RNA silencing encoded by cereal-infecting members of the family Luteoviridae. *Journal of General Virology, 93*, 1825-1830, doi:10.1099/vir.0.042135-0.

Llave, C., Kasschau, K. D., & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences of the United States of America, 97*(24), 13401-13406, doi:10.1073/pnas.230334397.

Loebenstein, G. (2001). *Potato leafroll virus* (PLRV;Genus Polerovirus; family Luteoviridae). In G. Loebenstein, P. H. Berger, A. A. Brunt, & R. H. Lawson (Eds.), *Virus and Virus-Like Diseases of Potatoes and Production of Seed-Potatoes* (pp. 69-75). Dordrecht: Kluwer Academic Publishers.

Longdon, B., Hadfield, J. D., Day, J. P., Smith, S. C. L., McGonigle, J. E., Cogni, R., et al. (2015). The causes and consequences of changes in virulence following pathogen host shifts. *PLoS Pathogens, 11*(3), doi:10.1371/journal.ppat.1004728.

Loniewska-Lwowska, A., Chelstowska, S., Zagorski-Ostoja, W., & Palucha, A. (2009). Elements regulating *Potato leafroll virus* sgRNA1 translation are located within the coding sequences of the coat protein and read-through domain. *Acta Biochimica Polonica, 56*(4), 619-625.

Lopez-Moya, J. J., Wang, R. Y., & Pirone, T. P. (1999). Context of the coat protein DAG motif affects potyvirus transmissibility by aphids. *Journal of General Virology, 80*, 3281-3288.

Lorenzen, J., Nolte, P., Martin, D., Pasche, J. S., & Gudmestad, N. C. (2008). NE-11 represents a new strain variant class of *Potato virus* Y. *Archives of Virology, 153*(3), 517-525.

Lorenzen, J. H., Meacham, T., Berger, P. H., Shiel, P. J., Crosslin, J. M., Hamm, P. B., et al. (2006). Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada. *Archives of Virology, 151*(6), 1055-1074.

Lu, J., Du, Z. X., Kong, J., Chen, L. N., Qiu, Y. H., Li, G. F., et al. (2012). Transcriptome analysis of *Nicotiana tabacum* infected by *Cucumber mosaic virus* during systemic symptom development. *PLoS ONE, 7*(8), doi:10.1371/journal.pone.0043447.

Lu, L., Wu, G., Xu, X., Luan, H., Zhi, H., Cui, J., et al. (2015). Soybean actin-depolymerizing factor 2 interacts with *Soybean mosaic virus*-encoded P3 protein. *Virus Genes, 50*(2), 333-339, doi:10.1007/s11262-014-1150-0.

Lucas, W. J., & Lee, J. Y. (2004). Plant cell biology - Plasmodesmata as a supracellular control network in plants. *Nature Reviews Molecular Cell Biology, 5*(9), 712-726, doi:10.1038/nrm1470.

Lutaladio, N., & Castaidi, L. (2009). Potato: The hidden treasure. *Journal of Food Composition and Analysis, 22*(6), 491-493, doi:10.1016/j.jfca.2009.05.002.

Lutaladio, N., Ortiz, O., & Haverkort, A. (2010). *Sustainable potato production: guidelines for developing countries*. Rome, Italy: Food & Agriculture Organization of the United Nations (FAO).

Machida-Hirano, R. (2015). Diversity of potato genetic resources. *Breeding Science, 65*(1), 26-40, doi:10.1270/jsbbs.65.26.

Maga, J. A. (1980). Potato Glycoalkaloids. *Crc Critical Reviews in Food Science and Nutrition, 12*(4), 371-405.

Maki-Valkama, T., Valkonen, J. P. T., Kreuze, J. F., & Pehu, E. (2000). Transgenic resistance to PVY^O associated with post-transcriptional silencing of P1 transgene is overcome by PVY^N strains that carry highly homologous P1 sequences and recover transgene expression at infection. *Molecular Plant-Microbe Interactions, 13*(4), 366-373, doi:10.1094/mpmi.2000.13.4.366.

Mandahar, C. L. (2006). *Multiplication of RNA plant viruses*. Dordrecht: Springer.

Manoj, P. (2016). Droplet digital PCR technology promises new applications and research areas. *Mitochondrial DNA, 27*(1), 742-746, doi:10.3109/19401736.2014.913168.

Marczewski, W., Flis, B., Syller, J., Schafer-Pregl, R., & Gebhardt, C. (2001). A major quantitative trait locus for resistance to *Potato leafroll virus* is located in a resistance hotspot on potato chromosome XI and is tightly linked to N-gene-like markers. *Molecular Plant-Microbe Interactions, 14*(12), 1420-1425, doi:10.1094/mpmi.2001.14.12.1420.

Marczewski, W., Flis, B., Syller, J., Strzelczyk-Zyta, D., Hennig, J., & Gebhardt, C. (2004). Two allelic or tightly linked genetic factors at the PLRV.4 locus on potato chromosome XI control resistance to potato leafroll virus accumulation. *Theoretical and Applied Genetics, 109*(8), 1604-1609, doi:10.1007/s00122-004-1780-z.

Martinez, F., & Daros, J.-A. (2014). *Tobacco etch virus* Protein P1 traffics to the nucleolus and associates with the host 60S ribosomal subunits during infection. *Journal of Virology, 88*(18), 10725-10737, doi:10.1128/jvi.00928-14.

Mayo, M. A., Robinson, D. J., Jolly, C. A., & Hyman, L. (1989). Nucleotide-sequence of Potato leafroll Luteovirus RNA. *Journal of General Virology, 70*, 1037-1051.

McDonald, J. G., & Singh, R. P. (1996a). Host range, symptomology, and serology of isolates of *Potato virus Y* (PVY) that share properties with both the PVY^N and PVY^O strain groups. *American Potato Journal, 73*(7), 309-315, doi:10.1007/bf02855210.

McDonald, J. G., & Singh, R. P. (1996b). Response of potato cultivars to North American isolates of PVY^{NTN}. *American Potato Journal, 73*(7), 317-323, doi:10.1007/bf02855212.

Menke, U., SchildeRentschler, L., Ruoss, B., Zanke, C., Hemleben, V., & Ninnemann, H. (1996). Somatic hybrids between the cultivated potato *Solanum tuberosum* L. and the 1EBN wild species *Solanum pinnatisectum* Dun: Morphological and molecular characterization. *Theoretical and Applied Genetics, 92*(5), 617-626.

Merits, A., Guo, D. Y., Jarvekulg, L., & Saarma, M. (1999). Biochemical and genetic evidence for interactions between potato A potyvirus-encoded proteins P1 and P3 and proteins of the putative replication complex. *Virology, 263*(1), 15-22, doi:10.1006/viro.1999.9926.

Merits, A., Guo, D. Y., & Saarma, M. (1998). VPg, coat protein and five non-structural proteins of potato A potyvirus bind RNA in a sequence-unspecific manner. *Journal of General Virology, 79*, 3123-3127.

Merits, A., Rajamaki, M. L., Lindholm, P., Runeberg-Roos, P., Kekarainen, T., Puustinen, P., et al. (2002). Proteolytic processing of potyviral proteins and polyprotein processing intermediates in insect and plant cells. [Article]. *Journal of General Virology, 83*, 1211-1221.

Mestre, P., Brigneti, G., & Baulcombe, D. C. (2000). An Ry-mediated resistance response in potato requires the intact active site of the NIa proteinase from *Potato virus Y*. *Plant Journal, 23*(5), 653-661, doi:DOI 10.1046/j.1365-313x.2000.00834.x.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., et al. (2008). Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. *Cell, 133*(1), 116-127, doi:http://dx.doi.org/10.1016/j.cell.2008.02.034.

Mihovilovich, E., Aponte, M., Lindqvist-Kreuze, H., & Bonierbale, M. (2014). An RGA-derived SCAR marker linked to PLRV resistance from *Solanum tuberosum* ssp *andigena*. *Plant Molecular Biology Reporter, 32*(1), 117-128, doi:10.1007/s11105-013-0629-5.

Milbourne, D., Meyer, R. C., Collins, A. J., Ramsay, L. D., Gebhardt, C., & Waugh, R. (1998). Isolation, characterisation and mapping of simple sequence repeat loci in potato. *Molecular and General Genetics, 259*(3), 233-245.

Miller, J. S., & Mayo, M. A. (1991). The location of the 5′ end of the potato leafroll luteovirus subgenomic coat protein mRNA. *Journal of General Virology, 72*(11), 2633-2638, doi:10.1099/0022-1317-72-11-2633.

Miller, M. R., White, A., & Boots, M. (2006). The evolution of parasites in response to tolerance in their hosts: The good, the bad, and apparent commensalism. *Evolution, 60*(5), 945-956, doi:10.1554/05-654.1.

Miller, W. A., Dineshkumar, S. P., & Paul, C. P. (1995). Luteovirus gene expression. *Critical Reviews in Plant Sciences, 14*(3), 179-211, doi:10.1080/713608119.

Missiou, A., Kalantidis, K., Boutla, A., Tzortzakaki, S., Tabler, M., & Tsagris, M. (2004). Generation of transgenic potato plants highly resistant to *Potato virus Y* (PVY) through RNA silencing. *Molecular Breeding, 14*(2), 185-197, doi:Doi 10.1023/B:Molb.0000038006.32812.52.

Mochizuki, T., Ogata, Y., Hirata, Y., & Ohki, S. T. (2014). Quantitative transcriptional changes associated with chlorosis severity in mosaic leaves of tobacco plants infected with *Cucumber mosaic virus*. *Molecular Plant Pathology, 15*(3), 242-254, doi:10.1111/mpp.12081.

Mohan, B. R., Dinesh-Kumar, S. P., & Miller, W. A. (1995). Genes and cis-acting sequences involved in replication of *Barley yellow dwarf Virus*-PAV RNA. *Virology, 212*(1), 186-195, doi:10.1006/viro.1995.1467.

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods, 59*(2), 145-157, doi:http://dx.doi.org/10.1016/j.jbbm.2003.12.005.

Morroni, M., Jacquemond, M., & Tepfer, M. (2013). Deep sequencing of recombinant virus populations in transgenic and nontransgenic plants infected with *Cucumber mosaic virus*. *Molecular Plant-Microbe Interactions, 26*(7), 801-811, doi:10.1094/Mpmi-02-13-0057-R.

Motré, A., Li, Y., & Kong, H. (2008). Enhancing helicase-dependent amplification by fusing the helicase with the DNA polymerase. *Gene, 420*(1), 17-22, doi:10.1016/j.gene.2008.04.017.

Moury, B. (2010). A new lineage sheds light on the evolutionary history of *Potato virus Y*. *Mol Plant Pathol, 11*(1), 161-168, doi:10.1111/j.1364-3703.2009.00573.x.

Moury, B., Caromel, B., Johansen, E., Simon, V., Chauvin, L., Jacquot, E., et al. (2011). The helper component proteinase cistron of *Potato virus Y* induces hypersensitivity and resistance in potato genotypes carrying dominant resistance genes on chromosome IV. *Molecular Plant-Microbe Interactions, 24*(7), 787-797, doi:10.1094/mpmi-10-10-0246.

Moury, B., Morel, C., Johansen, E., & Jacquemond, M. (2002). Evidence for diversifying selection in *Potato virus Y* and in the coat protein of other potyviruses. *The Journal of general virology, 83*(Pt 10), 2563-2573, doi:10.1099/0022-1317-83-10-2563.

Mueller, E., Gilbert, J., Davenport, G., Brigneti, G., & Baulcombe, D. C. (1995). Homology-dependent resistance - Transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant Journal, 7*(6), 1001-1013, doi:10.1046/j.1365-313X.1995.07061001.x.

Mukherjee, K., Verma, Y., Chakrabarti, S. K., Singh, M. N., & Khurana, S. M. P. (2003). Cloning and sequencing of coat protein gene of an Indian *Potato leaf roll virus* (PLRV) isolate and its similarity with other members of *Luteoviridae*. *Virus Genes, 26*(3), 247-253.

Mullis, K. B., Erlich, H. A., Arnheim, N., Horn, G. T., Saiki, R. K., & Scharf, S. J. (1987). Process for amplifying, detecting, and/or-cloning nucleic acid sequences. Patent US4683195 A.

Nanayakkara, U. N., Singh, M., Pelletier, Y., & Nie, X. (2012). Investigation of *Potato virus Y* (PVY) strain status and variant population in potatoes in New Brunswick, Canada. *American Journal of Potato Research, 89*(3), 232-239, doi:10.1007/s12230-012-9247-2.

National Center for Biotechnology Information: NCBI GenBank. http://www.ncbi.nlm.nih.gov/.

Navarro, E., Serrano-Heras, G., Castano, M. J., & Solera, J. (2015). Real-time PCR detection chemistry. *Clinica Chimica Acta, 439*, 231-250, doi:10.1016/j.cca.2014.10.017.

Naveed, K., Mitter, N., Harper, A., Dhingra, A., & Pappu, H. R. (2014). Comparative analysis of virus-specific small RNA profiles of three biologically distinct strains of *Potato virus Y* in infected potato (*Solanum tuberosum*) cv. Russet Burbank. *Virus Res, 191*, 153-160, doi:10.1016/j.virusres.2014.07.005.

Neergaard, E. d., Munk, L., & Nielsen, S. L. (2014). First report of *Potato leafroll virus, Potato virus A*, *Potato virus X* and *Potato virus Y* in potato in Greenland. *New Disease Reports, 30*, 20-20.

Ng, J. C. K., & Falk, B. W. (2006). Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology, 44*, 183-212, doi:10.1146/annurev.phyto.44.070505.143325.

Nickel, H., Kawchuk, L., Twyman, R. M., Zimmermann, S., Junghans, H., Winter, S., et al. (2008). Plantibody-mediated inhibition of the *Potato leafroll virus* P1 protein reduces virus accumulation. *Virus Research, 136*(1-2), 140-145, doi:10.1016/j.virusres.2008.05.001.

Nicolia, A., Proux-Wera, E., Ahman, I., Onkokesung, N., Andersson, M., Andreasson, E., et al. (2015). Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts. *Journal of Biotechnology, 204*, 17-24, doi:10.1016/j.jbiotec.2015.03.021.

Nie, B., Singh, M., Murphy, A., Sullivan, A., Xie, C., & Nie, X. (2012). Response of potato cultivars to five isolates belonging to four strains of *Potato virus Y*. *Plant Disease, 96*(10), 1422-1429, doi:10.1094/pdis-01-12-0018-re.

Nie, X., & Molen, T. A. (2015). Host recovery and reduced virus level in the upper leaves after *Potato virus Y* infection occur in tobacco and tomato but not in potato plants. *Viruses-Basel, 7*(2), 680-698, doi:10.3390/v7020680.

Nie, X., & Singh, R. P. (2002a). Probable geographical grouping of PVY(^{N}) and PVY(^{NTN}) based on sequence variation in P1 and 5'-UTR of PVY genome and methods for differentiating North American PVY(^{NTN}). *Journal of Virological Methods, 103*(2), 145-156.

Nie, X., & Singh, R. P. (2003a). Evolution of North American PVY(NTN) strain Tu 660 from local PVY(N) by mutation rather than recombination. *Virus Genes, 26*(1), 39-47.

Nie, X. Z., & Singh, R. P. (2002b). A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RT-PCR. *Journal of Virological Methods, 104*(1), 41-54.

Nie, X. Z., & Singh, R. P. (2003b). Evolution of North American PVY^{NTN} strain Tu 660 from local PVY^N by mutation rather than recombination. *Virus Genes, 26*(1), 39-47, doi: C:\Dokumente und Einstellungen\Anja.Huehnlein\Eigene Dateien\Literatur\PVY

Nie, X. Z., Singh, R. P., & Singh, M. (2004). Molecular and pathological characterization of N : O isolates of the *Potato virus Y* from Manitoba, Canada. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 26*(4), 573-583.

Nolte, P., Wihitworth, J. L., Thornton, M. K., & McIntosh, C. S. (2004). Effect of seedborne *Potato virus Y* on performance of Russet Burbank, Russet Norkotah, and Shepody potato. *Plant Disease, 88*, 248-252.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research, 28*(12), doi:10.1093/nar/28.12.e63.

Novina, C. D., & Sharp, P. A. (2004). The RNAi revolution. *Nature, 430*(6996), 161-164, doi:10.1038/430161a.

Nutrition Data (2015). Nutrition Facts - Potatoes, boiled, cooked in skin, flesh, without salt. http://nutritiondata.self.com/. Accessed 23rd September 2015.

Oerke, E. C., & Dehne, H. W. (2004). Safeguarding production - losses in major crops and the role of crop protection. *Crop Protection, 23*(4), 275-285, doi:10.1016/j.cropro.2003.10.001.

Ogawa, T., Nakagawa, A., Hataya, T., & Ohshima, K. (2012). The genetic structure of populations of *Potato virus Y* in Japan; based on the analysis of 20 full genomic sequences. *Journal of Phytopathology, 160*(11-12), 661-673, doi:10.1111/j.1439-0434.2012.01959.x.

Ogawa, T., Tomitaka, Y., Nakagawa, A., & Ohshima, K. (2008). Genetic structure of a population of *Potato virus Y* inducing potato tuber necrotic ringspot disease in Japan; comparison with North American and European populations. *Virus Research, 131*(2), 199-212, doi:10.1016/j.virusres.2007.09.010.

Oyarzun, P. J., Ordones, M. E., Forbes, G. A., & Fry, W. E. (1997). First report of *Phytophthora infestans* A2 mating type in Ecuador. *Plant Disease, 81*(3), 311-311, doi:10.1094/pdis.1997.81.3.311c.

Pagan, I., Alonso-Blanco, C., & Garcia-Arenal, F. (2007). The relationship of within-host multiplication and virulence in a plant-virus system. *PLoS ONE, 2*(8), doi:10.1371/journal.pone.0000786.

Pallas, V., & Garcia, J. A. (2011). How do plant viruses induce disease? Interactions and interference with host components. *The Journal of general virology, 92*(Pt 12), 2691-2705, doi:10.1099/vir.0.034603-0.

Pałucha, A., Sadowy, E., Kujawar, A., Juszczcuk, M., Zagórski, W., & Hulanicka, D. (1994). Nucleotide sequence of RNA of a Polish isolate of potato leafroll luteovirus. *Acta Biochimica Polonica, 41*(4), 405-414.

Pandey, S. K. (2007). Approaches for breaching yield stagnation in potato. *Potato Journal (Shimla), 34*(1-2), 1-9.

Parry, H. M., Zuo, J., Frumento, G., Mirajkar, N., Inman, C., Edwards, E., et al. (2016). Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years. *Immunity & Ageing, 13*, doi:10.1186/s12979-015-0056-6.

Pasin, F., Simon-Mateo, C., & Antonio Garcia, J. (2014). The hypervariable amino-terminus of P1 protease modulates potyviral replication and host defense responses. *PLoS Pathogens, 10*(3), doi:10.1371/journal.ppat.1003985.

Patterson, E., Guo, T.-C., Evensen, O., Haugland, O., & Mikalsen, A. B. (2015). In vitro adaptation of SAV3 in cell culture correlates with reduced in vivo replication capacity and virulence to Atlantic salmon (Salmo salar L.) parr. *Journal of General Virology, 96*, 3023-3034, doi:10.1099/jgv.0.000242.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., et al. (2006). F-box-like domain in the polerovirus protein P0 is required for silencing

suppressor function. *Proceedings of the National Academy of Sciences of the United States of America, 103*(6), 1994-1999, doi:10.1073/pnas.0510784103.

Peng, Y. H., Kadoury, D., Gal-On, A., Wang, Y., & Raccah, B. (1998). Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. *Journal of General Virology, 79*, 897-904.

Pereira, A. B., Villa Nova, N. A., Ramos, V. J., & Pereira, A. R. (2008). Potato potential yield based on climatic elements and cultivar characteristics. *Bragantia, 67*(2), 327-334, doi:10.1590/s0006-87052008000200008.

Peter, K., Palukaitis, P., Gildow, F., & Gray, S. (2008a). Phloem limitation of *Potato leafroll virus* is an asset not a liability. *Phytopathology, 98*(6), S124-S125.

Peter, K. A., Gildow, F., Palukaitis, P., & Gray, S. M. (2009). The C terminus of the polerovirus P5 readthrough domain limits virus infection to the phloem. *Journal of Virology, 83*(11), 5419-5429, doi:10.1128/jvi.02312-08.

Peter, K. A., Liang, D. L., Palukaitis, P., & Gray, S. M. (2008b). Small deletions in the *Potato leafroll virus* readthrough protein affect particle morphology, aphid transmission, virus movement and accumulation. [Article]. *Journal of General Virology, 89*, 2037-2045, doi:10.1099/vir.0.83625-0.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In S. A. Bustin (Ed.), *A-Z of quantitative PCR* (pp. 89-120). La Jolla, USA: International University Line.

PGSC (2011). Genome sequence and analysis of the tuber crop potato. *Nature, 475*(7355), 189-195, doi:10.1038/nature10158.

Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W., & Sontheimer, E. J. (2004). A Dicer-2-dependent 80S complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell, 117*(1), 83-94, doi:10.1016/s0092-8674(04)00258-2.

Phan, D., & Wodarz, D. (2015). Modeling multiple infection of cells by viruses: Challenges and insights. *Mathematical Biosciences, 264*, 21-28, doi:10.1016/j.mbs.2015.03.001.

Piche, L. M., Singh, R. P., Nie, X., & Gudmestad, N. C. (2004). Diversity among *Potato virus Y* isolates bbtained from potatoes grown in the United States. *Phytopathology, 94*(12), 1368-1375, doi:10.1094/PHYTO.2004.94.12.1368.

Pieterse, L., & Judd, J. (2014). *World Catalogue of Potato Varieties* (6ed.). Clenze, Germany: Erling Verlag.

Poma, H. R., Davies, C., Gutierrez Cacciabue, D., Mora, M. C., Basombrio, M. A., & Rajal, V. B. (2012). Comparison of nucleic acid extraction efficiency using different commercial kits and qPCR. Effect of inhibitors. *Revista Argentina De Microbiologia, 44*(3), 144-149.

Pospieszny, H., Hasiow-Jaroszewska, B., Rymelska, N., & Borodynko, N. (2012). Using the IC real-time RT-PCR technique for the detection of *Tomato torrado virus* (ToTV) in tomato seedling from infected seeds. *Progress in Plant Protection, 52*(3), 515-517.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., & Tepfer, M. (2008). Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology, 9*(1), 73-83, doi:10.1111/j.1364-3703.2007.00447.x.

Prüfer, D., Kawchuk, L., Monecke, M., Nowok, S., Fischer, R., & Rohde, W. (1999). Immunological analysis of potato leafroll luteovirus (PLRV) P1 expression identifies a 25 kDa RNA-binding protein derived via P1 processing. *Nucleic Acids Research, 27*(2), 421-425.

Prufer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., & Rohde, W. (1992). Ribosomal frameshifting in plants - a novel signal directs the -1 frameshift in the synthesis of the putative viral replicase of potato leafroll Luteovirus. *EMBO Journal, 11*(3), 1111-1117.

Pruss, G., Ge, X., Shi, X. M., Carrington, J. C., & Vance, V. B. (1997). Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that

transactivates replication of heterologous viruses. *Plant Cell, 9*(6), 859-868, doi:10.1105/tpc.9.6.859.

Puustinen, P., & Makinen, K. (2004). Uridylylation of the potyvirus VPg by viral replicase NIb correlates with the nucleotide binding capacity of VPg. *Journal of Biological Chemistry, 279*(37), 38103-38110, doi:10.1074/jbc.M402910200.

Quenouille, J., Vassilakos, N., & Moury, B. (2013). *Potato virus Y*: a major crop pathogen that has provided major insights into the evolution of viral pathogenicity. *Molecular Plant Pathology, 14*(5), 439-452, doi:10.1111/mpp.12024.

Rajamäki, M.-L., & Valkonen, J. P. T. (1999). The 6K2 protein and the VPg of potato virus A are determinants of systemic infection in Nicandra physaloides. *Molecular Plant-Microbe Interactions, 12*(12), 1074-1081, doi:10.1094/mpmi.1999.12.12.1074.

Rajamäki, M.-L., & Valkonen, J. P. T. (2009). Control of nuclear and nucleolar localization of nuclear inclusion protein a of picorna-like *Potato virus A* in *Nicotiana* species. *Plant Cell, 21*(8), 2485-2502, doi:10.1105/tpc.108.064147.

Rakosy-Tican, E., Thieme, R., Nachtigall, M., Molnar, I., & Denes, T.-E. (2015). The recipient potato cultivar influences the genetic makeup of the somatic hybrids between five potato cultivars and one cloned accession of sexually incompatible species *Solanum bulbocastanum* Dun. *Plant Cell Tissue and Organ Culture, 122*(2), 395-407, doi:10.1007/s11240-015-0777-4.

Rantalainen, K. I., Eskelin, K., Tompa, P., & Makinen, K. (2011). Structural Flexibility Allows the Functional Diversity of Potyvirus Genome-Linked Protein VPg. *Journal of Virology, 85*(5), 2449-2457, doi:10.1128/jvi.02051-10.

Reinbold, C., Gildow, F. E., Herrbach, E., Ziegler-Graff, V., Goncalves, M. C., van den Heuvel, J., et al. (2001). Studies on the role of the minor capsid protein in transport of Beet western yellows virus through Myzus persicae. *Journal of General Virology, 82*, 1995-2007.

Restrepo-Hartwig, M. A., & Carrington, J. C. (1994). The tobacco etch Potyvirus 6-kilodalton protein is membrane-associated and involved in viral replication. *Journal of Virology, 68*(4), 2388-2397.

Restrepo, M. A., Freed, D. D., & Carrington, J. C. (1990). Nuclear transport of plant potyviral proteins. *Plant Cell, 2*(10), 987-998.

Revers, F., & García, J. A. (2015). Chapter Three - Molecular Biology of Potyviruses. In M. Karl, & C. M. Thomas (Eds.), *Advances in Virus Research* (Vol. Volume 92, pp. 101-199): Academic Press.

Ristaino, J. B. (2002). Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. *Microbes and Infection, 4*(13), 1369-1377, doi:10.1016/S1286-4579(02)00010-2.

Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., & Casse-Delbart, F. (1989). Nucleotide sequence of *Potato virus Y* (N Strain) genomic RNA. *The Journal of general virology, 70 (Pt 4)*, 935-947, doi:10.1099/0022-1317-70-4-935.

Roberts, I. M., Wang, D., Findlay, K., & Maule, A. J. (1998). Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (CIs) show that the CI protein acts transiently in aiding virus movement. *Virology, 245*(1), 173-181, doi:10.1006/viro.1998.9132.

Rodriguezcerezo, E., Klein, P. G., & Shaw, J. G. (1991). A determinant of disease symptom severity is located in the 3'-terminal noncoding region of the RNA of a plant virus. *Proceedings of the National Academy of Sciences of the United States of America, 88*(21), 9863-9867, doi:10.1073/pnas.88.21.9863.

Rogers‑Broadway, K. R., & Karteris, E. (2015). Amplification efficiency and thermal stability of qPCR instrumentation: Current landscape and future perspectives.

Experimental and therapeutic medicine, 10(4), 1261-1264, doi:10.3892/etm.2015.2712.

Rohde, W., Gramstat, A., Schmitz, J., Tacke, E., & Prüfer, D. (1994). Plant viruses as model systems for the study of noncanonical translation mechanisms in higher plants. *Journal of General Virology, 75*, 2141-2149, doi:10.1099/0022-1317-75-9-2141.

Rojas, M. R., Zerbini, F. M., Allison, R. F., Gilbertson, R. L., & Lucas, W. J. (1997). Capsid protein and helper component proteinase function as potyvirus cell-to-cell movement proteins. *Virology, 237*(2), 283-295, doi:10.1006/viro.1997.8777.

Rolland, M., Lacroix, C., Blanchard, A., Baldwin, T., Kerlan, C., & Jacquot, E. (2008). *Potato virus Y* (PVY): from its discovery to the latest outbreaks. *Virologie, 12*(4), 261-273, doi:10.1684/vir.2008.0190.

Romon, M., Soustre-Gacougnolle, I., Schmitt, C., Perrin, M., Burdloff, Y., Chevalier, E., et al. (2013). RNA Silencing Is Resistant to Low-Temperature in Grapevine. *PLoS ONE, 8*(12), doi:10.1371/journal.pone.0082652.

Rosner, A., & Raccah, B. (1988). Nucleotide sequence of the capsid protein gene of *Potato virus Y* (PVY). *Virus Genes, 1*(3), 255-260.

Ross, H. (1959). On the distribution of tobacco veinal necrosis strain of *Potato virus Y* in Germany and other countries. *Phytopathologische Zeitschrift, 35*(1), 97-102.

Ross, H. (1986). Potato breeding - problems and perspectives. *Fortschritte der Pflanzenzuchtung*(13), 132pp.-132pp.

Rossmann, M. G., & Johnson, J. E. (1989). Icosahedral RNA virus structure. *Annual Review of Biochemistry, 58*, 533-573.

Roudet-Tavert, G., Michon, T., Walter, J., Delaunay, T., Redondo, E., & Le Gall, O. (2007). Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro. *Journal of General Virology, 88*, 1029-1033, doi:10.1099/vir.0.82501-0.

Rowhani, A., Maningas, M. A., Lile, L. S., Daubert, S. D., & Golino, D. A. (1995). Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. *Phytopathology, 85*(3), 347-352, doi:10.1094/Phyto-85-347.

Ruiz de Galarreta, J. I., Lopez-Pardo, R., Tierno, R., Alor, N., Barandalla, L., Haase, N. U., et al. (2015). Disease resistance and nutritional properties of tuber-bearing native potato species and old Spanish cultivars. *Journal of Agricultural Science and Technology, 17*(4), 935-947.

Sadowy, E., Juszczuk, M., David, C., Gronenborn, B., & Hulanicka, M. D. (2001a). Mutational analysis of the proteinase function of *Potato leafroll virus*. *Journal of General Virology, 82*, 1517-1527.

Sadowy, E., Maasen, A., Juszczuk, M., David, C., Zagórski-Ostoja, W., Gronenborn, B., et al. (2001b). The ORF0 product of *Potato leafroll virus* is indispensable for virus accumulation. *Journal of General Virology, 82*(6), 1529-1532.

Safarnejad, M. R., Jouzani, G. S., Tabatabaie, M., Twyman, R. M., & Schillberg, S. (2011). Antibody-mediated resistance against plant pathogens. *Biotechnology Advances, 29*(6), 961-971, doi:10.1016/j.biotechadv.2011.08.011.

Salazar, L. F. (2003). Potato viruses after the XXth century: effects, dissemination and their control. www.crawfordfund.org/wp-content/uploads/2014/03/Salazar-Potato-Viruses.pdf. Accessed 2nd February 2016.

Salazar, L. F., Bartolini, I., & Flores, V. (2000). Evidence for the existence of PVY[NTN] in the Andes and a hypothesis towards its origin. *Fitopatologia, 35*(2), 87-90.

Salvador, B., Saenz, P., Yanguez, E., Quiot, J. B., Quiot, L., Delgadillo, M. O., et al. (2008). Host-specific effect of P1 exchange between two potyviruses. *Molecular Plant Pathology, 9*(2), 147-155, doi:10.1111/j.1364-3703.2007.00450.x.

Sato, M., Nishikawa, K., Komura, K., & Hosaka, K. (2006). *Potato virus Y* resistance gene, *Ry(chc)*, mapped to the distal end of potato chromosome 9. *Euphytica, 149*(3), 367-372, doi:10.1007/s10681-006-9090-y.

Sawazaki, H. E., Caram de Souza-Dias, J. A., Lorenzen, J. H., Jeffries, C., Modolo, D. G., & Machado, D. S. (2009). *Potato Virus Y*-NTN: A coat and P1 protein sequences analysis of a Brazilian isolate. *Potato Research, 52*(4), 379-392, doi:10.1007/s11540-009-9141-6.

Schaad, M. C., Jensen, P. E., & Carrington, J. C. (1997). Formation of plant RNA virus replication complexes on membranes: Role of an endoplasmic reticulum-targeted viral protein. *EMBO Journal, 16*(13), 4049-4059, doi:10.1093/emboj/16.13.4049.

Schmitz, J., StussiGaraud, C., Tacke, E., Prufer, D., Rohde, W., & Rohfritsch, O. (1997). In situ localization of the putative movement protein (pr17) from potato leafroll luteovirus (PLRV) in infected and transgenic potato plants. *Virology, 235*(2), 311-322, doi:10.1006/viro.1997.8679.

Schoelz, J. E., & Wintermantel, W. M. (1993). Expansion of viral host-range through complementation and recombination in transgenic plants. *Plant Cell, 5*(11), 1669-1679.

Schouten, H. J., Krens, F. A., & Jacobsen, E. (2006). Cisgenic plants are similar to traditionally bred plants. *International regulations for genetically modified organisms should be altered to exempt cisgenesis, 7*(8), 750-753, doi:10.1038/sj.embor.7400769.

Schubert, J., Fomitcheva, V., & Sztangret-Wiśniewska, J. (2007). Differentiation of *Potato virus Y* strains using improved sets of diagnostic PCR-primers. *Journal of Virological Methods, 140*(1-2), 66-74.

Schubert, J., Thieme, T., Thieme, R., Cuong Viet, H., & Giang Thi, H. (2015). Molecular and biological characterization of *Potato virus Y* isolates from Vietnam. *Journal of Phytopathology, 163*(7-8), 620-631, doi:10.1111/jph.12362.

Schubert, S., Grünweller, A., Erdmann, V. A., & Kurreck, J. (2005). Local RNA target structure influences siRNA efficacy: Systematic analysis of intentionally designed binding regions. *Journal of Molecular Biology, 348*(4), 883-893, doi:http://dx.doi.org/10.1016/j.jmb.2005.03.011.

Secor, G. A., Rivera, V. V., Abad, J. A., Lee, I. M., Clover, G. R. G., Liefting, L. W., et al. (2009). Association of '*Candidatus Liberibacter solanacearum*' with Zebra Chip Disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. *Plant Disease, 93*(6), 574-583, doi:10.1094/pdis-93-6-0574.

Senthil, G., Liu, H., Puram, V. G., Clark, A., Stromberg, A., & Goodin, M. M. (2005). Specific and common changes in *Nicotiana benthamiana* gene expression in response to infection by enveloped viruses. *Journal of General Virology, 86*, 2615-2625, doi:10.1099/vir.0.81043-0.

Seo, J.-K., Kang, S.-H., Seo, B. Y., Jung, J. K., & Kim, K.-H. (2010). Mutational analysis of interaction between coat protein and helper component-proteinase of *Soybean mosaic virus* involved in aphid transmission. *Molecular Plant Pathology, 11*(2), 265-276, doi:10.1111/j.1364-3703.2009.00603.x.

Shen, W. T., Wang, M. Q., Yan, P., Gao, L., & Zhou, P. (2010). Protein interaction matrix of *Papaya ringspot virus* type P based on a yeast two-hybrid system. *Acta Virologica, 54*(1), 49-54, doi:10.4149/av_2010_01_49.

Shi, B. J., Palukaitis, P., & Symons, R. H. (2002). Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. *Molecular Plant-Microbe Interactions, 15*(9), 947-955, doi:10.1094/mpmi.2002.15.9.947.

Shi, X. M., Miller, H., Verchot, J., Carrington, J. C., & Vance, V. B. (1997). Mutations in the region encoding the central domain of helper component-proteinase (HC-Pro)

eliminate *Potato virus X*/potyviral synergism. *Virology, 231*(1), 35-42, doi:10.1006/viro.1997.8488.

Shiboleth, Y. M., Haronsky, E., Leibman, D., Arazi, T., Wassenegger, M., Whitham, S. A., et al. (2007). The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. *Journal of Virology, 81*(23), 13135-13148, doi:10.1128/jvi.01031-07.

Shukla, D. D., Ward, C. W., & Brunt, A. A. (1994). *The Potyviridae* (The Potyviridae.).

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., et al. (2015). Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research, 22*(1), 5-34, doi:10.1007/s11356-014-3470-y.

Singh, M., & Singh, R. P. (1996). Nucleotide sequence and genome organization of a Canadian isolate of the common strain of *Potato virus Y* (PVY^O). *Canadian Journal of Plant Pathology, 18*(3), 209-224.

Singh, R. P., McLaren, D. L., Nie, X., & Singh, M. (2003). Possible escape of a recombinant isolate of *Potato virus Y* by serological indexing and methods of its detection. *Plant Disease, 87*(6), 679-685, doi:10.1094/pdis.2003.87.6.679.

Singh, R. P., Valkonen, J. P. T., Gray, S. M., Boonham, N., Jones, R. A. C., Kerlan, C., et al. (2008). Discussion paper: The naming of *Potato virus Y* strains infecting potato. *Archives of Virology, 153*(1), 1-13, doi:10.1007/s00705-007-1059-1.

Singsit, C., & Hanneman, R. E. (1991). Rescuing abortive inter-EBN potato hybrids through double pollination and embryo rescue. *Plant Cell Reports, 9*(9), 475-478.

Small, I., & Puchta, H. (2014). Emerging tools for synthetic biology in plants. *The Plant Journal, 78*(5), 725-726, doi:10.1111/tpj.12462.

Smirnova, E., Firth, A. E., Miller, W. A., Scheidecker, D., Brault, V., Reinbold, C., et al. (2015). Discovery of a Small Non-AUG-Initiated ORF in Poleroviruses and Luteoviruses That Is Required for Long-Distance Movement. *PLoS Pathogens, 11*(5), e1004868, doi:10.1371/journal.ppat.1004868.

Smith, K. M. (1924). On a curious effect of mosaic disease upon the cells of the Potato leaf. *Annals of Botany, 38*(150), 385-388.

Smith, K. M. (1931). Composite nature of certain potato viruses of the mosaic group. *Nature Australia, 127*(3210), 702, doi:10.1038/127702a0.

Smith, O. P., & Harris, K. F. (1990). *Potato leaf roll virus* 3'genome organization: Sequence of the coat protein gene and identification of a viral subgenomic RNA. *Molecular Plant Pathology, 80*(7), 609-614.

Solomon-Blackburn, R. M., & Barker, H. (2001a). Breeding virus resistant potatoes *(Solanum tuberosum)*: a review of traditional and molecular approaches. *Heredity, 86*, 17-35, doi:10.1046/j.1365-2540.2001.00799.x.

Solomon-Blackburn, R. M., & Barker, H. (2001b). A review of host major-gene resistance to potato viruses X, Y, A and V in potato: genes, genetics and mapped locations. *Heredity, 86*, 8-16, doi:10.1046/j.1365-2540.2001.00798.x.

Song, Y. S., Hepting, L., Schweizer, G., Hartl, L., Wenzel, G., & Schwarzfischer, A. (2005). Mapping of extreme resistance to PVY (*Ry(sto)*) on chromosome XII using anther-culture-derived primary dihaploid potato lines. *Theoretical and Applied Genetics, 111*(5), 879-887, doi:10.1007/s00122-005-0010-7.

Song, Y. S., & Schwarzfischer, A. (2008). Development of STS markers for selection of extreme resistance (*Ry (sto)*) to PVY and maternal pedigree analysis of extremely resistant cultivars *American Journal of Potato Research, 85*(5), 392-393, doi:10.1007/s12230-008-9044-0.

Song, Y. Z., Han, Q. J., Jiang, F., Sun, R. Z., Fan, Z. H., Zhu, C. X., et al. (2014). Effects of the sequence characteristics of miRNAs on multi-viral resistance mediated by single

amiRNAs in transgenic tobacco. *Plant Physiol Biochem, 77*, 90-98, doi:10.1016/j.plaphy.2014.01.008.

Sos-Hegedus, A., Lovas, A., Kondrak, M., Kovacs, G., & Banfalvi, Z. (2005). Active RNA silencing at low temperature indicates distinct pathways for antisense-mediated gene-silencing in potato. *Plant Molecular Biology, 59*(4), 595-602, doi:10.1007/s11103-005-0354-z.

Sotelo, A., Contreras, E., Sousa, H., & Hernandez, V. (1998). Nutrient composition and toxic factor content of four wild species of Mexican potato. *Journal of Agricultural and Food Chemistry, 46*(4), 1355-1358, doi:10.1021/jf970773f.

Soumounou, Y., & Laliberte, J. F. (1994). Nucleic acid-binding properties of the P1 protein of Turnip mosaic Potyvirus produced in *Escherichia coli*. *Journal of General Virology, 75*, 2567-2573, doi:10.1099/0022-1317-75-10-2567.

Souto, S., Olveira, J. G., & Bandin, I. (2015). Influence of temperature on Betanodavirus infection in Senegalese sole (*Solea senegalensis*). *Veterinary Microbiology, 179*(3-4), 162-167, doi:10.1016/j.vetmic.2015.07.004.

Spetz, C., & Valkonen, J. P. T. (2004). Potyviral 6K2 protein long-distance movement and symptom-induction functions are independent and host-specific. *Molecular Plant-Microbe Interactions, 17*(5), 502-510, doi:10.1094/mpmi.2004.17.5.502.

Spök, A., Karner, S., & Gaugitsch, H. (2002). *Introduction.* Paper presented at the conference "Evaluating substantial equivalence: A step towards improving the risk/safety evaluation of GMOs", Vienna, Austria, October 19th–20th 2001, Conference papers/Tagungsberichte of the Federal Environment Agency – Austria*, 32*, 5-10.

Spooner, D. M., & Bamberg, J. B. (1994). Potato genetic resources - Sources of resistance and systematics. *American Potato Journal, 71*(5), 325-337, doi:10.1007/bf02849059.

Srinivasan, R., & Alvarez, J. M. (2007). Effect of mixed viral infections (*Potato virus Y-Potato leafroll virus*) on biology and preference of vectors *Myzus persicae* and *Macrosiphum euphorbiae* (Hemiptera : *Aphididae*). *Journal of Economic Entomology, 100*(3), 646-655.

Stace-Smith, R., & Tremaine, J. H. (1970). Purification and composition of *Potato virus Y*. *Phytopathology, 60*(12), 1785-1789.

Sun, Y., Zhang, X., Liu, L., Liu, X., & Liu, H. (2008). High temperature activate RNA silencing-mediated antiviral defense in tobacco plants. *Acta Phytopathologica Sinica, 38*(1), 58-63.

Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M. W., & Kubista, M. (2015). How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification, 3*, 9-16, doi:10.1016/j.bdq.2015.01.005.

Swiezynski, K. M., Dziewonska, M. A., & Ostrowska, K. (1989). Resistance to the *Potato leafroll virus* (PLRV) in diploid potatoes. *Plant Breeding, 103*(3), 221-227, doi:10.1111/j.1439-0523.1989.tb00375.x.

Szajko, K., Chrzanowska, M., Witek, K., Strzelczyk-Zyta, D., Zagorska, H., Gebhardt, C., et al. (2008). The novel gene Ny-1 on potato chromosome IX confers hypersensitive resistance to *Potato virus Y* and is an alternative to Ry genes in potato breeding for PVY resistance. *Theoretical and Applied Genetics, 116*(2), 297-303, doi:10.1007/s00122-007-0667-1.

Szajko, K., Strzelczyk-Żyta, D., & Marczewski, W. (2014). *Ny*-1 and *Ny*-2 genes conferring hypersensitive response to *Potato virus Y* (PVY) in cultivated potatoes: mapping and marker-assisted selection validation for PVY resistance in potato breeding. *Molecular Breeding, 34*(1), 267-271, doi:10.1007/s11032-014-0024-4.

Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., et al. (2003). Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO Journal, 22*(3), 633-640, doi:10.1093/emboj/cdg74.

Sztuba-Solińska, J., Stollar, V., & Bujarski, J. J. (2011). Subgenomic messenger RNAs: Mastering regulation of (+)-strand RNA virus life cycle. *Virology, 412*(2), 245-255, doi:10.1016/j.virol.2011.02.007.

Tacahashi, Y., & Uyeda, I. (1999). Restoration of the 3′ End of Potyvirus RNA Derived from Poly(A)-Deficient Infectious cDNA Clones. *Virology, 265*(1), 147-152, doi:10.1006/viro.1999.0027.

Tacke, E., Prufer, D., Schmitz, J., & Rohde, W. (1991). The potato leafroll Luteovirus 17 K protein is a single-stranded nucleic acid-binding protein. *Journal of General Virology, 72*, 2035-2038, doi:10.1099/0022-1317-72-8-2035.

Tacke, E., Schmitz, J., Prufer, D., & Rohde, W. (1993). Mutational analysis of the nucleic acid-binding 17kDa phosphorprotein of potato leafroll Luteovirus identifies an amphipathic alpha-helix as the domain for protein-protein interactions. *Virology, 197*(1), 274-282, doi:10.1006/viro.1993.1588.

Taliansky, M., Mayo, M. A., & Barker, H. (2003). *Potato leafroll virus:* a classic pathogen shows some new tricks. *Molecular Plant Pathology, 4*(2), 81-89.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution, 30*(12), 2725-2729, doi:10.1093/molbev/mst197.

Tavert-Roudet, G., Abdul-Razzak, A., Doublet, B., Walter, J., Delaunay, T., German-Retana, S., et al. (2012). The C terminus of lettuce mosaic potyvirus cylindrical inclusion helicase interacts with the viral VPg and with lettuce translation eukaryotic initiation factor 4E. *Journal of General Virology, 93*, 184-193, doi:10.1099/vir.0.035881-0.

Tena Fernandez, F., Gonzalez, I., Doblas, P., Rodriguez, C., Sahana, N., Kaur, H., et al. (2013). The influence of cis-acting P1 protein and translational elements on the expression of *Potato virus Y* helper-component proteinase (HCPro) in heterologous systems and its suppression of silencing activity. *Molecular Plant Pathology, 14*(5), 530-541, doi:10.1111/mpp.12025.

Tenllado, F., Llave, C., & Diaz-Ruiz, J. R. (2004). RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Research, 102*(1), 85-96, doi:10.1016/j.virusres.2004.01.019.

Tepfer, M., Jacquemond, M., & Garcia-Arenal, F. (2015). A critical evaluation of whether recombination in virus-resistant transgenic plants will lead to the emergence of novel viral diseases. *New Phytologist, 207*(3), 536-541, doi:10.1111/nph.13358.

Terradot, L., Souchet, M., Tran, V., & Ducray-Bourdin, D. G. (2001). Analysis of a three-dimensional structure of *Potato leafroll virus* coat protein obtained by homology modeling. *Virology, 286*(1), 72-82, doi:10.1006/viro.2001.0900.

Thiele, G., Theisen, K., Bonierbale, M., & Walker, T. (2010). Targeting the poor and hungry with potato science. *Potato Journal (Shimla), 37*(3-4), 75-86.

Thieme, T., Hoffmann, U., & Heimbach, U. (2009). Efficacy of plant protection substances against virus transmission by aphids infesting potato. *Journal für Kulturpflanzen, 61*(1), 21-30.

Thivierge, K., Cotton, S., Dufresne, P. J., Mathieu, I., Beauchemin, C., Ide, C., et al. (2008). Eukaryotic elongation factor 1A interacts with *Turnip mosaic virus* RNA-dependent RNA polymerase and VPg-Pro in virus-induced vesicles. *Virology, 377*(1), 216-225, doi:10.1016/j.virol.2008.04.015.

Thole, V., Dalmay, T., Burgyan, J., & Balazs, E. (1993). Cloning and sequencing of *Potato virus Y* (Hungarian isolate) genomic RNA. *Gene, 123*(2), 149-156.

Thomas-Sharma, S., Abdurahman, A., Ali, S., Andrade-Piedra, J. L., Bao, S., Charkowski, A. O., et al. (2016). Seed degeneration in potato: the need for an integrated seed health strategy to mitigate the problem in developing countries. *Plant Pathology, 65*(1), 3-16, doi:10.1111/ppa.12439.

Thomas, J. E. (1987). Potato leafroll *Luteovirus*. Plant viruses online, Descriptions and lists from the VIDE Database. http://sdb.im.ac.cn/vide/descr644.htm. Accessed 1st December 2015.

Thomas, P. E., Kaniewski, W. K., & Lawson, E. C. (1997). Reduced field spread of *Potato leafroll virus* in potatoes transformed with the *Potato leafroll virus* coat protein gene. *Plant Disease, 81*(12), 1447-1453.

Tian, Y.-P., & Valkonen, J. P. T. (2013). Genetic determinants of *Potato virus Y* required to overcome or trigger hypersensitive resistance to PVY strain group O controlled by the gene *Ny* in potato. *Molecular Plant-Microbe Interactions, 26*(3), 297-305, doi:10.1094/mpmi-09-12-0219-r.

Tingey, W. M. (1984). Glycoalkaloids as pest resistance factors. *American Potato Journal, 61*(3), 157-167, doi:10.1007/bf02854036.

Tiwari, J. K., Gopal, J., & Singh, B. P. (2012). Marker-assisted selection for virus resistance in potato: Options and challenges. *Potato Journal (Shimla), 39*(2), 101-117.

Torrance, L., Liu, H., Cowan, G., Bradshaw, J., & MacFarlane, S. (2009). Extreme resistance to potyviruses in *Solanum tuberosum* group *phureja*. *Aspects of Applied Biology*(94), 1-4.

Toth, I. K., Torrance, L., Fenton, B., & Lees, A. K. (2008). Impact of climate change on pests and diseases of potatoes in Scotland: risks and recommendations. *The Dundee Conference. Crop Protection in Northern Britain, 2008, Dundee, UK, 26-27 February 2008*, 187-192.

Tu, Y., Jin, Y., Ma, D., Li, H., Zhang, Z., Dong, J., et al. (2015). Interaction between PVY HC- Pro and the NtCF(1)beta-subunit reduces the amount of chloroplast ATP synthase in virus- infected tobacco. *Scientific Reports, 5*, doi:10.1038/srep15605.

Turturo, C., Friscina, A., Gaubert, S., Jacquemond, M., Thompson, J. R., & Tepfer, M. (2008). Evaluation of potential risks associated with recombination in transgenic plants expressing viral sequences. *Journal of General Virology, 89*, 327-335, doi:10.1099/vir.0.83339-0.

Ummad-ud-Din, U., Khan, M. A., Nazir, J., & Bushra, S. (2011). Evaluation of resistance against PLRV in potato cultivars. *Pakistan Journal of Phytopathology, 23*(1), 14-19.

UNECE (2006). UNECE standard S-1 concerning the marketing and commercial quality control of seed potatoes (pp. 30). New York, Geneva: United Nations.

UNECE (2014). Virus tolerances. Working party on agricultural quality standards, Annex II. (Vol. GE.6/BUR/2014/6, pp. 5). Melbourne, Australia: United Nations.

Valkonen, J. P. (2007). Viruses: Economical Losses and Biotechnological Potential In D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D. K. L. McKarren, M. A. Taylor, et al. (Eds.), *Potato Biology and Biotechnology - Advances and Perspectives* (pp. 619-641). Amsterdam: Elsevier.

Valli, A., Gallo, A., Calvo, M., de Jesus Perez, J., & Antonio Garcia, J. (2014). A novel role of the potyviral helper component proteinase contributes to enhance the yield of viral particles. *Journal of Virology, 88*(17), 9808-9818, doi:10.1128/jvi.01010-14.

Vance, V. B., Berger, P. H., Carrington, J. C., Hunt, A. G., & Shi, X. M. (1995). 5'-proximal potyviral sequences mediate potato-virus-x potyviral synergistc disease in transgenic tobacco. *Virology, 206*(1), 583-590.

Varrelmann, M., Maiss, E., Pilot, R., & Palkovics, L. (2007). Use of pentapeptide-insertion scanning mutagenesis for functional mapping of the *Plum pox virus* helper component

proteinase suppressor of gene silencing. *Journal of General Virology, 88*, 1005-1015, doi:10.1099/vir.0.82200-0.

Varveri, C. (2006). Biological, serological and molecular characterization of *Potato virus Y* isolates in Greece. *Annals of the Benaki Phytopathological Institute, 20*(2), 67-81.

Velasquez, A. C., Mihovilovich, E., & Bonierbale, M. (2007). Genetic characterization and mapping of major gene resistance to *Potato leafroll virus* in *Solanum tuberosum* ssp *andigena*. *Theoretical and Applied Genetics, 114*(6), 1051-1058, doi:10.1007/s00122-006-0498-5.

Verbeek, M., Piron, P. G. M., Dullemans, A. M., Cuperus, C., & van der Vlugt, R. A. A. (2010). Determination of aphid transmission efficiencies for N, NTN and Wilga strains of *Potato virus* Y. *Annals of Applied Biology, 156*(1), 39-49, doi:10.1111/j.1744-7348.2009.00359.x.

Verchot, J., & Carrington, J. C. (1995). Debilitation of plant potyvirus infectivity by P1 proteinase-inactivating mutations and restoration by 2nd-site modifications. *Journal of Virology, 69*(3), 1582-1590.

Verchot, J., Koonin, E. V., & Carrington, J. C. (1991). The 35-kDa protein from the N-terminus of the potyviralpolyproteinfunctions as a 3rd virus-encoded proteinase. *Virology, 185*(2), 527-535, doi:10.1016/0042-6822(91)90522-d.

Vijayapalani, P., Maeshima, M., Nagasaki-Takekuchi, N., & Miller, W. A. (2012). Interaction of the trans-frame potyvirus protein P3N-PIPO with host protein PCaP1 facilitates potyvirus movement. *PLoS Pathogens, 8*(4), doi:10.1371/journal.ppat.1002639.

Vincent, M., Xu, Y., & Kong, H. M. (2004). Helicase-dependent isothermal DNA amplification. *Embo Reports, 5*(8), 795-800, doi:10.1038/sj.embor.7400200.

Visser, J. C., & Bellstedt, D. U. (2015). *Potato virus Y in South Africa: isolate characterization and assessment of potato cultivar resistance* (Potato and sweetpotato in Africa: transforming the value chains for food and nutrition security).

Visser, J. C., Bellstedt, D. U., & Pirie, M. D. (2012). The recent recombinant evolution of a major crop pathogen, *Potato virus Y. PLoS ONE, 7*(11), doi:10.1371/journal.pone.0050631.

Vogel, F., Hofius, D., & Sonnewald, U. (2007). Intracellular trafficking of *Potato leafroll virus* movement protein in transgenic *Arabidopsis*. *Traffic, 8*(9), 1205-1214, doi:10.1111/j.1600-0854.2007.00608.x.

Volkov, Y. G., Kakareka, N. N., Balabanova, L. A., Kozlovskaya, Z. N., & Sapotsky, M. V. (2009). Characterization of a novel Far-Eastern *Potato Virus Y i*solates. *Plant Pathology Journal (Faisalabad), 8*(2), 62-67, doi:10.3923/ppj.2009.62.67.

van der Waals, J. E., Krueger, K., Franke, A. C., Haverkort, A. J., & Steyn, J. M. (2013). Climate change and potato production in contrasting South African agro-ecosystems 3. Effects on relative development rates of selected pathogens and pests. *Potato Research, 56*(1), 67-84, doi:10.1007/s11540-013-9231-3.

Waiblinger, H.-U., Graf, N., Broll, H., Grohmann, L., & Pietsch, K. (2011). Evaluation of real-time PCR results at the limit of detection. *Journal fur Verbraucherschutz und Lebensmittelsicherheit, 6*(4), 411-417, doi:10.1007/s00003-011-0669-4.

Waltermann, A., & Maiss, E. (2006). Detection of 6K1 as a mature protein of 6 kDa in *Plum pox virus*-infected *Nicotiana benthamiana*. *Journal of General Virology, 87*, 2381-2386, doi:10.1099/vir.0.81873-0.

Wang, D. W., & Maule, A. J. (1995). Inhibition of host gene expression associated with plant virus replication. *Science, 267*(5195), 229-231, doi:10.1126/science.267.5195.229.

Wang, K.-D., Empleo, R., Nguyen, T. T. V., Moffett, P., & Sacco, M. A. (2015a). Elicitation of hypersensitive responses in *Nicotiana glutinosa* by the suppressor of RNA silencing protein P0 from poleroviruses. *Molecular Plant Pathology, 16*(5), 435-448, doi:10.1111/mpp.12201.

Wang, S., Zhang, S., Wang, W., Xiong, X., Meng, F., & Cui, X. (2015b). Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. *Plant Cell Reports, 34*(9), 1473-1476, doi:10.1007/s00299-015-1816-7.

Wang, Y., Khatabi, B., & Hajimorad, M. R. (2015c). Amino acid substitution in P3 of *Soybean mosaic virus* to convert avirulence to virulence on Rsv4-genotype soybean is influenced by the genetic composition of P3. *Molecular Plant Pathology, 16*(3), 301-307, doi:10.1111/mpp.12175.

Watanabe, K. (2015). Potato genetics, genomics, and applications. *Breeding Science, 65*(1), 53-68, doi:10.1270/jsbbs.65.53.

Watanabe, K., & Peloquin, S. J. (1989). Occurrence of 2*n* pollen and *ps* gene frequencies in cultivated groups and their related wild species in tuber-bearing solanums. *Theoretical and Applied Genetics, 78*(3), 329-336.

Watanabe, K. N., Orrillo, M., Vega, S., Iwagana, M., Ortiz, R., Freyre, R., et al. (1995). Selection of diploid potato clones from diploid (haploid x wild-species) F1-hybrid families for short-day conditions. *Breeding Science, 45*(3), 341-347.

Waterhouse, P. M., Graham, H. W., & Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences of the United States of America, 95*(23), 13959-13964, doi:DOI 10.1073/pnas.95.23.13959.

Waterhouse, P. M., & Helliwell, C. A. (2003). Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews Genetics, 4*(1), 29-38, doi:10.1038/nrg982.

Wei, T., Huang, T.-S., McNeil, J., Laliberte, J.-F., Hong, J., Nelson, R. S., et al. (2010a). Sequential recruitment of the endoplasmic reticulum and chloroplasts for plant potyvirus replication. *Journal of Virology, 84*(2), 799-809, doi:10.1128/jvi.01824-09.

Wei, T., & Wang, A. (2008). Biogenesis of cytoplasmic membranous vesicles for plant Potyvirus replication occurs at endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. *Journal of Virology, 82*(24), 12252-12264, doi:10.1128/jvi.01329-08.

Wei, T., Zhang, C., Hong, J., Xiong, R., Kasschau, K. D., Zhou, X., et al. (2010b). Formation of complexes at plasmodesmata for Potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. *PLoS Pathogens, 6*(6), doi:10.1371/journal.ppat.1000962.

Weintraub, M., Ragetli, H. W. J., & Lo, E. (1974). *Potato virus Y* particles in plasmodesmata of tobacco leaf cells. *Journal of Ultrastructure Research, 46*(1), 131-148, doi:10.1016/s0022-5320(74)80027-4.

Wendt, T., & Mullins, E. (2011). Future challenges and prospects. In J. M. Bradeen, & C. Kole (Eds.), *Genetics, genomics and breeding of potato* (pp. 260-276). Enfield, USA: Science Publishers.

Were, H. K., Kabira, J. N., Kinyua, Z. M., Olubayo, F. M., Karinga, J. K., Aura, J., et al. (2013). Occurrence and distribution of potato pests and diseases in Kenya. *Potato Research, 56*(4), 325-342, doi:10.1007/s11540-013-9246-9.

Werner, R., Muhlbach, H. P., & Buttner, C. (1997). Detection of cherry leaf roll nepovirus (CLRV) in birch, beech and petunia by immuno-capture RT-PCR using a conserved primer pair. *European Journal of Forest Pathology, 27*(5), 309-318.

Whale, A. S., Huggett, J. F., Cowen, S., Speirs, V., Shaw, J., Ellison, S., et al. (2012). Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. *Nucleic Acids Research, 40*(11), doi:10.1093/nar/gks203.

Whitham, S. A., Quan, S., Chang, H. S., Cooper, B., Estes, B., Zhu, T., et al. (2003). Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant Journal, 33*(2), 271-283, doi:10.1046/j.1365-313X.2003.01625.x.

Whitham, S. A., Yang, C., & Goodin, M. M. (2006). Global impact: Elucidating plant responses to viral infection. *Molecular Plant-Microbe Interactions, 19*(11), 1207-1215, doi:10.1094/mpmi-19-1207.

van der Wilk, F., Houterman, P., Molthoff, J., Hans, F., Dekker, B., van den Heuvel, J., et al. (1997a). Expression of the *Potato leafroll virus* ORF0 induces viral-disease-like symptoms in transgenic potato plants. *Molecular Plant-Microbe Interactions, 10*(2), 153-159.

van der Wilk, F., Huisman, M. J., Cornelissen, B. J. C., Huttinga, H., & Goldbach, R. (1989). Nucleotide sequence and organization of *Potato leafroll virus* genomic RNA. *FEBS Letters, 245*(1-2), 51-56.

van der Wilk, F., Posthumus-Lutke Willink, D., Huisman, M. J., Huttinga, H., & Goldbach, R. (1991). Expression of the potato leafroll luteovirus coat protein gene in transgenic potato plants inhibits viral infection. *Plant Molecular Biology, 17*(3), 431-439.

van der Wilk, F., Verbeek, M., Dullemans, A. M., & vandenHeuvel, J. (1997b). The genome-linked protein of *Potato leafroll virus* is located downstream of the putative protease domain of the ORF1 product. *Virology, 234*(2), 300-303, doi:10.1006/viro.1997.8654.

Witek, K., Strzelczyk-Zyta, D., Hennig, J., & Marczewski, W. (2006). A multiplex PCR approach to simultaneously genotype potato towards the resistance alleles *Ry*-f(sto) and *Ns*. *Molecular Breeding, 18*(3), 273-275, doi:10.1007/s11032-006-9021-6.

Wright, N. S., & Cole, E. F. (1966). The occurrence of a mild strain of *Potato leaf roll virus* in commercial potato varieties. *American Potato Journal, 43*, 347.

Wright, N. S., MacCarthy, H. R., & Cole, E. F. (1967). Detection and control of mild strains of *Potato leaf roll virus*. [Article]. *American Potato Journal, 44*(7), 245-248, doi:10.1007/bf02862520.

Wu, X.-L., Hou, W.-C., Wang, M.-M., Zhu, X.-P., Li, F., Zhang, J.-D., et al. (2008). RNA silencing-mediated resistance is related to biotic/abiotic stresses and cellular RdRp expression in transgenic tobacco plants. *Bmb Reports, 41*(5), 376-381.

Xu, H., Nie, J., & De Boer, S. H. (2005). Differentiation and molecular detection of Canadian necrotic strains of *Potato virus Y*. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie, 27*(1), 125-131.

Yang, L., Du, Z., Gao, F., Wu, K., Xie, L., Li, Y., et al. (2014). Transcriptome profiling confirmed correlations between symptoms and transcriptional changes in RDV infected rice and revealed nucleolus as a possible target of RDV manipulation. *Virology journal, 11*, doi:10.1186/1743-422x-11-81.

Yasumoto, S., Seki, H., & Muranaka, T. (2014). Changes and improvement of metabolism in plant by using TALEN genome editing. *Seibutsu-kogaku Kaishi, 92*(10), 553-555.

Young, M. J., & Filichkin, S. A. (1999). Luteovirus interactions with aphid vector cellular components. *Trends in Microbiology, 7*(9), 346-347, doi:10.1016/s0966-842x(98)01434-6.

Young, M. J., Kelly, L., Larkin, P. J., Waterhouse, P. M., & Gerlach, W. L. (1991). Infectious in vitro transcripts from a cloned cDNA of *Barley yellow dwarf virus*. *Virology, 180*(1), 372-379, doi:10.1016/0042-6822(91)90042-A.

Zarghani, S., Shams-Bakhsh, M., Zand, N., Sokhandan-Bashir, N., & Pazhouhandeh, M. (2012). Genetic analysis of Iranian population of *Potato leafroll virus* based on ORF0. *Virus Genes, 45*(3), 567-574, doi:10.1007/s11262-012-0804-z.

Zhang, J., Roberts, R., & Rakotondrafara, A. M. (2015). The role of the 5' untranslated regions of Potyviridae in translation. *Virus Research, 206*, 74-81, doi:10.1016/j.virusres.2015.02.005.

Zhou, X., Harrington, R., Woiwod, I. P., Perry, J. N., & Clark, S. J. (1996). Impact of climate change of aphid flight phenology. *Aspects of Applied Biology*(45), 299-305.

Zhuo, T., Li, Y.-Y., Xiang, H.-Y., Wu, Z.-Y., Wang, X.-B., Wang, Y., et al. (2014). Amino acid sequence motifs essential for P0-mediated suppression of RNA silencing in an isolate of *Potato leafroll virus* from inner Mongolia. *Molecular Plant-Microbe Interactions, 27*(6), 515-527, doi:10.1094/mpmi-08-13-0231-r.

Ziegler, A., Klingebeil, K., Papke, V., & Kastirr, U. (2014). Quantification of *Wheat spindle streak mosaic virus* and *Soil borne cereal mosaic virus* in resistance testing of field samples of triticale using real-time RT-PCR. *Journal of Plant Diseases and Protection, 121*(4), 149-155.

Ziegler, A., & Torrance, L. (2002). Applications of recombinant antibodies in plant pathology. *Molecular Plant Pathology, 3*(5), 401-407, doi:DOI 10.1046/j.1364-3703.2002.00130.x.

Zilian, E., & Maiss, E. (2011). Detection of plum pox potyviral protein-protein interactions in planta using an optimized mRFP-based bimolecular fluorescence complementation system. *Journal of General Virology, 92*, 2711-2723, doi:10.1099/vir.0.033811-0.

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