Role of the *Rdr1* gene family in the black spot resistance in roses

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Ina Röhrs (geb. Menz), M. Sc.

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Referent: Prof. Dr. rer. nat. Thomas Debener
Korreferentin: Prof. Dr. rer. hort. Traud Winkelmann
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Abstract

Black spot disease, caused by the hemibiotrophic ascomycete *Diplocarpon rosae*, is the most severe disease in field-grown roses. The *Rdr1* locus, comprising nine highly similar TNL-genes, was previously characterised in roses and it is known to confer resistance to black spot. To identify the active *Rdr1* gene, we analysed stable transgenic roses harbouring single members of the *Rdr1* locus in a disease assay. *muRdr1A* was identified as the functional *Rdr1* and it provides resistance to 13 different single-spore isolates of *Diplocarpon rosae* belonging to six different races; so far, *Rdr1* is only overcome by two races. The identification and phylogenetic analysis of *Rdr1*-family members from the two recently available genomes of the diploid old Chinese *Rosa chinensis* cultivar 'Old Blush', together with nine different rose species, resulted in a genomic organisation of the *Rdr1*-family in two major clusters at the distal end of chromosome 1 with different ancient origins. Genes belonging to cluster 2, like the functional *muRdr1A*, were subjected to a faster evolution compared to genes from cluster 1 due to known processes, such as higher rates of recombination, gene conversion, and birth and death processes. In addition, phylogenetic analysis with additional *Rdr1* homologues identified in other Rosaceae, i.e. *Fragaria*, *Malus*, *Prunus* and *Rubus*, resulted in the hypothesis that the *Rdr1*-family moved to its current position after the split of Rubeae from other groups within Rosoideae. Transcriptomic analysis during the compatible interaction of roses and *D. rosae* indicated an initial PTI reaction which is either insufficient, avoided or suppressed by *D. rosae*. As for the incompatible interaction of roses and *D. rosae* caused by *Rdr1,* two genes (peroxidase superfamily protein and Kunitz family trypsin and protease inhibitor protein) showed significant higher expressions in the incompatible interaction compared to the compatible interaction, independently of the genetic background.

In conclusion, *Rdr1* can be used as the starting point for the breeding of rose varieties with a durable broad spectrum resistance against *D. rosae*. Furthermore, the genomic organisation and the sequence information of the *Rdr1*-family provided in this study is a valuable source to analyse the role of *Rdr1* homologs in disease resistance in other species.

Keywords: *Diplocarpon rosae*, TIR-NBS-LRR, *R*-gene, evolution, RT-qPCR

Zusammenfassung

Sternrußtau, hervorgerufen durch den hemibiotrophen Ascomyceten *Diplocarpon rosae*, gilt als eine der bedeutendsten Krankheiten an Gartenrosen. Der erste kartierte Resistenzlokus gegen Sternrußtau in Rosen, der *Rdr1*- Lokus, wurde bereits als ein Cluster von neun Resistenzgenanaloga der TIR- NBS- LRR-Familie charakterisiert. Eine Analyse von stabilen transgenen Rosen, die einzelne Mitglieder des *Rdr1*- Lokus tragen, ergab, dass *muRdr1A* das funktionelle *Rdr1*- Gen ist und eine breite Resistenz gegenüber 13 *D. rosae* Isolate aus mindestens sechs verschiedenen Rassen vermittelt. Die kürzlich veröffentlichten Rosegenome der diploiden Sorte ‘Old Blush’, zusammen mit Sequenzdaten neun weiterer Rosenarten, erlaubten eine Identifizierung von weiteren *Rdr1*- Familienmitgliedern, die in zwei großen Clustern am distalen Ende von Chromosom 1 organisiert sind. Eine phylogenetische Analyse der *Rdr1*- Homologen ergab, dass die zwei großen Cluster unterschiedliche Ursprünge haben, wobei *muRdr1A* in dem sich schneller entwickelnden Cluster liegt. Eine Analyse mit zusätzlichen *Rdr1*- Homologen aus anderen Rosengewächsen, wie *Fragaria*, *Malus*, *Prunus* und *Rubus*, führt zu der Annahme, dass die *Rdr1*- Familie nach der Spaltung von Rubeae von anderen Gruppen innerhalb der Rosoideae an ihre derzeitige Position verschoben wurde. Die Analyse von transkriptomischen Unterschieden während einer kompatiblen Interaktion von Rosen und *D. rosae* mittels MACE- Analyse zeigte eine Hochregulierung von Genen, die auf eine erste PTI-Reaktion hindeuten, die entweder unzureichend ist oder von *D. rosae* umgangen wird. Mittels Standard RT-qPCR konnte während der inkompatiblen Interaktion von Rosen und *D. rosae* eine Induktion von zwei Genen, wovon eines für ein Peroxidase-Superfamily-Protein und das andere für ein Trypsin- und Protease-Inhibitor-Protein der Kunitz-Familie kodiert, beobachtet werden. Durch die Analyse in transgenen Rosen (PC::muRdr1A) und deren Nachkommen konnte die Induktion beider Gene unabhängig vom genetischen Hintergrund in Verbindung mit der *Rdr1* vermittelten Resistenz gebracht werden. Die transgenen Rosen bieten somit eine gute Basis für weitere transkriptomische Analysen um die inkompatible Interaktion von Rosen und *D. rosae* besser zu verstehen. Weiterhin kann *Rdr1* als Startpunkt für die Züchtung von Rosensorten mit einer dauerhaften breiten Sternrußtauresistenz verwendet werden.

Schlagwörter: *Diplocarpon rosae*, TIR-NBS-LRR, *R*-gene, Evolution, RT-qPCR

Content

Abbreviations

A	
AB	Rose variety ‘Arthur Bell’
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
Avr	avirulence
B	
BAC	Bacterial Artificial Chromosome
C	
CC	coiled-coil
CNL	CC-NBS-LRR
COMT	caffeic acid 3-O-methyltransferase
D	
DEGs	differentially expressed genes
DRR	Disease Resistance Response
E	
EFR	EF-Tu receptor
ET	ethylene
ETI	effector-triggered immunity
F	
FLS2	fagellin-sensing 2
G	
GA	Gibberellin
H	
Hsf	heat shock factors
J	
JA	jasmonic acid
K	
Ka	non-synonymous nucleotide substitution
Ks	synonymous substitution
L	
LPS	lipopolysaccharides
LRR	leucine rich repeat
LysM	lysine motif

M	
MACE	Massive Analysis of cDNA Ends
MAMP	microbe-associated molecular pattern
MUSCLE	Multiple sequence comparison by log- expectation

N	
NBS	nucleotide binding site
NLR	nucleotide-binding leucine-rich repeat receptors

O	
OB	Rose variety 'Old Blush'

P	
PAMP	pathogen-associated molecular pattern
PC	Rose variety 'Pariser Charme'
PER	peroxidase
PG	polygalacturonase
PGN	peptidoglycans
PRRs	pattern recognition receptors
PTI	PAMP-triggered immunity

R	
R-gene	resistance gene
RK	receptor kinases
RLKs	receptor-like kinases
RLPs	receptor-like transmembrane proteins

S	
SA	salicylic acid
SI	self-incompatibility
STAND	signal transduction ATPases with numerous domains
STKs	serine-theorine kinases

T	
TF	transcription factor
TIR	Toll/interleukin-1 receptor
TNL	TIR-NBS-LRR
TTSS	type III secretion system

1 General Introduction

1.1 Roses

Among ornamentals, roses are the most popular plants due to a high symbolic and cultural value. They are characterized by a high diversity in flower characteristics like flower colour and petal number. As cultivated ornamentals, e.g. as cut flowers, potted plants and garden plants, they are economically very important. FloraHolland (https://www.royalfloraholland.com/en), the world biggest auction company for ornamentals, ranks roses on the first place of cut-flowers in the year 2016, with a turnover of 746 million € and 3,377 million sold units. In the top 10 house plants, potted roses are ranked on place three, with a turnover of 60 million € and 47 million sold units. In 2003, approximately 220 million garden roses were sold (Roberts, 2003). Besides that, roses are also used as sources for perfume and vitamin C (Zlesak, 2007).

1.1.1 Taxonomy and geographic distribution

Together with species from the genera *Fragaria*, *Rubus*, *Potentilla*, and *Geum,* roses belong to the Rosaceae family and are therefore related to economically important fruit crops, such as apple, pear, cherry and peach (Table 1.1) (Debener and Linde, 2009). The genus *Rosa* comprises about 200 species and is subdivided in *Hulthemia, Platyrhodon, Hesperrhodos* and *Rosa*, with the latter comprising around 95 % of all the species (Wissemann, 2003; Wissemann and Ritz, 2005). Many interspecific hybridizations resulted in thousands of cultivars, classified as old garden roses (before 1867) and modern roses (after 1867), which are further subdivided into horticultural classes. With regard to the cultivar numbers, the most popular horticultural class of modern roses today is the hybrid tea with over 10,000 registered cultivars. Interestingly, in most of the modern rose cultivars only about seven to ten rose species are found in their genetic background (Cairns, 2000; Zlesak, 2007). The natural distribution of the genus *Rosa* originally lies in the northern hemisphere, but today, roses are present in Canada, the United States and Mexico. In Europe, they are spread across the entire continent as far as the Arctic Circle. The Near East and Asia are also homes to numerous rose species, whereas China is undoubtedly the greatest (Brichet, 2003). Nonetheless, cultivated roses are grown all around the world in almost all climates (Debener and Linde, 2009).

Table 1.1: Taxonomic classification of roses (acc. to Brands (1989-present))..

Kingdom	Plantae
Subkingdom	Viridiplantae
Phylum	Tracheophyta
Subphylum	Euphyllophytina
Class	Spermatopsida
Order	Rosales
Family	Rosaceae
Genus	*Rosa*

1.1.2 Genetics

Rose species have a basic chromosome number of seven and ploidy levels ranging from diploid (2n=2x=14) to octoploid (2n=8x=56), whereas the majority of modern rose cultivars are tetraploid, highly heterozygous and can be genetically classified as 'segmental' allopolyploids - a mixture between allopolyploid and autopolyploid (Bourke *et al.* 2017; Cairns, 2000; Crane and Byrne, 2003; Gudin, 2000; Krüssmann and Hemer, 1974).

Raymond *et al.* (2018) assembled a rose genome (513 Mb) from a homozygous genotype, generated from a heterozygous diploid modern rose progenitor, *Rosa chinensis* 'Old Blush', using single-molecule real-time sequencing and a meta-assembly approach. The final assembly was composed of 82 contigs with an N50 of 24 Mb.

In addition, Hibrand Saint-Oyant *et al.* (2018) also developed a high-quality reference genome sequence for the genus Rosa by sequencing a doubled haploid rose line ('HapOB') from *Rosa chinensis* 'Old Blush'. The generated rose genome assembly anchored to seven pseudo-chromosomes (512 Mb with N50 of 3.4 Mb and 564 contigs), whereas the length of 512 Mb represents 90.1–96.1% of the estimated haploid genome size and 95% of the assembly is contained in only 196 contigs.

Moreover, the rose genome displays extensive synteny with the *Fragaria vesca* genome with only two major rearrangements (Hibrand Saint-Oyant *et al.* 2018).

Gametophytic self-incompatibility (SI), characterized by the lack of seeds after self-pollination, is present in roses and many diploid species. However, within the group of tetraploid modern cultivars and several polyploid species self-fertilization is a common phenomenon, indicating a breakdown of the SI system in tetraploids (Debener and Linde, 2009; Rajapakse *et al.* 2001; Zlesak, 2007).

1.1.3 Breeding and propagation of cultivars

Rose plants can be propagated either by seed or vegetatively. The modern rose is a product of many years of selection and their progeny tend to segregate widely for traits; therefore rose cultivars are propagated asexually by bud-grafting or cuttings (Mattock, 2003; Zlesak, 2007). For most of the genotypes, an easy and rapid vegetative *in vitro* propagation is also possible (Davies, 1980).

In contrast, sexual propagation of roses is primarily used for the development of new cultivars and for the production of some rootstocks (Zlesak, 2007).

In the past, breeding objectives became more specific and are quite different depending on their use as garden, cut-flower, landscape, pot or rootstock roses. Ornamental characters, productivity and long vase-life are more important breeding goals of cut roses, whereas, apart from ornamental values, disease resistance became one of the most important breeding goals for garden roses (Gudin, 2000).

Depending on the trait, the use of different wild species as crossing partners might be valuable to broaden the genetic base of cultivated roses (Smulders *et al.* 2011).

1.1.4 Genetic transformation

Successful transformation of roses with genes encoding for diverse traits (such as disease resistance, plant habit, flower colour and root characteristics), as well as different transformation protocols (*Agrobacterium tumefaciens*-mediated gene transfer and particle bombardment), has been established for several rose cultivars (Dohm, 2003). However, these transformation protocols are restricted to specific genotypes and are highly dependent on the *in vitro* regeneration system. Dohm *et al.* (2001) observed a regeneration rate of 69 % via somatic embryogenesis in 50 tested genotypes. Somatic embryogenesis in roses is a very time-consuming process, where the regeneration of transformed plants takes around one year and shows a low transformation frequency of approximately 3 % (Dohm, 2003).

1.2 *Diplocarpon rosae*

1.2.1 Discovery and Taxonomy

The hemibiotrophic ascomycete *Diplocarpon rosae* Wolf (conidial stage: *Marssonina rosae* (Lib.) Died.) causes rose black spot, a disease of major importance for field

grown roses (Drewes-Alvarez, 2003; Horst *et al.* 2007). The conidial stage was first reported by Fries in 1815 in Sweden, with further reports in Belgium, France and Germany in the second half of the nineteenth century (Aronescu, 1934; Drewes-Alvarez, 2003). *Diplocarpon rosae* is classified as Ascomycota and belongs, together with other Rosaceae family pathogens such as *D. mali* (host: *Malus*), *D. mespili* (host: *Mespili*) or *D. earlianum* (host: *Fragaria*), to the genus *Diplocarpon* (Table 1.2) (Ainsworth, 2008).

Table 1.2: Genomic classification of *D. rosae* (acc. to Brands (1989-present)).

Kingdom	Fungi
Subkingdom	Dikarya
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Leotiomycetes
Order	Helotiales
Family	Dermateaceae
Genus	*Diplocarpon*
Species	*Diplocarpon rosae* F. A. Wolf

1.2.2 Life cycle of *D. rosae*

D. rosae infected plants typically show dark spots at the site of infection, often followed by the development of heavy chlorosis and loss of foliage very early in the vegetation period. Repeated infection cycles can lead to reduced growth and eventually death of the plant (Malek and Debener, 1998). First infections in spring are caused by spores that overwintered in fallen leaf material or in acervuli - fruiting body structures where new conidia are formed - on stems. The spores stick to the leaf surface and start to germinate after 8 hours under favourable conditions. The penetration of the cuticle can be either direct or via the development of an appressorium at the distal end of the germ tube. After two days and after the penetration into the epidermal cells, the fungus grows deeper into the leaf with subcuticular and intercellular hyphae, where the latter is responsible for sending nutrient-absorbing structures called haustoria into the plant cells. Around six days after the infection, the formation of necrotrophic intracellular hyphae is followed by a fungal reproductive stage in which acervuli with new conidia are formed. A new infection cycle begins with conidia released from acervuli through the ruptured cuticle (Figure 1.1) (Blechert and Debener, 2005; Drewes-Alvarez, 2003; Gachomo *et al.* 2006; Gachomo and Kotchoni, 2007). In contrast to this asexual

lifecycle, the sexual life cycle of *D. rosae*, which involves the formation of ascospores via meiosis in apothecia, is rarely described and it is assumed to occur only under unfavourable conditions, e.g. during winter (Frick, 1943).

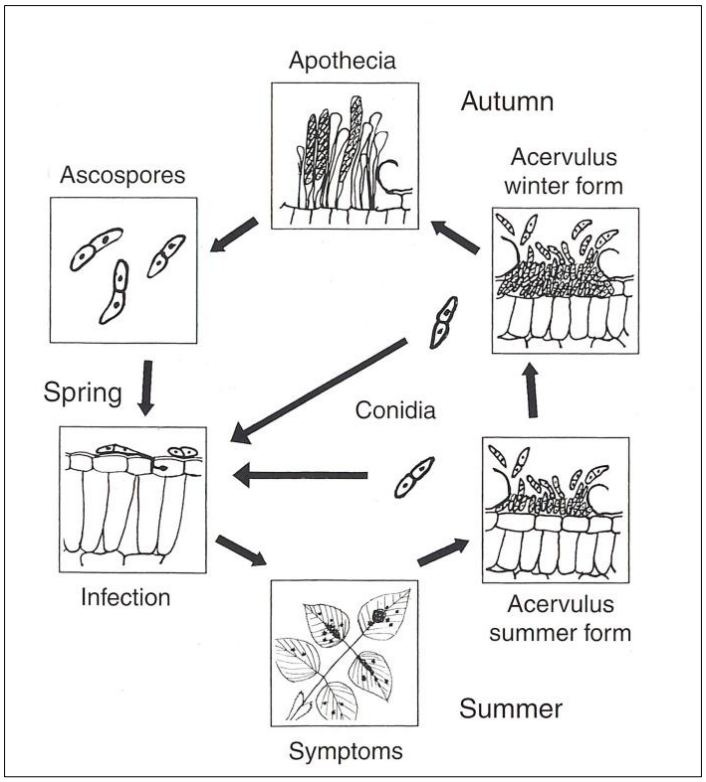

Figure 1.1: Life cycle of black spot (Drewes-Alvarez, 2003).

1.2.3 Distribution, genetics and control of black spot

The conidia of *D. rosae* are disseminated mainly by splash water or direct contact, thus causing the distribution to be localized and thus reducing the risks for the evolution of new races (Lühmann *et al.* 2010). However, to date, eleven different pathogenic races of *D. rosae* can be distinguished and it is assumed that more races exist (Table 1.3, Whitaker *et al.* (2010b)). The control of black spot in the field relies on fungicide treatment and cultivation of resistant varieties (Malek and Debener, 1998; Reddy *et al.* 1992). The application of fungicides is often used as a preventive measure and leads to high costs, environmental contamination and the development of pesticide resistant pathogens (Debener and Byrne, 2014).

Table 1.3: New race nomenclature for 11 races of *Diplocarpon rosae* (1–11) using nine differential rose genotypes (Whitaker *et al.* 2010b). Compatible interactions = +; incompatible interactions = -.

	Race										
Host	1	2	3	4	5	6	7	8	9	10	11
Pariser Charme®	–	+	+	+	+	+	+	+	+	+	+
Honeybee™	–	–	+	+	+	+	+	0	0	+	0
Caramba®	–	–	–	+	+	+	+	+	+	+	+
cv. Surrey	–	–	–	–	–	+	+	+	+	+	+
Love and Peace™	–	–	–	–	–	–	+	+	+	+	+
cv. George Vancouver	–	–	+	–	–	–	–	–	+	+	+
Knock Out®	–	–	–	–	+	–	–	–	–	+	–
Baby Love™	–	–	–	–	–	–	–	–	–	–	+
cv. Mrs Doreen Pike	+	–	–	–	–	–	–	–	–	–	–
Isolate	HSN	2402 E1	GVH	Dü A3	B 005	Dort E4	I 001R6	ACTDA1	AR1IGWAZM1	KOMN	CW1

The genome size of the isolate DortE4 is estimated to range from 70 to 90 Mb and contains 4,004 predicted gene models, with 88.5% of the predicted genes being expressed during the early stages of infection. The genome size is outstandingly large for a fungal genome, which is explained with a relatively recent whole genome duplication event (Neu *et al.* 2017). This provides a useful working tool to study the plant-host interaction mechanisms of *D. rosae*.

Analysis of global black spot populations for their gene diversity revealed no unique alleles from any of the locations, indicating a slow evolution of new alleles in the *D. rosae*/rose pathosystem and that global trading of rose varieties led to an admixture of the pathogen. In Germany, the highest gene diversity was observed in older rose collections managed without fungicide application and the lowest diversity occurred in the two- to three-year-old testing sites of German rose breeders. Thus, gene diversities of the *D. rosae* populations are dependent on the age of the host populations and the application of fungicides. Therefore, breeding of new cultivars with broad spectrum resistances against *D. rosae* is a major goal for rose production, since they could be successfully used over several years in different countries throughout the world (Münnekhoff *et al.* 2017).

1.3 Characterised resistances against *D. rosae*

Breeding rose cultivars which are resistant to *D. rosae* is a time-consuming process due to the complex genetic constitution of roses (Drewes-Alvarez, 2003). Information on the genetic complexity of black spot disease resistance would simplify the breeding for resistant varieties; however, this information is scarce. Cultivated and wild roses have been evaluated in the field and laboratory for their black spot resistance in

previous studies (Byrne *et al.* 2010; Debener *et al.* 1998; Mangandi *et al.* 2013; Schulz *et al.* 2009). A detached leaf assay, using different single spore isolates and field collected samples of *D. rosae*, demonstrated that the highest level of resistance occurs in wild species, especially within the subgenus *Cinnemomeae* (Schulz *et al.* 2009). The resistance levels classified as 'good' to 'excellent' observed in cultivated roses are assumed to originate from a wide range of rose species, including *Rosa wichurana*, *R. rugosa*, *R. multiflora*, *R. carolina*, *R. virginiana*, *R. laxa*, and *R. spinossisima*. In contrast, the popular cultivated rose groups, like hybrid teas or floribundas, lack the resistance against black spot (Byrne *et al.* 2010). Up to now, three resistance loci, named *Rdr1*, *Rdr2*, and *Rdr3*, have been found, with *Rdr1* being the best characterised one (Hattendorf *et al.* 2004; Malek and Debener, 1998; Whitaker *et al.* 2010a).

1.4 Discovery and cloning of *Rdr1*

As the highest level of black spot resistance occurs in wild species, a diploid *R. multiflora* Thunb. (2n=2x=14) was crossed with a tetraploid garden rose (2n=4x=28) to introgress black spot resistance from wild species into garden roses. The resulting triploid hybrid `Goldfinch` was also crossed with a diploid *R. multiflora* and after several rounds of open pollination, progeny were selected based on garden rose characteristics, e. g. lack of thorns, high petal number and black spot resistance. Due to the high level of observed black spot resistance, the diploid *R. multiflora* hybrid 88/124-46 was selected for a colchicine treatment in order to double the chromosomal set. The resulting tetraploid line CT50-4 was crossed with several varieties with different susceptibilities against black spot to generate a set of genotypes differing in black spot resistance. From the crossing with the susceptible hybrid tea variety 'Caramba', the genotype 91/100-5 was selected out of ten hybrids. The genotype 91/100-5 was tested to be resistant against the *D. rosae* isolate DortE4, but still displayed many unwanted characteristics of the wild species *R. multiflora*. Therefore, 91/100-5 was crossed with the susceptible varieties 'Caramba' (progeny 95/1), 'Pariser Charme' (progeny 95/2) and 'Heckenzauber' (progeny 95/3) (Debener *et al.* 1998; Drewes-Alvarez, 1992; Malek and Debener, 1998).

The segregation ratios for black spot resistance in the BC, F1 and F2 progenies of 91/100-5 provided evidence for the presence of a single dominant resistance gene in duplex configuration (RRrr) in the rose genotype 91/100-5 (Figure 1.2). This gene,

named *Rdr1*, was the first resistance gene described in roses (Malek and Debener, 1998) and it has shown to confer resistance against five races of *D. rosae* (Debener *et al.* 1998).

Malek *et al.* (2000) identified seven AFLP (amplified fragment length polymorphism) markers linked to *Rdr1*. The most closely linked marker was used to locate *Rdr1* on a chromosome map of diploid roses, indicating a position near the telomere. Later, *Rdr1* was finely mapped to a telomeric position in rose linkage group 1 (Biber *et al.* 2010; Kaufmann *et al.* 2003).

With the application of the AFLP markers, the resistant genotype 95/3-23 with a small donor fraction was selected for further crosses as a male parent with 'Caramba', resulting in the population 99/18, and 'Heckenzauber', resulting in the population 99/20 (Debener *et al.* 2003).

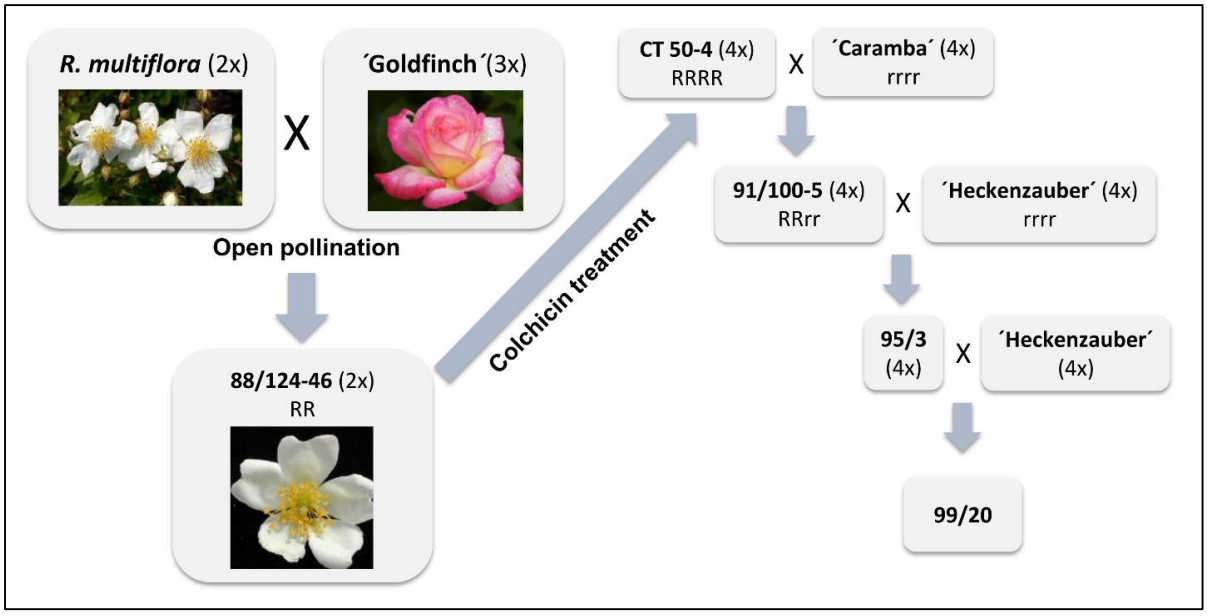

Figure 1.2: Crossing scheme for the introgression of black spot resistance from wild species into garden roses (modified after Debener *et al.* (2003)).

In order to clone *Rdr1*, BAC clones were isolated from a *R. rugosa* insert library and then assembled to a contig spanning the genomic region around the gene locus (Kaufmann *et al.* 2003). However, the *R. rugosa* contig did not contain the resistant allele, and another BAC contig from a new BIBAC library of *R. muliflora* spanning the *Rdr1* locus was constructed (Biber *et al.* 2010). High-resolution mapping narrowed down the region around the *Rdr1* locus to four overlapping BAC clones (29O9, 94G8, 20F5 and 69E24) and by sequencing these four BAC clones a complete sequence of

265,477 bp was obtained (Biber *et al.* 2010; Kaufmann *et al.* 2010). Out of 40 predicted genes with significant matches to GenBank entries, nine showed highly significant similarities to resistance genes of the TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide binding site-leucine rich repeat, TNL) type (Kaufmann *et al.* 2010; Terefe-Ayana *et al.* 2011), thus, being considered to be the most likely candidates for *Rdr1* and designated as *muRdr1A*- *muRdr1I* (Figure 1.3).

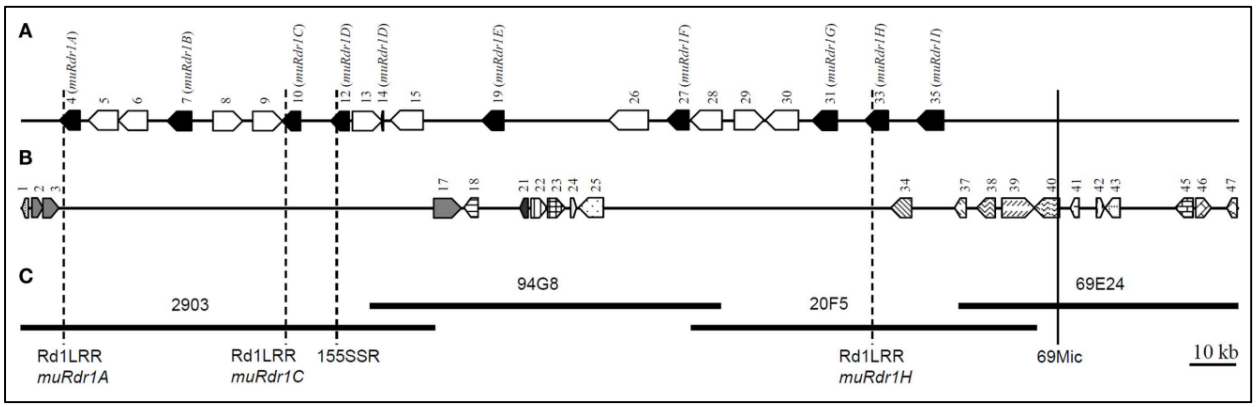

Figure 1.3: Physical positions of the *Rdr1* gene region and schematic representation of positions of predicted genes in the *R. multiflora* genotype (acc. to Terefe-Ayana *et al.* (2011)). (A) Nine TNLs (black pentagon) and 10 transposable-elements (unfilled pentagon). (B) Other genes distributed along the *Rdr1* region. (C) The completely sequenced four overlapping BAC clones carrying the *Rdr1* gene. The broken-line indicates *Rdr1*-linked SSR markers. The vertical unbroken line indicates a recombination break point (69Mic) on the right side of the region.

Except for *muRdr1D*, all genes have a size in the range of 4085 to 5920 bp and sequence similarities of 87.8 to 99.5 % with four exons and three introns. The first exon contains a toll/interleukin-1 receptor (TIR) domain, the second one has a nucleotide-binding site (NBS) domain and the fourth one has a leucine- rich repeat (LRR) domain (Figure 1.4) (Kaufmann *et al.* 2010; Terefe-Ayana *et al.* 2011).

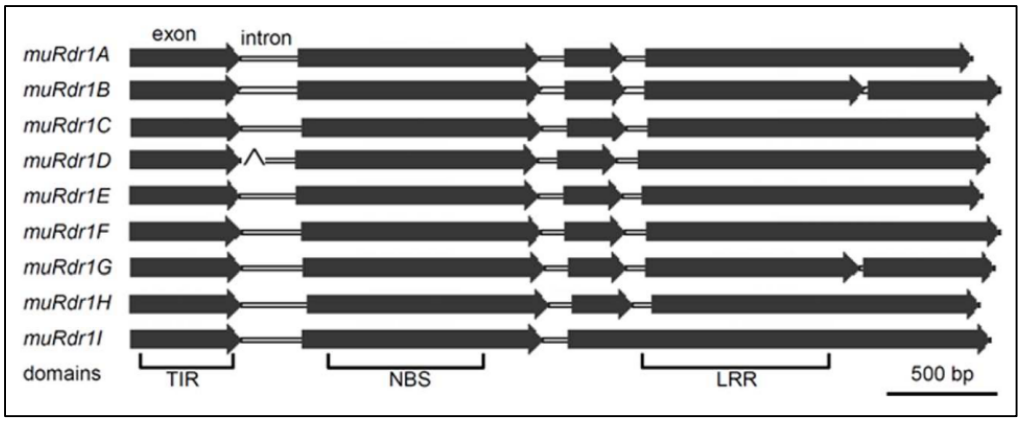

Figure 1.4: The structure of the nine TNLs (acc. to Terefe-Ayana *et al.* (2011)). The majority of the TNLs are characterised by four exons and three introns. The fourth TNL (*muRdr1D*) is interrupted by a 6957-bp transposable-element at intron 1.

For the identification of the functional *Rdr1*, the expression of all nine *muRdr1*- family members was tested in different rose organs (Terefe-Ayana *et al.* 2011). Due to no detectable expression in leaves and petals, *muRdr1B, D, E,* and *F* were excluded as likely candidates for *Rdr1*. On the other hand, *muRdr1A, C, G, H,* and *I* were expressed in both leaves and petals, and thus were further analysed in transient infiltration experiments. Each of the genes were subcloned, transformed and co-infiltrated with *D. rosae* single spore isolates DortE4 and R6 in the susceptible genotype 'Pariser Charme' (PC). While none of the *muRdr1* genes showed effects against the isolate R6, leaf areas infiltrated with DortE4 and the *muRdr1H* gene showed significant reduction of fungal growth in each of the nine experiments (Figure 1.5). Although *muRdr1A* also showed effects in six of the nine experiments, *muRdr1H* was considered to be the most likely candidate for *Rdr1*.

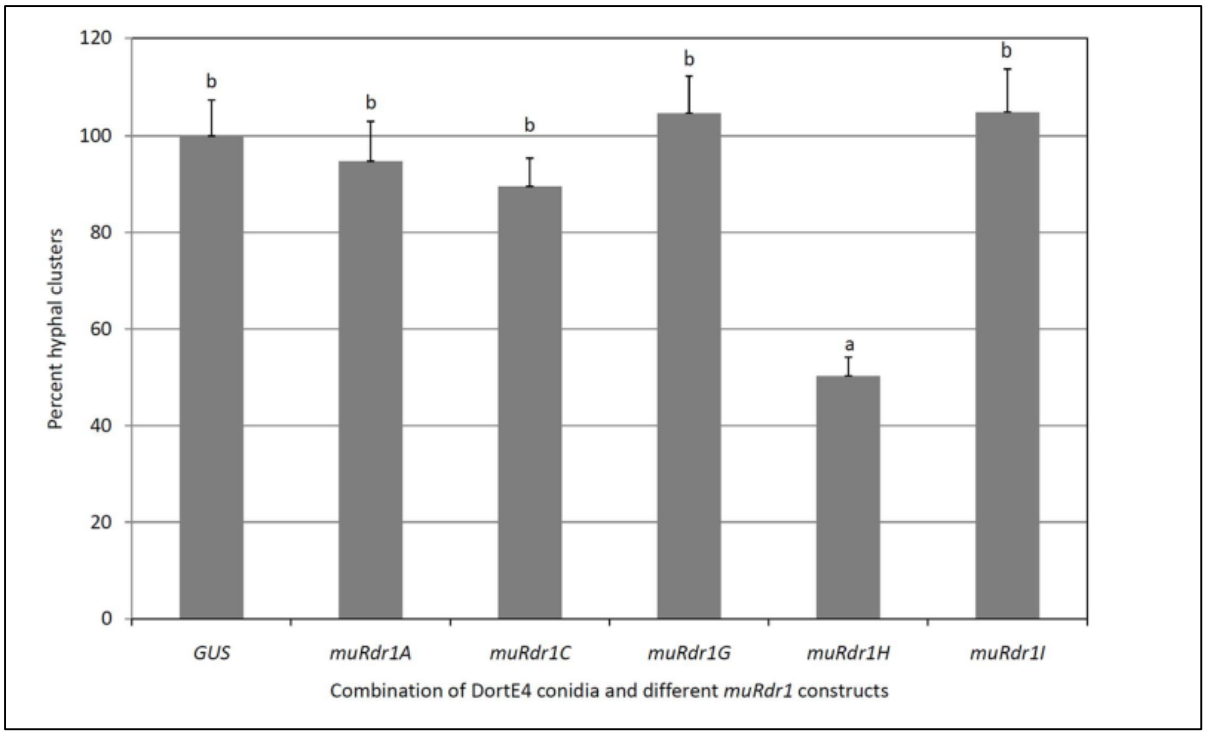

Figure 1.5: Transient complementation disease assay with individual *muRdr1* TNLs and DortE4 (acc. to Terefe-Ayana *et al.* (2011)). This graph represents average data of nine independent experiments ± SEM. Results with same letters are not significantly different ($P < 0.05$).

1.5 Plant defence mechanisms

Due to their sessile way of living, plants are constantly exposed to a number of potential pathogens, such as fungi, bacteria, nematodes and insects. Therefore, plants evolved mechanisms to perceive such attacks, and to translate this perception into an adaptive response. Passive protection against pathogens that are not specialized to a specific host is provided by barriers like the cell wall, the waxy cuticle or preformed anti-microbial compounds (Dangl and Jones, 2001). To initiate immunity, the ability to recognize and respond to potential pathogens is necessary and two different forms of immunity in plants are described; the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and the effector-triggered immunity (ETI).

PTI is elicited by the activation of pattern recognition receptors (PRRs) by microbe-associated molecular patterns (MAMP; or also called pathogen-associated molecular pattern (PAMP)), which are highly conserved molecular signatures characteristic for a whole class of microbes, such as fungal chitin or bacterial lipopolysaccharides (LPS), peptidoglycans (PGN) and flagellins (Boller and Felix, 2009). PRRs are typically leucine-rich repeat kinases and lysine motif (LysM) kinases, with leucine-rich-repeat receptor kinases (LRR-RKs) flagellin-sensing 2 (FLS2) and EF-Tu receptor (EFR)

being the two best characterised PRRs (Boller and Felix, 2009). For fungi and oomycetes, fungal chitin or the Pep13-domain of the cell wall transglutaminase and heptaglucosides of oomycetes are known (Zipfel and Felix, 2005). PRRs do not act alone but rather function as part of multi-protein complexes at the plasma membrane, where the activation of PRRs leads to intracellular signalling, transcriptional reprogramming, and biosynthesis of a complex response that limits microbial colonization (Dangl *et al.* 2013; Monaghan and Zipfel, 2012) (Figure 1.6, step 1).

To suppress PRR-dependent responses, pathogens with diverse life styles use effectors to facilitate nutrient acquisition and to contribute to pathogen dispersal. Effectors are virulence factors (proteins or toxins) which do not have a “housekeeping” function in microbial growth and development outside of the host (Bent and Mackey, 2007). They are secreted into host cells from extracellular plant bacterial pathogens by the type III secretion system (TTSS). For fungi or oomycetes, this is done by haustoria, and for aphids and nematodes, the secretion occurs during the feeding process (Dangl *et al.* 2013) (Figure 1.6, step 2).

If an effector is recognized by a host defense receptor, the intended virulence function is often overshadowed by a dominant avirulence function (Bent and Mackey, 2007). Some effectors from evolutionary different pathogens target an overlapping subset of plant proteins, including well-connected cellular hubs, so that they are likely to suppress effective host defence and facilitate pathogen fitness (Mukhtar *et al.* 2011) (Figure 1.6, step 3).

The recognition of pathogen effectors by plant receptors, encoded by resistance genes (*R*-genes), induces ETI and limits the pathogen proliferation (Figure 1.6, step 5). Most of the *R*-genes encode nucleotide-binding leucine-rich repeat receptors (NLR), whereas specific NLR proteins are activated by specific pathogen effectors. The interaction between NLR and effector can be direct, as receptor or ligand (Figure 6, step 4a), but such interactions have been rarely reported (Caplan *et al.* 2008; Dangl *et al.* 2013; McHale *et al.* 2006). Direct interactions have been described in flax (*Linum usitatissimum*) for the NLR proteins L5, L6 and L7 and the corresponding AvrL567 avirulence proteins from flax rust (Dodds *et al.* 2006), and in rice for the pi-ta protein and the AVR-Pita(176) protein from rice blast fungus (Jia *et al.* 2000).

In an indirect interaction, the effectors modify its host cellular target (or a molecular decoy of that target), and this modification activates a specific NLR (Dangl *et al.* 2013; Dangl and Jones, 2001; van der Hoorn, R. A. L. and Kamoun, 2008) (Figure 1.6, steps

4b and 4c). *Cladosporium fulvum*, for example, secretes the Avr2 protein during infection into the apoplast of tomato leaves, which is a protease inhibitor of the Rcr3 protein and necessary for the Cf2-mediated resistance (Rooney *et al.* 2005). RIN4 is a negative regulator of basal defence that is targeted by multiple *Pseudomonas syringae* effectors (AvrRpm1, AvrRpt2, and AvrB) and monitored by *RPM1* and *RPS2* (Mackey *et al.* 2002; van der Hoorn, R. A. L. and Kamoun, 2008).

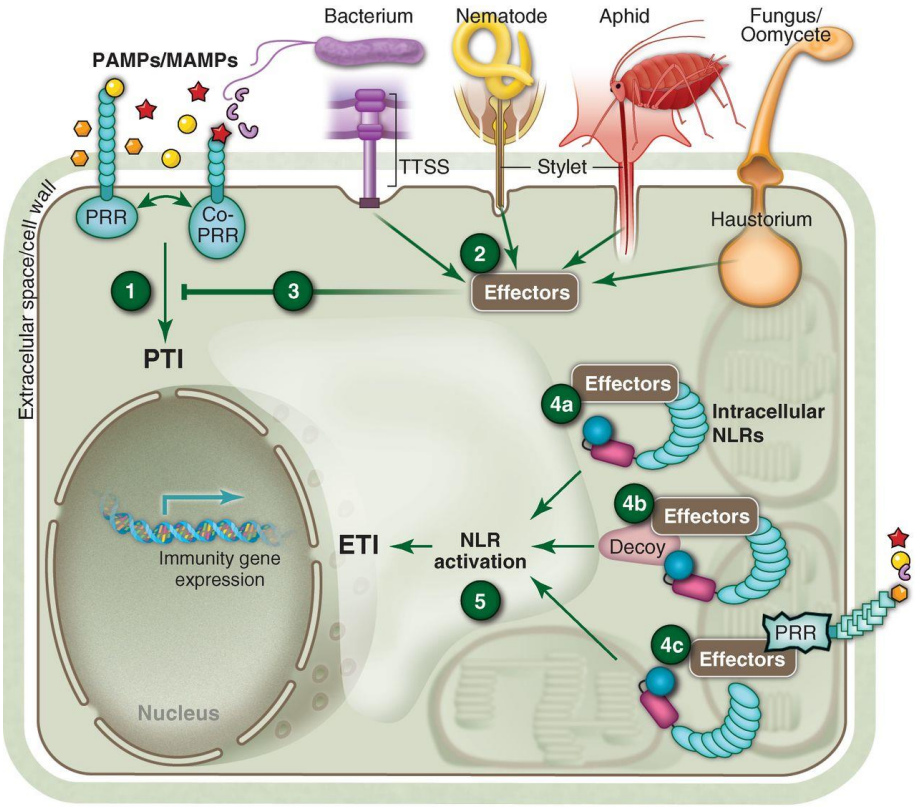

Figure 1.6: Schematic overview of the plant immune system (acc. to Dangl *et al.* (2013)). Pathogens of all lifestyle classes express PAMPs and MAMPs as they colonize plants. Plants perceive these via extracellular PRRs and initiate PRR-mediated immunity (PTI; step 1). Pathogens deliver virulence effectors to the plant cell (step 2). These effectors are addressed to specific subcellular locations where they can suppress PTI and facilitate virulence (step 3). Intracellular NLR receptors can sense effectors in three ways (steps 4a, 4b and 4c), each results in NLR-dependent effector-triggered immunity (ETI) (step 5).

The recognition of a pathogen attack either by PTI or ETI, triggers common defence responses such as oxidative burst, hormonal changes and transcriptional reprogramming in varying magnitude (Tao, 2003; Tsuda and Katagiri, 2010).

1.5.1 Hormone signalling

Plant hormones, like salicylic acid (SA), ethylene (ET), brassinosteroids, auxin, cytokinins, gibberellins, abscisic acid, and jasmonic acid (JA), are key determinants in

plant-pathogen interactions (Vleesschauwer *et al.* 2013). However, the effect of each hormone on the defense response depends on the pathogen lifestyle (Robert-Seilaniantz *et al.* 2011). Auxin induces susceptibility to (hemi-)biotrophic rice pathogens in a SA- and JA-independent fashion, whereas cytokinins enhance SA-dependent rice immunity (Vleesschauwer *et al.* 2013). The SA-dependent response is triggered by biotrophic and hemibiotrophic pathogens, which obtain nutrients from living plant tissue. In contrast, the ET–JA-dependent defense is activated by necrotrophic pathogens, which kill plant cells to obtain nutrients (Glazebrook, 2005; McDowell and Dangl, 2000). It is suggested that JA and ET pathways usually work together in a cooperative way and that they are antagonistic to the SA-pathway (Derksen *et al.* 2013).

1.5.2 Pathogen related (PR) proteins

PR-proteins, pathogen or related stress induced plant proteins, are main indicators of a defence response (Bowles, 1990) and are classified into 17 families based on their structural or functional similarities. For example, the PR-2 family consists of β-1,3-endoglucanases and the PR-3, -4, -8, and -11 families are endochitinases, whereas the PR-6 family are proteinase inhibitors. Additionally, there are defensins (PR-12), thionins (PR-13), lipid transfer proteins (PR-14), thaumatin-like (PR-5), ribonuclease-like (PR-10) and peroxidases (PR-9) (van Loon *et al.* 2006).

The peroxidase superfamily is divided in three distantly related structural classes: i) mitochondrial yeast cytochrome c peroxidase, chloroplast and cytosol ascorbate peroxidases, and gene duplicated bacterial peroxidase (class I); ii) secretory fungal peroxidases (class II); and iii) secretory plant peroxidases (class III) (Welinder, 1992). Proteases cleave internal peptide bonds of polypeptides or single amino acid residues from the terminal ends and have been classified into four major groups based on their active site: serine proteinase, cysteine or sulphydryl proteinases, metalloproteinases and acid proteinases (Casaretto and Corcuera, 1995; Hartley, 1960; Ryan, 1973). Proteins that form complexes with proteases and inhibit their proteolytic activity are widespread in nature and have been found to be specific for each of the four protease groups (Ryan, 1990).

It has been shown that both peroxidase superfamily proteins and protease inhibitor proteins are involved in plant defence responses to pathogen/pest attacks. In

Arabidopsis, resistance to *B. cinerea* was observed in transformed plants overexpressing class III peroxidase (PER) family proteins and protease inhibitor (PI) family proteins. However, no correlation between the level of expression and the degree of resistance could be observed (Chassot *et al.* 2007).

1.5.3 Transcription factors

Transcription factors (TFs) are essential for the regulation of gene expression and several TFs have been identified as key regulators of various plant functions, including growth, development and stress signalling. They are divided in proteins containing a DNA-binding domain (DBD), which regulate the first step of gene expression, and proteins without a DBD, which interact with a DNA-binding protein to form a transcriptional complex (Mitsuda and Ohme-Takagi, 2009).

A single TF can control the expression of many target genes through specific binding of the TF to the cis-acting element in the promoters of respective target genes (Nakashima *et al.* 2009). To alter the activation of physiological and metabolic responses, complex regulatory networks are established that lead to stress tolerance or enhanced disease resistance (Buscaill and Rivas, 2014; Erpen *et al.* 2018; Nakashima *et al.* 2009). In plants, different TFs, such as WRKYs (Pandey and Somssich, 2009), heat shock factors (Hsf) (Pajerowska-Mukhtar *et al.* 2012), zinc finger proteins (Ciftci-Yilmaz and Mittler, 2008; Yu *et al.* 2016), MYBs (Vailleau *et al.* 2002) and NACs (Sun *et al.* 2013) are known to be important regulators of plant defence responses.

1.6 NBS-LRR resistance genes

Plant *R*-genes encode for proteins which specifically interact with Avr-proteins of the pathogens and elicit a defence response. *R*-genes encode only five classes of proteins: NBS-LRRs, RLKs (receptor-like kinases), RLPs (receptor-like transmembrane proteins), STKs (serine-theorine kinases) and a final class containing all other types (Dangl and Jones, 2001; Zhong *et al.* 2015). The largest class of *R*-genes encodes for NBS-LRR proteins, which are also some of the largest proteins known in plants, ranging from about 860 to 1,900 amino acids (Dangl and Jones, 2001; McHale *et al.* 2006). They are characterised by three domains, all sharing a nucleotide-binding site (NBS) domain and a leucine-rich repeat (LRR) domain. By their domain at

the N-terminus, they can be subdivided in two classes: many have a domain with homology to the intracellular signalling domains of the *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR-NBS-LRR, or: TNL), whereas others contain putative coiled-coil domains (CC-NBS-LRR, or: CNL) (Dangl and Jones, 2001). CNL proteins are generally encoded in single exons, whereas TNL proteins are encoded in modular exons with conserved intron positions separating distinct protein domains (Meyers, 2003).

The three domains of a NBS-LRR protein have different functions (Figure 1.7). The N-termini are thought to be involved in protein-protein interactions with an adaptor protein, probably with the proteins being guarded or with downstream signalling components (Belkhadir *et al.* 2004; McHale *et al.* 2006). The NBS domain contains several defined motifs of the 'signal transduction ATPases with numerous domains' (STAND) family of ATPases and is required for adenosine triphosphate (ATP) binding and hydrolysis (Leipe *et al.* 2004; McHale *et al.* 2006). ATP hydrolysis is thought to result in conformational changes that regulate downstream signalling (McHale *et al.* 2006). The LRR domain is a common motif found in various species, from viruses to eukaryotes, and is involved in protein-protein interactions and ligand binding (Jones and Jones, 1997). LRRs comprise a core of about 26 amino acids containing the Leu-xx-Leu-xx-Leu-x-Leu-xx-Cys/Asn-xx motif (where x is any amino acid), which forms a ß-sheet, and each core region is separated by a section of variable length that varies from zero to 30 amino acids (McHale *et al.* 2006). The N-terminal and C-terminal domains within the LRRs appear to have different functions. The N-terminal domain of the LRR appears to modulate activation, whereas the C-terminal domain of the LRR works as an interaction platform for upstream activators (Belkhadir *et al.* 2004).

Compared to CNL proteins, TNL proteins contain larger and more variable C-terminal domains. CNL proteins have conserved motifs present in the 40– to 80–amino acid C-terminal domain. In contrast, the C-termini of the TNL proteins have a large number of non-LRR conserved motifs spanning 200 to 300 amino acids, corresponding to the size of the LRR domain. Several TNLs have extensions with similarity to other proteins; nevertheless, the functional roles of these C-terminal motifs are still unclear. Due to their conservation and wide distribution, it is assumed that these domains are important for protein function (McHale *et al.* 2006; Meyers, 2003).

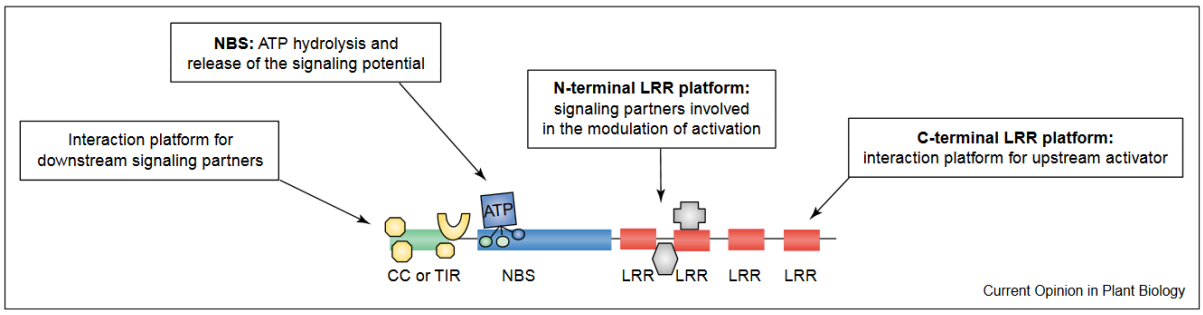

Figure 1.7: Domain structure of NBS–LRR proteins (acc. to Belkhadir *et al.* (2004)). A schematic representation of NBS–LRR proteins shows a domain-based platform for the assembly of various putative regulatory factors (i.e. CC, TIR, NBS and amino-terminal [N-terminal] LRR regions) necessary for controlled signalling. These domains also link to a possible intramolecular regulatory region on the carboxy-terminal (C-terminal) LRR. The cartoons in yellow represent putative interactors assembled on and carboxyl to the CC/TIR domains. The blue square represents ATP, but could also be GTP. The gray cartoons that are associated with the amino-terminal part of the LRR domain represent another set of putative interactors that might be positive regulators.

1.6.1 Evolution of NBS-LRRs

Compared to CNLs, TNLs are largely over-expressed in dicot genomes. For instance, the *Arabidopsis* genome comprises nearly double the number of TNLs than CNLs (Meyers, 2003). Furthermore, the presence of TNLs in pine and moss indicate an evolution prior to the angiosperm–gymnosperm split, which occurred at least 200 million years ago (Joshi and Nayak, 2013). According to Joshi and Nayak (2013) and Pan *et al.* (2000), the evolution of TNLs and CNLs occurred in two stages (Figure 1.8). In stage I, both CNLs and TNLs were present with broad-spectrum specificity, which evolved during the divergence of angiosperms and gymnosperms around 200 million years ago. Stage II took place after the monocot-dicot separation, around 100 million years ago and was dominated by gene duplication and diversification. This stage characterized the evolution of TNLs and CNLs and led to the degeneration of TNL genes in cereals and possibly in monocots in general. Across different plant species, there is a greater degree of diversity among non-TNLs than TNLs (Cannon *et al.* 2002).

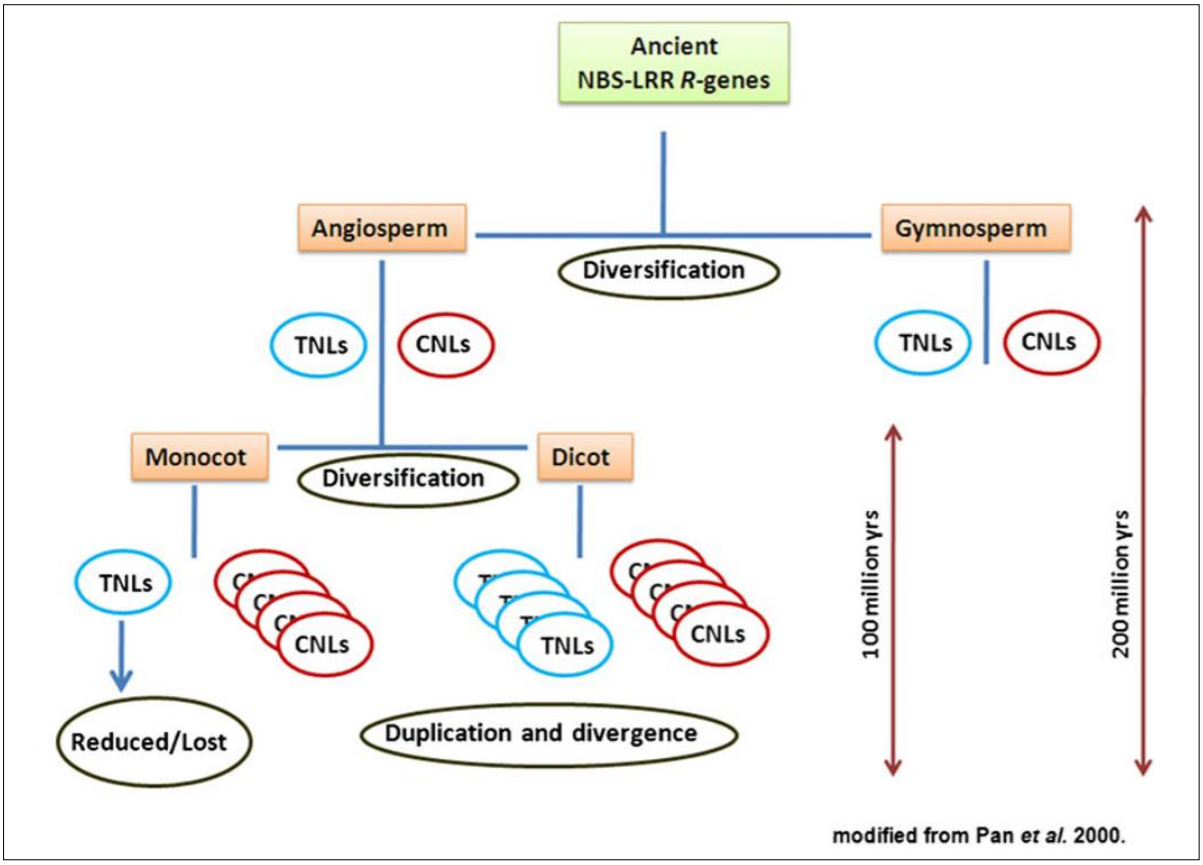

Figure 1.8: Evolutionary pattern of NBS-LRR class resistance genes in plants (acc. to Joshi and Nayak (2013)). Diversification of TIR-NBS-LRR (group I) and non-TIR-NBS-LRR (group II) took place during differentiation of angiosperms and gymnosperms. The separation of monocot and dicot was followed by extensive gene duplication and diversification resulting in NBS-LRR genes with diverse recognition specificities

Many NBS-LRR genes reside in local multigene families, but they can be also be present in the genome as single-copy genes (Dangl and Jones, 2001; van der Biezen, E. A. and Jones, 1998). The organisation of NBS-LRR genes can be either as tight clusters with little intervening sequence, like the 90 kb spanning *RPP5* cluster in *Arabidopsis thaliana* (Noel, 1999), or they can be spread over several megabases, like the resistance gene candidates 2 (*RGC2*) locus in lettuce (Meyers, 1998). The *R*-gene clustering may facilitate diversity to keep pace with newly evolving virulent races of a pathogen and to counteract with their newly emerging Avr-protein variants (Hulbert *et al.* 2001; Terefe-Ayana *et al.* 2012). The *R*-gene diversity is generated by genetic mechanisms such as unequal crossing-over, insertions/deletions, gene conversion, point mutations and illegitimate recombination (Kuang *et al.* 2004; Wicker *et al.* 2007). The rate of evolution of NBS-LRR-encoding genes can be rapid or slow, even within an individual cluster of similar sequences (McHale *et al.* 2006).

Kuang *et al.* (2004) reported two patterns of evolution for genes of the major cluster of NBS-LRR encoding genes in lettuce. Type I genes evolve rapidly with frequent

sequence exchanges between them, whereas type II genes evolve slowly with infrequent sequence exchange between paralogous sequences. This heterogeneous rate of evolution within the same cluster is consistent with a birth- and death-model of *R*-gene evolution (Kuang *et al.* 2004; McHale *et al.* 2006; Michelmore and Meyers, 1998).

Protein variation among NBS-LRR genes can be assessed by comparing non-synonymous nucleotide substitution (Ka) and synonymous substitution (Ks) in the nucleotide sequence from orthologues or paralogues. Non-synonymous nucleotide substitutions result in changes at the amino acid level, whereas synonymous substitutions do not. A ratio between Ka and Ks significantly greater than one, leads to amino acid diversity (Joshi and Nayak, 2013; Leister, 2004). In this case, a positive selection for amino acid substitution can be realized (Stahl and Bishop, 2000). Experiments in various plant species, such as tomato, rice and lettuce, showed that LRR-encoding regions in many NBS-LRR genes are subject to strong diversifying selection due to high ratios of non-synonymous compared to synonymous nucleotide substitutions (Meyers, 1998; Parniske *et al.* 1997; Wang, 1998). Compared to the other species, only moderate increased Ka/Ks ratios could be observed in the LRR regions of roses (Terefe-Ayana *et al.* 2012). The same authors identified a 340.4-kb region from *R. rugosa* orthologous to the *Rdr1* locus in *R. multiflora* and the two loci, except for some deletions and rearrangements, displayed a high degree of synteny. An analysis of 20 TNL genes obtained from both species revealed illegitimate recombination, gene conversion, unequal crossing over, indels, point mutations and transposable elements as mechanisms of diversification.

Additionally, strawberry, peach and apple were screened for *Rdr1* orthologous regions. In strawberry, an orthologous locus with less pronounced synteny was found, whereas in peach and apple genes from the *Rdr1* locus are distributed on two different chromosomes. Furthermore, a complete set of 53 TNL genes from the five *Rosaceae* species was used in a phylogenetic analysis. The resulting species-specific clusters indicate that recent TNL gene diversification began prior to the split of *Rosa* from *Fragaria* in the Rosoideae, and peach from apple in the Spiraeoideae and continued after the split in individual species.

1.7 Objectives

Black spot is the most severe disease in field-grown roses in temperate regions with a worldwide distribution. Intensive analyses resulted in the identification of the first resistance gene - *Rdr1*. Since *Rdr1* belongs to a cluster of nine highly similar TNL-genes, various experiments have been already performed to identify the functional *Rdr1* gene. Initial transient expression assays indicated that either *muRdr1H* or *muRdr1A* is the most likely candidate for *Rdr1.* Previous experiments showed that *Rdr1* cluster genes do not segregate within the progeny. Therefore, stable transgenic roses were generated to analyse the function of the *muRdr1A* and *muRdr1H* single *Rdr1* family members.

Due to these findings, the first part of the thesis focuses on the following objectives:

- Expression analysis and copy number identification of *muRdr1A* and *muRdr1H* genes in stable transgenic roses
- Identification of the functional *Rdr1* gene
- Specificity analysis of the *Rdr1* conferred resistance in a disease assay with a broad spectrum of *D. rosae* isolates
- Analysis of the *Rdr1* function in different genetic backgrounds

Furthermore, an *Rdr1* homologous locus was identified in *R. rugosa* and strawberry. Thus, the second part of the thesis aimed to identify and phylogenetically analyse *Rdr1* family members in a large set of rose species in comparison to other Rosaceae family members.

The last parts of the thesis focus on the characterisation of transcriptomic changes in rose leaves during *D. rosae* infection. Stable transgenic roses and their progeny harbouring *Rdr1* allow transcriptomic analysis of compatible and incompatible interaction in the same and also in a different genetic background.

2 The TNL gene *Rdr1* confers broad-spectrum resistance to *Diplocarpon rosae*

Ina Menz[1], Jannis Straube[1], Marcus Linde[1] and Thomas Debener[1]

[1]Institute for Plant Genetics, Leibniz University Hannover, Hannover, Germany

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Type of article:	Research article
Contribution to the article:	Planned and performed most of the experiments, analysed the data, prepared all tables and figures and wrote most of the manuscript.
Contribution of the other authors:	Jannis Straube performed some of the Southern Blot analysis and inoculation experiments. Marcus Linde helped in planning the experimental designs, writing and reviewing the manuscript. Thomas Debener contributed in planning of the experimental design, data analysis, writing and reviewing of the manuscript.

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The TNL gene *Rdr1* confers broad-spectrum resistance to *Diplocarpon rosae*

INA MENZ, JANNIS STRAUBE, MARCUS LINDE AND THOMAS DEBENER*

Department of Molecular Plant Breeding, Institute for Plant Genetics, Leibniz Universität Hannover, Hannover 30419, Germany

SUMMARY

Black spot disease, which is caused by the ascomycete *Diplocarpon rosae*, is the most severe disease in field-grown roses in temperate regions and has been distributed worldwide, probably together with commercial cultivars. Here, we present data indicating that *muRdr1A* is the active *Rdr1* gene, a single-dominant TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide binding site-leucine rich repeat) (TNL)-type resistance gene against black spot disease, which acts against a broad range of pathogenic isolates independent of the genetic background of the host genotype. Molecular analyses revealed that, compared with the original donor genotype, the multiple integrations that are found in the primary transgenic clone segregate into different integration patterns in its sexual progeny and do not show any sign of overexpression. *Rdr1* provides resistance to 13 different single-spore isolates belonging to six different races and broad field mixtures of conidia; thus far, *Rdr1* is only overcome by two races. The expression of *muRdr1A*, the active *Rdr1* gene, leads to interaction patterns that are identical in the transgenic clones and the non-transgenic original donor genotype. This finding indicates that the interacting avirulence (Avr) factor on the pathogen side must be widespread among the pathogen populations and may have a central function in the rose–black spot interaction. Therefore, the *Rdr1* gene, pyramided with only a few other *R* genes by sexual crosses, might be useful for breeding roses that are resistant to black spot because the spread of new pathogenic races of the fungus appears to be slow.

Keywords: black spot, disease resistance, effector-triggered immunity, NBS-LRR, plant immunity, *R* gene, roses.

INTRODUCTION

Rose black spot disease, which is caused by the hemibiotrophic ascomycete *Diplocarpon rosae*, is a disease of major importance for field-grown roses (Horst and Cloyd, 2007). Infected plants typically show dark spots at the site of infection that are mostly surrounded by heavily chlorotic areas. This infection can lead to reduced growth and, eventually, the death of the plant (von Malek and Debener, 1998). Currently, 11 different pathogenic races of *D. rosae* can be distinguished by tests that are based on a differential set of host genotypes (Whitaker *et al.*, 2010). Blechert and Debener (2005) characterized eight different interaction types between roses and *D. rosae*. Compatible interactions are characterized by weak to strong mycelium development, followed by sporulation. Incompatible interactions vary from no visible fungal growth to a fungal penetration of the epidermal cells, followed by necrosis of single cells or larger cell clusters, which indicates a response similar to a hypersensitive reaction.

Münnekhoff *et al.* (2017) showed low gene diversities in *D. rosae* populations depending on the age and diversity of the host population and the application of fungicides. The conidia of *D. rosae* are distributed mainly through splash water; the distribution is therefore localized, which reduces the risk of the evolution of new races (Lühmann *et al.*, 2010), in contrast with fungi with airborne conidia (Debener and Byrne, 2014). The control of black spot disease in the field relies on fungicide treatments and the cultivation of resistant varieties (von Malek and Debener, 1998; Reddy *et al.*, 1992).

Previous studies have evaluated black spot resistance in cultivated and wild roses in the field and laboratory (Byrne *et al.*, 2010; Debener *et al.*, 1998; Mangandi *et al.*, 2013; Schulz *et al.*, 2009). Schulz *et al.* (2009) used different single-spore isolates and field-collected samples of the pathogen in a detached-leaf assay. This assay revealed that the highest level of resistance occurs in wild species, particularly within the subgenus *Cinnamomeae*. An analysis of cultivated roses with good resistance to black spot indicated that their resistance originated from a wide variety of rose species. However, certain cultivated rose groups, such as hybrid teas and floribundas, lack black spot resistance (Byrne *et al.*, 2010).

To introgress black spot resistance genes from wild species into tetraploid garden roses, a diploid *Rosa multiflora* was crossed with a tetraploid garden rose (Drewes-Alvarez, 1992). After several rounds of open pollination, the diploid *R. multiflora* hybrid 88/124-46 was selected because of its high level of resistance and the chromosomal set was doubled via colchicine treatment. The resulting tetraploid line was crossed with the susceptible variety 'Caramba' leading to the genotype 91/100-5. This genotype was further crossed with the susceptible variety 'Heckenzauber'

* *Correspondence*: Email: debener@genetik.uni-hannover.de

resulting in the population 95/3 (Debener *et al.*, 1998; Drewes-Alvarez, 1992; von Malek and Debener, 1998).

Using amplified fragment length polymorphism (AFLP) markers, the resistant genotype 95/3-23 with a small donor fraction was selected and backcrossed as a male parent to 'Heckenzauber' leading to the population 99/20 (Debener *et al.*, 2003). Inoculation experiments provided evidence of the presence of a single dominant resistance locus in the duplex configuration in the hybrid 91/100-5. This gene was the first resistance gene described in roses and was named *Rdr1* (von Malek and Debener, 1998). Thus far, *Rdr1* has been characterized to confer resistance to five races of *D. rosae* (Debener *et al.*, 1998). High-resolution mapping confined the region surrounding the *Rdr1* locus in *R. multiflora* to four overlapping BAC clones with a complete sequence of 265 477 bp (Biber *et al.*, 2010; Kaufmann *et al.*, 2010; Terefe-Ayana *et al.*, 2011). Amongst 40 predicted genes, nine showed highly significant similarities to resistance genes of the TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide binding site-leucine rich repeat) (TNL) type. These genes were considered to be the most likely candidates for *Rdr1* and were designated as *muRdr1A–muRdr1I*. Except for *muRdr1D*, all ranged in size between 4085 and 5920 bp and had sequence similarities of 87.8%–99.5% with four exons and three introns. The first exon contains a TIR domain, the second contains an NBS domain and the fourth contains an LRR domain (Kaufmann *et al.*, 2010; Terefe-Ayana *et al.*, 2011).

To identify the function of the *Rdr1* family members, the expression of all nine *muRdr1* genes was assessed in different rose organs by Terefe-Ayana *et al.* (2011). Because there was no detectable expression of *muRdr1B*, *D*, *E* and *F* in the leaves and petals, these genes were considered to be unlikely candidates for *Rdr1*. In contrast, *muRdr1A*, *C*, *G*, *H* and *I* were expressed in both the leaves and petals, and thus were further analysed in several transient infiltration experiments. Each of these five genes was subcloned, transformed and co-infiltrated with the *D. rosae* single-spore isolates DortE4 and R6 in a susceptible genotype, as described in Terefe-Ayana *et al.* (2011). Between 15 and 20 infiltration sites were analysed in each of the nine experiments. In all nine conducted experiments, the leaf areas that were infiltrated with the *muRdr1H* gene showed a significant reduction in the number of fungal colonies formed (Terefe-Ayana *et al.*, 2011). In contrast, *muRdr1A* showed effects in only six of the experiments, and was therefore not considered to be the most likely candidate for *Rdr1*. None of the *muRdr1* genes showed effects against the isolate R6.

The goal of the current work was to further analyse the *Rdr1* family members *muRdr1A* and *muRdr1H* in stable transgenic rose plants and their sexual progeny. Furthermore, the specificity of the resistance, particularly the number of isolates to which *Rdr1* confers resistance, was analysed in inoculation experiments with a broad selection of isolates and isolate mixtures.

RESULTS

Complementation of *muRdr1A* and *muRdr1H*

To further analyse the genes of the *Rdr1* cluster described by Terefe-Ayana *et al.* (2011), we performed stable transformation experiments in the susceptible genotype 'Pariser Charme' (PC) with genomic clones of the two genes *muRdr1A* and *muRdr1H*. The number of embryo clusters used for transformation varied from 4926 in *muRdr1A* to 6295 in *muRdr1H*. From the regenerated shoots, 22 (*muRdr1A*) and 14 (*muRdr1H)* were positive for the integration of the transformation constructs by polymerase chain reaction (PCR). Not all plants survived the original selection and only 13 (*muRdr1A*) and five (*muRdr1H*) could be tested for the copy number of the transgene in a Southern blot analysis. The transgenic shoots tested in the Southern blot analysis showed that, for each of the two constructs, only one integration pattern was detected. For *muRdr1A*, we detected one integration pattern with seven copies (Figs 1 and S1, see Supporting Information) and, for *muRdr1H*, one integration pattern with two copies (Fig. 1), indicating that the transgenic clones were derived from a single event per gene. Surprisingly, the inoculation with isolate DortE4 revealed that the five clones obtained from the transformations with *muRdr1A* were resistant, whereas the five tested clones that contained *muRdr1H* were susceptible (Table 1). Both groups of clones were susceptible to isolate R6, which is also virulent against the *Rdr1* donor genotype 88/124-46 and its hybrids (Table 1).

Microscopic analysis

The microscopic analysis showed that the stage at which the pathogen is stopped is identical between PC containing *muRdr1A* and the *Rdr1* donor 88/124-46. In these clones, conidia germinate and form appressoria and a few short hyphae, but are not able to establish haustoria (Fig. 2c,d). In contrast, the fungal development in the susceptible cultivar PC is indistinguishable from that in the transgenic clones that harbour *muRdr1H*, and extensive mycelia with long-range hyphae and numerous haustoria are formed (Fig. 2a,b).

Expression analysis

An analysis of all clones with gene-specific simple sequence repeats (SSRs) (Terefe-Ayana and Debener, 2011) confirmed the correct identity of the transgenes, and reverse transcription-polymerase chain reaction (RT-PCR) revealed that the transgenes are expressed in both groups of clones (data not shown).To rule out artefacts from the potential overexpression of the transgenes in the clones derived from the *muRdr1A* transformation, the expression levels in these clones were compared with those in the diploid homozygous donor genotype 88/124-46 and the

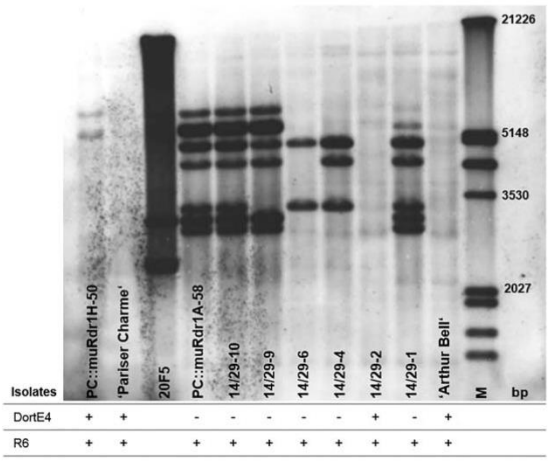

Fig. 1 Southern blot analysis and resistance data of population 14/29. For Southern blot analysis, DNA from 'Arthur Bell' and PC::muRdr1A-58, their sexual progeny 14/29 (-1, −2, −4, −6, −9, −10), a transgenic clone harbouring *muRdr1H* (PC::muRdr1H-50), a non-transgenic control ('Pariser Charme') and a plasmid harbouring the *NPTII* sequence (Plasmid 20F5) was digested with *Hind*III and hybridized with an *NPTII* probe. The integration patterns of PC::muRdr1A-58 and PC::muRdr1H-50 are representative for all *muRdr1A* and *muRdr1H* transgenics. The interaction between the genotypes and the single-spore isolates DortE4 and R6 of *Diplocarpon rosae* are represented in the table by '+' for compatible interactions and by '–' for incompatible interactions. M, DNA molecular weight marker.

Table 1 Interaction between the transgenic and non-transgenic rose genotypes and the single-spore isolates DortE4 and R6 of *Diplocarpon rosae*.

	Isolates of *D. rosae*	
Genotype	DortE4	R6
88/124–46	–	+
91/100–5	–	+
95/3–23	–	+
99/20–35	–	+
PC	+	+
PC::GUS	+	+
PC::muRdr1A	–	+
PC::muRdr1H	+	+

The tetraploid hybrids 91/100-5, 95/3-23 and 99/20-35 are progeny from the diploid donor of *Rdr1* (88/124-46) and carry the *Rdr1* locus. PC ('Pariser Charme') not carrying the *Rdr1* locus was transformed with the *Rdr1* family members *muRdr1A* (PC::muRdr1A = PC::muRdr1A-38, -43, -53, -57, -58) and *muRdr1H* (PC::muRdr1H = PC::muRdr1H-50, -62, -63, -65, -66) and the *GUS* gene as control (PC::GUS). Compatible interactions are represented by '+'; incompatible interactions are represented by '−'.

heterozygous (duplex configuration) tetraploid hybrid 91/100-5. Therefore, specific primers for the TIR region of *muRdr1A* were used (Fig. S2, see Supporting Information); in addition, specific primers amplifying a part of the LRR region were employed to show that the entire gene is expressed (Fig. S3, see Supporting Information). As shown in Fig. 3, the relative expression values of *muRdr1A* in all analysed transgenic clones were lower than those in the genotype 91/100-5, a non-transgenic carrier of *muRdr1A*. The results were not as clear for the original *Rdr1* donor 88/124-46 as a result of high variation in the expression analyses. However, the mean expression values of the transgenics were much lower than the mean of genotype 88/124-46.

Analysis of sexual progeny

Because the transformation of roses with large DNA inserts is very laborious as a result of the extremely low transformation efficiencies, we decided to analyse independent integration events by sexual segregation of the multiple integrations of PC::muRdr1A, rather than by conducting new transformation experiments. Therefore, the transgenic clone PC::muRdr1A-58 with seven integrations of *muRdr1A* was crossed to the tetraploid cultivar 'Arthur Bell' (AB), which is susceptible to most pathogenic races of black spot. Amongst the six progeny of the resulting population 14/29, five carried the transgene. A Southern blot analysis revealed the following five different integration patterns: one pattern was identical to the original clone, and four patterns had different numbers of the original seven integrations, ranging from two to six (Fig. 1). All clones carrying the transgene were resistant to DortE4, whereas progeny 14/29-2, which lacked the transgene, was susceptible (Fig. 1). This finding also indicates that the transgene is integrated into at least five different genomic locations.

Inoculation of several Rdr1 plants with different races and race mixtures

The observations of the resistance of the diploid donor genotype 88/124-46 and the tetraploid hybrid 91/100-5 (planted with three clones each) in a 140-m^2 field plot at Leibniz Universität Hannover Herrenhausen between 2005 and 2016 did not reveal any sign of infection, although highly infected genotypes grew within a distance of 5 m. Furthermore, 96 rose genotypes with three to four clones each were grown in a field plot within 250 m of the above-mentioned two genotypes and showed high infection rates for more than 90 genotypes at the end of the growing season. Because this observation indicated that these genotypes had a broad spectrum of resistance, we inoculated transgenic clones that harboured both *muRdr1A* and *muRdr1H*, transformation controls that carried the *GUS* reporter gene, a number of cultivars and clones with and without the *Rdr1* gene with several isolates and conidial mixtures from the field plots at Hannover Herrenhausen (Table 2).

The experiments revealed that all plants that harboured *Rdr1*, either by conventional cross-breeding or transformation of the isolated gene, were resistant to a diverse set of 13 single conidial

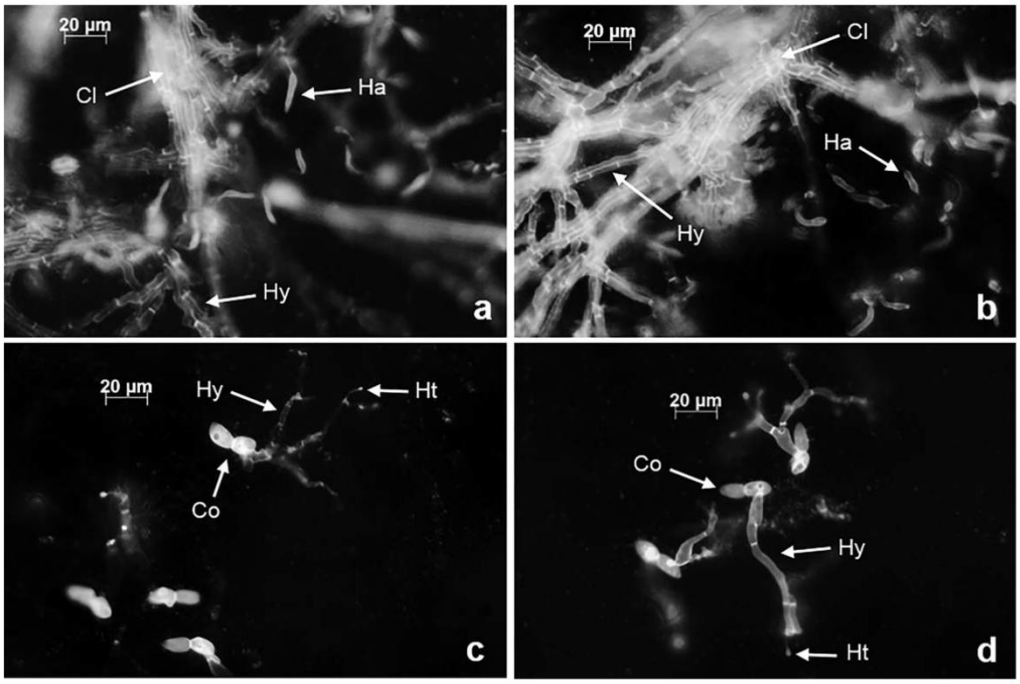

Fig. 2 Interaction between DortE4 and the different rose genotypes at 5 days post-inoculation (dpi). The diploid *Rdr1* donor 88/124–46, the susceptible genotype 'Pariser Charme' and the transgenic clones harbouring *muRdr1A* (PC::muRdr1A-58) and *muRdr1H* (PC::muRdr1H-66) were inoculated with the isolate DortE4 and stained with wheatgerm agglutinin (WGA) Alexa Fluor®488. (a) PC::muRdr1H-66; (b) 'Pariser Charme'; (c) PC::muRdr1A-58; (d) 88/124–46. Co, conidia; Cl, hyphal cluster; Ha, haustoria; Ht, haustorial tube; Hy, hyphae.

isolates and races, and mixtures of conidia from the field plots. These plants were only infected by two races (R6 and Ab13) that broke the *Rdr1* resistance regardless of whether it was the conventional source of *Rdr1* or a transgenic clone. Furthermore, progeny from the conventional crosses (95/3-23 and 99/20-35), progeny from crosses between the transgenic PC::muRdr1A-58 and 'Arthur Bell' (14/29) and three additional crosses of PC::muRdr1A-58 to susceptible genotypes (data not shown) were resistant to a subset of all the isolates to which the resistance donors were resistant and were susceptible to the same two isolates, indicating that the copy number of the transgenes does not have an influence on the resistance specificity.

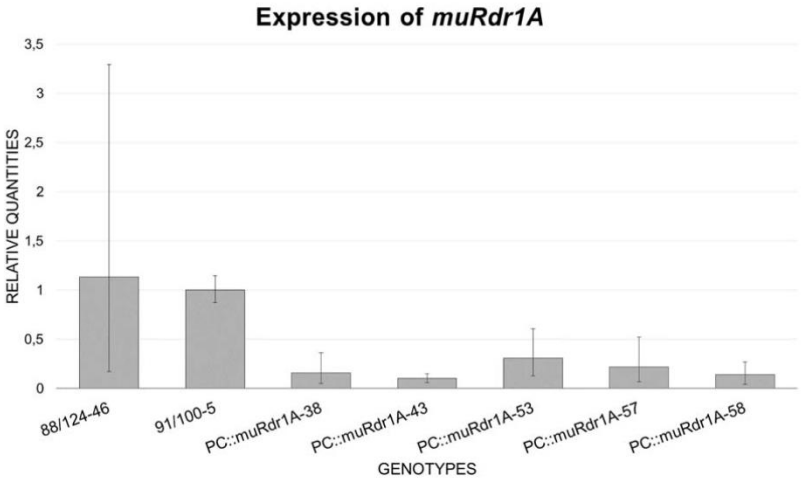

Fig. 3 Results of the quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *muRdr1A*. The tetraploid hybrid 91/100–5 and the diploid genotype 88/124–46 are *Rdr1* carriers. 'Pariser Charme' (PC), not carrying the *Rdr1* locus, was transformed with the *Rdr1* family member *muRdr1A*, resulting in the clones PC::muRdr1A-38, −43, −53, −57, −58. The expression values of *muRdr1A* are averages of three independent biological repeats with three technical repeats. The reference genes *UBC*, *TIP* and *SAND* were used for normalization. Error bars, RQ_{min} and RQ_{max} (RQ, relative expression quantity).

Table 2 Interaction of transgenic and non-transgenic rose genotypes and *Diplocarpon rosae*.

	Isolates of *D. rosae*															
Genotype	DortE4	X122	S009	F004	R6	Ab13	I001	X104	X130	Br26	Sp-A1	191	187/2	D002	DüA3	Field mix
PC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PC::GUS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
91/100–5	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
88/124–46	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
PC::muRdr1A	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
PC::muRdr1H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
95/3–23	–	–	–	–	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	–	–
99/20–35	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	–	–
AB	+	+	+	+	+	+	+	+	n.a.	n.a.	+	+	+	+	n.a.	n.a.
14/29–1	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	n.a.	n.a.
14/29–2	+	+	+	+	+	+	+	+	n.a.	n.a.	+	+	+	+	n.a.	n.a.
14/29–4	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	n.a.	n.a.
14/29–6	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	n.a.	n.a.
14/29–9	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	n.a.	n.a.
14/29–10	–	–	–	–	+	+	–	–	n.a.	n.a.	–	–	–	–	n.a.	n.a.

The tetraploid hybrids 91/100–5, 95/3–23, 99/20–35 and the diploid genotype 88/124–46 are carriers of *Rdr1*. PC ('Pariser Charme'), as a non-carrier of *Rdr1*, was transformed with the single *Rdr1* family members *muRdr1A* (PC::muRdr1A = PC::muRdr1A-38, −43, −53, −57, −58) and *muRdr1H* (PC::muRdr1H = PC::muRdr1H-50, −62, −63, −65, −66) and the *GUS* gene as control (PC::GUS). The genotypes 14/29 (-1, −2, −4, −6, −9, −10) are the sexual progeny of AB ('Arthur Bell') × PC::muRdr1A-58. Compatible interactions are represented by '+'; incompatible interactions are represented by '–'. Field mix = Hannover (2014), n.a., not analysed.

DISCUSSION

In the work presented here, we provide experimental evidence indicating that *muRdr1A* is the active *Rdr1* gene and that it acts against a broad set of pathogenic races of black spot fungus.

muRdr1A is the active *Rdr1* gene

The experiments described by Terefe-Ayana *et al.* (2011), based on the positional cloning of the *Rdr1* locus, revealed that a complex locus comprising nine TNL genes co-segregates with *Rdr1* resistance to the *D. rosae* isolate DortE4. The transient leaf infiltration assays indicated that one of the genes (*muRdr1H*) partially restored resistance, and therefore was the most likely candidate for the functional *Rdr1* gene; other genes of the cluster showed partial, but non-significant, effects on black spot colonization. However, *muRdr1A* also partially restored resistance in six of the nine transient leaf infiltration experiments.

In contrast with these previously conducted transient expression studies by Terefe-Ayana *et al.* (2011), our stable transgenic clones that harbour *muRdr1H* are not resistant to the isolate DortE4, whereas *muRdr1A* confers the resistance that was expected for the *Rdr1* gene. Our conclusions are based on several lines of evidence as follows:

1. The stable transgenic clones of *muRdr1A* display interaction patterns that are similar to those of the original donor genotypes that harbour *Rdr1*, whereas the transgenic clones that harbour *muRdr1H* are susceptible to all the tested isolates.
2. The expression studies that compared the original transgene with the donor genotype and a natural hybrid showed no evidence of higher expression levels of the transgene. This finding rules out the artefacts that were observed previously when the NBS-LRR sequences were expressed under the control of strong constitutive promoters (Bendahmane *et al.*, 2002; Tao *et al.*, 2000).
3. Alternative explanations for the resistance (e.g. effects caused by the transformation event) can be ruled out because sexual progeny segregating for different combinations of transgene integrations all display an interaction pattern that is similar to that of the original transgenic clone.

The reason for the difference in the stable transgenic events compared with the transient assay published by Terefe-Ayana *et al.* (2011) might be explained by artefacts, as a result of the overexpression of transiently expressed genes (Wroblewski *et al.*, 2005). It remains unknown why this is not the case for *muRdr1A*, but the quantitative assay used by Terefe-Ayana *et al.* (2011) was based on a relatively small number of transiently transformed mesophyll cells.

muRdr1A confers broad resistance to various black spot pathotypes

TNL resistance genes can be present in the genome as single-copy genes, but many reside in local multigene families, which are either heterogeneous with distantly related sequences, or homogeneous, such as *Rdr1*, comprising closely related sequences (van der Biezen and Jones, 1998; Dangl and Jones, 2001; Michelmore

et al., 2013). Distinct TNL family members can encode different race-specific resistances to a particular pathogen (Wroblewski *et al.*, 2007). The *L* locus in flax confers an allelic series of TNLs that encode NBS-LRR proteins, which interact with different avirulence (Avr) proteins from various strains of the flax rust fungus (Dodds *et al.*, 2006; Ellis *et al.*, 1999).

Our inoculation results using several single conidial isolates showed that a broad range of pathotypes of *D. rosae* are recognized by *Rdr1*. *Rdr1* confers resistance to 13 single conidial isolates and a broad field mixture of conidia collected from field plots at Leibniz Universität Hannover, both in its natural genomic context and as a transgene. Some isolates have been characterized and represent various pathogenic races (Whitaker *et al.*, 2010), whereas, to date, others have not been compared with the existing pathotype pattern. However, in the inoculation experiments using the isolates DortE4, R6, X122, F004, S009 and Ab13 in the tetraploid progeny (07/57) of the multi-resistant *Rosa majalis* genotype 93/09-01 crossed to the cultivar 'König Stanislaus', the differential reactions of individual progeny to all isolates indicate that these seven isolates belong to different pathogenic races (Table S1, see Supporting Information). Although we cannot make conclusions regarding the total number of pathogenic races in our dataset, there is evidence that *Rdr1* confers a broad-spectrum resistance to *D. rosae* that is only overcome by two of the pathogenic races and isolates tested thus far. This finding is underlined by the resistance of *Rdr1*-carrying plants against broad mixtures of conidia from field plots. In addition, the original donor genotype 88/124-46 and the hybrid 91/100-5 were grown in field plots that were close to heavily infected plants between 2005 and 2016 and did not show any signs of black spot infection.

Therefore, it is tempting to speculate that the *D. rosae* effector/*Avr* gene that is recognised by *Rdr1* must be of central importance to the black spot fungus because it is present in a broad collection of pathotypes. Future work targeting this effector might provide interesting information regarding black spot biology and provide tools for the screening of additional resistance specificities (Hein *et al.*, 2009; Rietman *et al.*, 2012; Vleeshouwers and Oliver, 2014).

Rdr1 probably acts as a single TNL gene. This can be concluded by the observation that progeny segregating for different *muRdr1A* integrations, as well as conventional progeny from crosses with genotypes carrying *Rdr1* with various susceptible genotypes, are all resistant. As roses are highly heterozygous, progeny from bi-parental crosses usually segregates for many characteristics, creating a large array of genetic backgrounds. Therefore, we conclude that *muRdr1A* acts independently of a second TNL, which is a situation that can be found in a number of *R* genes in the NBS-LRR class (Qu *et al.*, 2006; Shen *et al.*, 2002; Song *et al.*, 2003). The probability that these factors were present in the various progeny analysed here is very low.

Usually, multiple stacked race-specific *R* genes are required to achieve a broad-spectrum resistance (Kim *et al.*, 2012; Marone *et al.*, 2013). In roses, the broad-spectrum resistance is conferred by the single dominant *Rdr1* gene. The gap in the resistance to *D. rosae* as a result of pathogenic races overcoming *Rdr1* may be closed by *R* genes that are directed against these isolates. Therefore, *Rdr1* might be useful for the breeding of durable resistance in roses because the gene flow between pathogen populations appears to be low compared with that seen in pathogens that are distributed by airborne propagules. There are single resistance genes that are known from other pathogenic systems that encode broad-spectrum resistance. A broad-spectrum resistance to potato late blight is caused by the *RB* gene, a coiled coil (CC)-NBS-LRR plant resistance gene and a member of a four-gene family (Song *et al.*, 2003). Moreover, the *Arabidopsis thaliana RRS1-R* resistance gene is a TIR-NBS-LRR gene and confers resistance to several strains of *Ralstonia solanacearum*, which is the causal agent of bacterial wilt (Deslandes *et al.*, 1998, 2003). The *Hero* gene from tomato confers a high level of resistance to all pathotypes of the potato cyst nematode *Globodera rostochiensis* and a partial resistance to *G. pallida*. *Hero* is characterized as a non-TIR-NBS-LRR gene and a member of an NBS-LRR gene family with 14 highly homologous genes, but, as in the case of *Rdr1*, none of the other homologues confers resistance to *G. rostochiensis* pathotypes (Ernst *et al.*, 2002). The single dominant resistance gene *Pi65(t)* confers a broad-spectrum resistance against the fungus *Magnaporthe oryzae* in rice. A single nucleotide polymorphism (SNP) marker for *Pi65(t)* was used to develop a new rice variety that is highly resistant to rice blast and produces a high yield. That indicates that *Pi65(t)* could play a key role in the improvement of rice blast resistance (Zheng *et al.*, 2016).

In conclusion, *Rdr1* may be both an interesting *R* gene for research studies investigating plant–pathogen interactions in a pathosystem involving a broad-spectrum resistance gene and as the starting point for resistance breeding. The first combinations of *Rdr1* with *R* genes providing resistance to pathogenic races overcoming *Rdr1* are currently underway.

EXPERIMENTAL PROCEDURES

Plant material

The roses used in the present study are a part of the genotype collection at the Department of Molecular Plant Breeding, Institute for Plant Genetics, Leibniz Universität Hannover (Germany). The genotypes 88/124-46, 91/100-5, 95/3-23 and 99/20-35 and the population 07/57 have been described previously (Debener *et al.*, 1998, 2003; Schulz *et al.*, 2009). All rose genotypes were cultivated under semi-controlled conditions in the glasshouse at Leibniz Universität Hannover. Transgenic plants were cultivated in climate chambers under short-day conditions (8 h light/16 h darkness) at 22 °C. For multiplication of the fungal isolates and the transformation approaches, the susceptible cultivar PC was propagated

Table 3 Single-spore isolates and field mixtures of *Diplocarpon rosae* used for the inoculation experiments

Isolate	Race classified by Whitaker *et al.* (2010)	Collection location	Reference
DortE4	6	Germany, Dortmund	Debener *et al.* (1998)
X122	10	Norway, Elverum	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
S009		Sweden, Ausås	Münnekhoff *et al.* (2017)
F004		Frankreich, Cannet-de Maures	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
R6	7	Germany, Ahrensburg	Whitaker *et al.* (2010)
Ab13		Germany, Ahrensburg	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
I001		Italy, Lucca	Whitaker *et al.* (2010)
X104	5	South Africa, Johannesburg	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
X130	5	Australia	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
Br26	2	Germany, Bremen	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
Sp-A1	6	Germany, Sparrieshoop	Debener *et al.* (1998)
191		France, Avranches	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
187/2		France, St. Michel de Loupes	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany
D002		Germany, Sarstedt	Münnekhoff *et al.* (2017)
DüA3	4	Germany, Dusseldorf	Debener *et al.* (1998)
Field mix		Germany, Hanover (2014)	Institute of Plant Genetics, Leibniz Universität, Hannover, Germany

in vitro as described previously in Davies (1980) and Dohm *et al.* (2002), rooted, transplanted into 9-cm pots and cultivated under semi-controlled conditions with 12 h light/12 h darkness.

Plant transformation

For the transformation, constructs including large genomic fragments of the *muRdr1A* (10 728 bp) and *muRdr1H* (11 065 bp) genes (GenBank accession numbers for the genomic regions of the *Rdr1* clusters in *R. multiflora* and *R. rugosa*: HQ455834.1 and JQ791545.1), including the promoter and terminator regions, were transferred into the pBINPLUS plant transformation vector (van Engelen *et al.*, 1995) and a 35S:GUS-intron in pBin19 as described previously (Terefe-Ayana *et al.*, 2011; Yasmin, 2011). The constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101 using electroporation (Mattanovich *et al.*, 1989). The transformation of roses was performed as described previously (Dohm *et al.*, 2002) using somatic embryos of the susceptible cultivar PC. A cluster of mature somatic embryos was collected and wounded by stirring with sterile sea sand and minimal A medium (Temmerman *et al.*, 2000) in a beaker on a magnetic stirrer for 4 min at 750–1000 rpm. The wounded embryos were covered with a bacterial suspension containing 100 mM acetosyringone and incubated on a shaker (60 rpm) for 1 h at room temperature. After 2 days of co-cultivation with agrobacteria on embryo proliferation medium (Dohm *et al.*, 2001), the embryos were collected and washed with an antibiotic solution containing 500 mg/L cefotaxime and 50 mg/L carbenicillin three to four times, and subsequently transferred again to an embryo proliferation medium containing 500 mg/L cefotaxime and 50 mg/L carbenicillin. After 1 month, the explants were transferred to embryo proliferation medium containing 60 mg/L kanamycin and 150 mg/L ticarcillin, and subcultured every 4–5 weeks. The emerging shoots were cut off and transferred to multiplication medium [1 × MS (Murashige and Skoog, 1962) with 0.1 g/L Ethylenediamine di-2-hydroxyphenyl acetate ferric (FeEDDHA), 0.004 mg/L 1-Naphthaleneacetic acid (NAA), 2.0 mg/L 6-Benzylaminopurine (BAP), 0.1 mg/L Gibberellic acid (GA_3), 8 g plant agar] containing 6 mg/L kanamycin and cultivated at 25 °C (16 h light/8 h darkness). Rooting was induced by subculture on half-strength MS medium containing 0.05 mg/L Indole-3-butyric acid (IBA).

DNA and RNA extraction

For the DNA extraction from the transgenic and non-transgenic rose genotypes, 70 mg of tissue from young leaves were dried on silica gel overnight and ground to a fine powder using a bead mill. DNA was extracted using the DNA extraction kit from a NucleoSpin® Plant II kit by Macherey-Nagel (Düren, Germany) according to the manufacturer's instructions. For the Southern blot analysis, DNA was extracted from 2 g of young leaves according to Kobayashi *et al.* (1998).

RNA was extracted from 20–30 mg of fresh leaf tissue, frozen in liquid nitrogen and disrupted using bead mills. RNA was extracted using the Invisorb Spin® Plant RNA Mini Kit from STRATEC Molecular GmbH (Berlin, Germany) according to the manufacturer's protocol, with an additional DNAse digestion using the Ambion® DNA-free™ Kit from Life Technologies (Carlsbad, CA, USA) to remove the remaining genomic DNA. Approximately 300 ng of total RNA were used for the cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems® (Carlsbad, CA, USA) according to the manufacturer's instructions.

PCR and quantitative RT-PCR

Stable integration of the transgene and possible contamination with persistent bacteria in the transgenic plants were tested using PCR with primers for the *NPT*II gene (forward, GAGGCTATTCGGCTATGACTG; reverse, ATCGGGAGCGGCGATACCGTA) and the *A. tumefaciens* chromosome (forward, ATGCGGATGAGGCTCGTCTTCGAG; reverse, GACGCAACGCATCC TCGATCAGCT). To amplify different regions of the *muRdr1A* gene, specific primers were used (180-forward, TGCCATCTCTCCGAAGCTCCA; 428-reverse, TCCTTATTACCTTGCCCAAG; 3071-forward, GCGGAGTTTAA GGGTGACAA; 3485-reverse, AGTACCGTACGCGTTCCAAG), which were designed based on the sequences of *Rdr1* (Terefe-Ayana *et al.*, 2011). The gene expression levels were determined using quantitative PCR with the specific primers for *muRdr1A* (180-forward, 428-reverse). As internal

control, specific primers for ubiquitin conjugating protein (*UBC*) (JN399227.1), TIP41-like protein (*TIP*) (JN399221.1) and SAND-family protein (*SAND*) (JN399228.1) (Klie and Debener, 2011) were used. The amplification was performed in a 10-μL volume with 1 μL cDNA (1 : 5 diluted) using Taykon™ Rox SYBR® MasterMix dTTP Blue from Eurogentec (Liège, Belgium) according to the manufacturer's instructions. The reactions were performed for one cycle of 3 min at 95 °C, 40 cycles of 95 °C for 10 s, and 60 s at 64 °C in a StepOnePlus™ System (Applied Biosystems, Austin, TX, USA). Baseline correction was performed using StepOne™ Software, and a common threshold of 0.5 was set for the quantification cycle (Cq). The primer efficiency was estimated for each reaction using LinRegPCR 11.1 (Ruijter *et al.*, 2009), and the relative expression quantities (RQs) were calculated according to Pfaffl (2001).

Southern blotting

The Southern blot analysis was performed as described previously (Terefe and Tatlioglu, 2005) using digoxigenin (DIG)-labelled probes (Roche, Basel, Switzerland). Primers for the *NPT*II gene (forward, GAGGCTATTCGGCTATGACTG; reverse, ATCGGGAGCGGCGATACCGTA) were used to amplify the probe from pCLD04541 containing 20F5 (Biber *et al.*, 2010). The DNA was digested with *Hind*III (NEB, Ipswich, MA, USA) and transferred to positively charged nylon membranes (Roche, Mannheim, Germany) in 20 × Saline Sodium Citrate (SSC) using a vacuum system (90 mbar, 90 min). The DNA Molecular Weight Marker III (Roche) was used as a molecular weight standard.

Fungal isolates

Single conidial isolates were maintained on detached leaves of the susceptible cultivar PC as described previously (von Malek and Debener, 1998). The isolates of *D. rosae* used in this study are listed in Table 3.

Disease assays

The disease assays were performed by inoculating the detached leaves with 10-μL droplets of conidial suspensions of 10^5 conidia/mL, as described previously (von Malek and Debener, 1998). The inoculated leaves were kept on moist filter paper in translucent plastic boxes in an air-conditioned laboratory at 20 °C. After 2 days, the droplets were carefully blotted, and the leaves were inspected at 7 and 10 days after inoculation. The genotypes in which mycelia and acervuli could be observed in the inoculation area were considered to be susceptible. In turn, the genotypes in which no mycelium and acervuli could be observed were considered to be resistant. A minimum of three independent biological repeats, with at least 10 infection sites for each genotype and isolate, were performed.

Microscopic analysis

For the microscopic analysis, the genotypes 91/100-5, PC, PC::muRdr1A and PC::muRdr1H were inoculated with the isolate DortE4 with 10^5 conidia/mL, as described previously. At 5 dpi, the infected areas were fixed in 75% ethanol and 15% acetic acid. The solution was replaced with 1 M KOH and incubated for 2 h at room temperature, and the leaf parts were subsequently washed five times with distilled water. The water was removed, and 1 mL of 10 × phosphate-buffered saline (PBS) (1.4 M NaCl, 27 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 18 mM KH_2PO_4, pH 7.3), with 20 μg of wheatgerm agglutinin (WGA) Alexa Fluor®488 (Invitrogen, Carlsbad, CA, USA), was added and stained overnight. The staining solution was substituted with 10 × PBS, and the infestation of the leaf samples was observed under an Axio Scope.A1 fluorescence microscope using filter set 38 HE (Carl Zeiss AG, Oberkochen, Germany).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Interaction of population 07/57 with different single-spore isolates of *Diplocarpon rosae*. The tetraploid population 07/57 is the sexual progeny of the multi-resistant *Rosa majalis* genotype 93/09-01 and the susceptible cultivar 'König Stanislaus'. The subset of genotypes shown is representative of the segregation of the resistance against the different isolates in the population 07/57. Compatible interactions are represented by '+'; incompatible interactions are represented by '−'.

Fig. S1 Southern blot analysis of *muRdr1A* transgenics. For Southern blot analysis, DNA from the non-transgenic 'Pariser Charme' and 'Pariser Charme' transformed with *muRdr1A* (PC::muRdr1A-38, -43, -53, -57, -58) was digested with *Hind*III and hybridized with an *NPT*II probe. All PC::muRdr1A transgenics show the same integration pattern with seven copies of *muRdr1A*. The patterns of PC::muRdr1A-38 and PC::muRdr1A-

43 are shifted in their position because of the high content of carbohydrates. M, DNA molecular weight marker.

Fig. S2 Results from *muRdr1A*-specific polymerase chain reaction (PCR). The susceptible genotype 'Pariser Charme', the *Rdr1* donors 88/124-46 and 91/100-5, and the transgenic clones harbouring *muRdr1A* (PC::muRdr1A-38) and *muRdr1H* (PC::muRdr1H-50) were used in a PCR with the specific primers 180-forward and 428-reverse. The products (248 bp) were separated on a 1.5% agarose gel. 100 bp, Gene Ruler^{TM} 100-bp DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Fig. S3 Results from *muRdr1A*-specific polymerase chain reaction (PCR). The cDNAs of the *Rdr1* donors 88/124-46 and 91/100-5, the susceptible genotype 'Pariser Charme' and the transgenic clones harbouring *muRdr1A* (PC::muRdr1A-38, -43, -53, -57, -58) and *muRdr1H* (PC::muRdr1H-50) were used in a PCR with the specific primers 3071-forward and 3485-reverse. The products (414 bp) were separated on a 1.5% agarose gel. 1 kb, Gene Ruler^{TM} 1-kb Plus DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.1 Supporting Information

Table S1: Interaction of population 07/57 with different single-spore isolates of Diplocarpon rosae. The tetraploid population 07/57 is the sexual progeny of the multi-resistant Rosa majalis genotype 93/09-01 and the susceptible cultivar 'König Stanislaus'. The subset of genotypes shown is representative of the segregation of the resistance against the different isolates in the population 07/57. Compatible interactions are represented by '1'; incompatible interactions are represented by '–'.

	Isolates of *D. rosae*					
Genotype	**DortE4**	**X122**	**S009**	**F004**	**R6**	**Ab13**
***Rosa majalis* (93/09-01)**	-	-	-	-	-	-
`König Stanislaus`	+	+	+	+	+	+
07/57-2	+	+	+	+	+	+
07/57-4	+	-	-	+	+	+
07/57-9	-	-	-	-	+	+
07/57-10	-	-	-	-	-	-
07/57-12	-	-	-	-	+	-
07/57-21	-	-	-	+	+	+
07/57-64	-	-	-	+	-	-
07/57-67	-	-	-	-	+	-
07/57-75	+	-	-	+	+	-
07/57-82	-	-	-	+	+	-
07/57-84	-	+	+	+	+	+
07/57-86	+	-	+	+	+	-
07/57-100	+	-	-	-	+	-

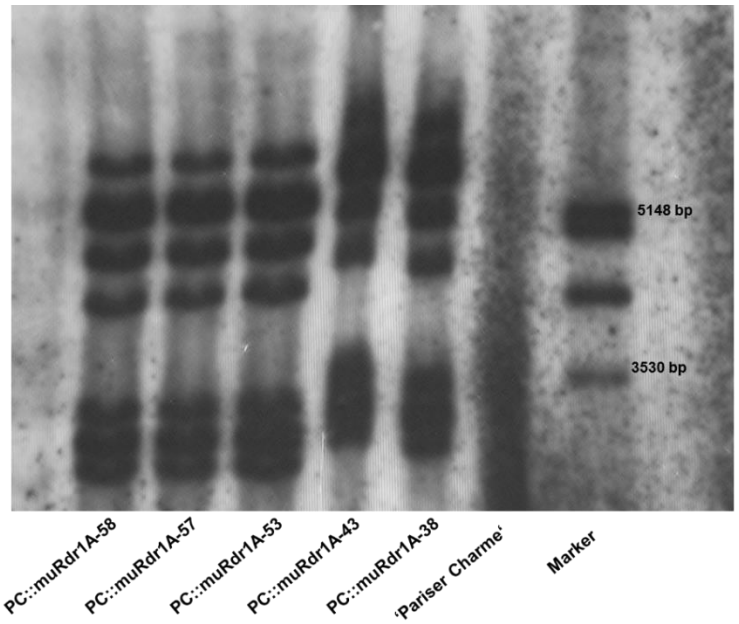

Figure S1: Southern blot analysis of *muRdr1A* transgenics. For Southern blot analysis, DNA from the non-transgenic 'Pariser Charme' and 'Pariser Charme' transformed with *muRdr1A* (PC::muRdr1A-38, -43, -53, -57, -58) was digested with *Hind*III and hybridized with an *NPT*II probe. All PC::muRdr1A transgenics show the same integration pattern with seven copies of *muRdr1A*. The patterns of PC::muRdr1A-38 and PC::muRdr1A- 43 are shifted in their position because of the high content of carbohydrates. M, DNA molecular weight marker.

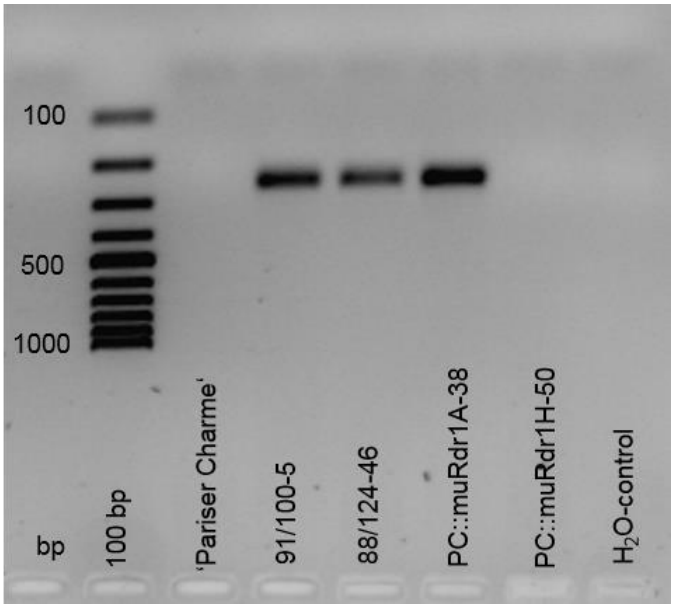

Figure S2: Results from muRdr1A-specific polymerase chain reaction (PCR). The susceptible genotype 'Pariser Charme', the *Rdr1* donors 88/124-46 and 91/100-5, and the transgenic clones harbouring *muRdr1A* (PC::muRdr1A-38) and *muRdr1H* (PC::muRdr1H-50) were used in a PCR with the specific primers 180-forward and 428-reverse. The products (248 bp) were separated on a 1.5% agarose gel. 100 bp, Gene Ruler™ 100-bp DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

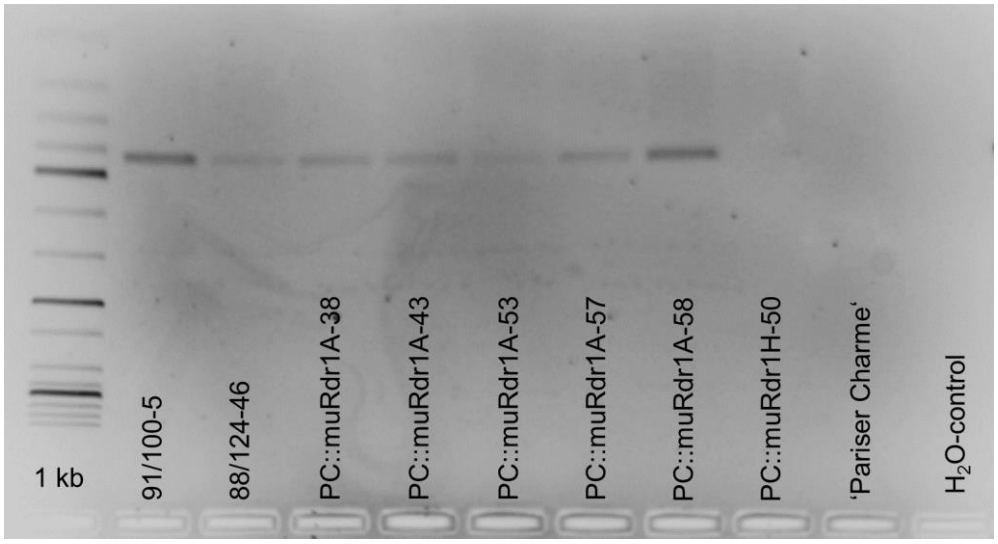

Figure S3: Results from *muRdr1A*-specific polymerase chain reaction (PCR). The cDNAs of the Rdr1 donors 88/124-46 and 91/100-5, the susceptible genotype 'Pariser Charme' and the transgenic clones harbouring *muRdr1A* (PC::muRdr1A-38, -43, -53, -57, -58) and *muRdr1H* (PC::muRdr1H-50) were used in a PCR with the specific primers 3071-forward and 3485-reverse. The products (414 bp) were separated on a 1.5% agarose gel. 1 kb, Gene Ruler™ 1-kb Plus DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

3 Analysis of the *Rdr1* gene family in different Rosaceae genomes reveals an origin of an *R*-gene cluster after the split of Rubeae within the Rosoideae subfamily

Ina Menz[1], Deepika Lakhwani[2], Jérémy Clotault[2], Marcus Linde[1], Fabrice Foucher[2], Thomas Debener[1]

[1] Institute for Plant Genetics, Leibniz University Hannover, Hannover, Germany
[2] IRHS, Agrocampus-Ouest, INRA, Université d'Angers, SFR 4207 QuaSaV, 49071 Beaucouzé, France

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Contribution to the article:	Wrote the manuscript, conducted most of the experiments, analysed the data and prepared all tables and figures.
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Abstract

The *Rdr1* gene confers resistance to black spot in roses and belongs to a large TNL gene family, which is organized in two major clusters at the distal end of chromosome 1. We used the recently available chromosome scale assemblies for the *R. chinensis* 'Old Blush' genome, re-sequencing data for nine rose species and genome data for *Fragaria, Rubus, Malus* and *Prunus* to identify *Rdr1* homologs from different taxa within Rosaceae.

Members of the *Rdr1* gene family are organized into two major clusters in *R. chinensis* and at a syntenic location in the *Fragaria* genome. Phylogenetic analysis indicates that the two clusters existed prior to the split of Rosa and Fragaria and that one cluster has a more recent origin than the other. Genes belonging to cluster 2, such as the functional *Rdr1* gene *muRdr1A*, were subject to a faster evolution than genes from cluster 1. As no *Rdr1* homologs were found in syntenic positions for *Prunus persica*, *Malus* x *domestica* and *Rubus occidentalis,* a translocation of the *Rdr1* clusters to the current positions probably happened after the Rubeae split from other groups within the Rosoideae approximately 70-80 million years ago during the Cretaceous period.

3.1 Introduction

Roses, together with species from the genera Fragaria, Rubus, Potentilla, and Malus, belong to the family Rosaceae and are therefore related to economically important fruit crops such as apple and peach [1, 2]. The genus Rosa, which includes approximately 200 species, shows a complex evolutionary history due to frequent hybridizations, multiple polyploidizations and recent radiation. The genus is subdivided into four subgenera (Hulthemia, Plathyrhodon, Hesperhodos and Rosa) by some authors, whereas others question the subgeneric status of Hulthemia and Plathyrhodon and propose to include them with the subgenus Rosa. The subgenus Rosa itself includes up to 10 sections and approximately 95 % of the species [1–5].

Until recently, only fragmented rose genomes were available [6,7]. Recently, two chromosome scale reference sequences for the diploid Rosa chinensis cultivar ‘Old Blush’ have been published [8,9]. The rose genome displays extensive synteny with the Fragaria vesca genome with only two major rearrangements [9]. Synteny between Fragaria and Rosa genes has been observed for TNL genes (TIR (Drosophila Toll and mammalian interleukin (IL)-1 receptors), NBS (nucleotide-binding site) and LRR (leucine- rich repeat)) [10].

NBS-LRR genes, which include TNLs and CNLs (CC (coiled-coil)-NBS-LRR), are the largest classes of R-genes in plants. They are characterized by three domains with different functions: the N-termini are thought to be involved in protein-protein interactions, the NBS domain is required for ATP (adenosine triphosphate) binding and hydrolysis, and the LRR-domain is involved in protein-protein interactions and ligand binding [11–14]. NBS-LRR genes have been detected in organisms from green algae to flowering plants and often occur in clusters of related paralogues or as single loci. The number of NBS coding genes in the genome varies widely between different species within the dicots and monocots. Whereas CNLs are found in both monocots and dicots, TNLs occur only in dicots [15]. Among the dicots, the Caricaceae (Carica papaya: 54) and Cucurbitaceae (Cucumis sativus: 59-71, C. melo: 80, C. lanatus: 45) families have very low numbers of NBS-encoding genes, whereas the number seems to be greater for some members of Rosaceae (198 NBS genes in F. vesca [16] and up to 1303 NBS genes in Malus x domestica [17,18]). The number of NBS-encoding genes also varies considerably within species, as shown for Oryza sativa lines (328-1120 NBS genes) or Gossypium herbaceum (268-1465 NBS genes) [19]. Different

evolutionary dynamics have been postulated, with some clusters comprising fast-evolving genes and others comprising slow-evolving genes [20,14].

In grapevine and poplar, the number of NBS-LRR genes in multi-gene clusters varies between 2 and 10 (mean 4.43) and between 2 and 23 (mean 5.33), respectively [21]. In Medicago truncatula approximately 50% of NBS domains occur in clusters of at least five genes; the largest cluster (14 genes) occurs on chromosome 6, with a sliding window size of 100 kb. The phylogenetic tree for the 333 non-redundant NBS-LRRs of M. truncatula showed that most groups were dominated by sequences from one chromosome and usually from one or a small number of genomic clusters [22]. Molecular characterization of the soybean Rsv3 resistance locus against multiple soybean mosaic virus strains revealed a cluster of seven highly homologous CNL genes intermixed with 16 other genes in the genotype Williams 82. All seven were also identified in the same order in the genotype Zaoshu 18. The five most likely resistance gene candidates (NBS_A-E) were also sequenced in ten additional soybean cultivars and showed very high sequence similarities [23].

In an R. multiflora hybrid (88/124-46), the single dominant TNL gene Rdr1 (muRdr1A), a member of a multigene family of at least nine highly similar clustered TNLs (muRdr1A-muRdr1I), confers broad-spectrum resistance against black spot [24,25]. The sizes of all TNLs for the Rdr1 locus, except muRdr1D (interrupted by 6957-bp transposable-element insertion within intron), range from 4085 to 5920 bp with sequence similarities between 78.0 % and 99.5 %. The domain structure of typical TNL proteins is reflected by the following intron-exon structure: the first exon contains the TIR domain, the second exon contains the NBS domain, and the fourth (or in case of TNL–muRdr1I, the third exon) contains an LRR domain [25].

A region from R. rugosa (subsection Cinnamomeae), homologous to the Rdr1 locus in R. multiflora (subsection Synstylae), was identified with a high degree of synteny that included some flanking non-TNL genes coding for a yellow stripe-like protein, ubiquitin and a TOPLESS-RELATED protein [10]. An analysis of 20 TIR-NBS-LRR (TNL) genes obtained from R. rugosa and R. multiflora revealed illegitimate recombination, gene conversion, unequal crossing over, indels, point mutations and transposable elements as mechanisms of diversification. Additionally, an orthologous locus in F. vesca (strawberry) was identified that contains a homologous TNL gene family and the flanking genes. In contrast, in Prunus persica (peach) and Malus x domestica (apple),

only the flanking genes can be detected in syntenic positions, and the genes homologous to the Rdr1 family are distributed on two different chromosomes. Phylogenetic analysis of TNL genes from five Rosaceae species showed that most of the genes occur in single species clades, indicating that recent TNL gene diversification began prior to the split of the Rosoideae (Rosa, Fragaria) from the Spiraeoideae (Malus, Prunus) [26,18].

With the availability of chromosome scale assemblies of the R. chinensis 'Old Blush' genome, we were interested in analysing the full complexity of the Rdr1 gene family at the genomic level and elucidating the dynamics of Rosaceae using data from different taxonomic levels, including re-sequencing data for nine rose species recently published along the with the 'Old Blush' genome sequences.

3.2 Results

3.2.1 *Rdr1* homologs in 'Old Blush' and *F. vesca*

The screening of the haploid genomes derived from 'Old Blush' for TNLs homologous to Rdr1 from R. multiflora resulted in seven complete TNLs for HapOB1 (OB1-A-G) and 21 for HapOB2 (OB2-A-U). A comparative analysis of TNLs from HapOB1 and HapOB2 showed that the following are identical: OB1-B and OB2-D, OB1-C and OB2-I and OB1-D and OB2-G. The sequences of all Rdr1 homologs are listed in Table S 3.3. Phylogenetic analysis of the TNLs from R. multiflora, HapOB1 and HapOB2 using the maximum likelihood method resulted in the tree shown in Figure S3.1. The phylogram shows that a group of three TNLs (OB1-G, OB2-T, OB2-U) are clearly separated from all other TNLs. OB1-G is located on chromosome 5, and OB2-T and OB2-U are located on chromosome 2. All other TNLs from HapOB1 and HapOB2 are located on chromosome 1 and are clustered in two distinct groups (1 and 2) that are highly supported by a bootstrap value of 100 %. TNLs from the R. multiflora Rdr1 cluster are clustered in group 2.

The genomic organization of HapOB1- and HapOB2-TNLs on chromosome 1 is shown in Figure 3.1. For HapOB2, all but three (OB2-A, -B, -I) of the 16 complete TNLs are organized in two clusters at the distal end of the chromosome. Cluster 1 (with a size of 76 kb) contains 37 protein-coding genes, of which 28 displayed significant similarities to entries in the GenBank database, including six complete TNL genes (OB1-C to OB1-H) and some truncated TNL genes (three TIR-domains, one LRR-domain and two NBS-LRR genes). Cluster 2 (with a size of 163 kb) contains 28 protein coding genes, of which 23 displayed significant similarities to entries in the GenBank database, including ten TNL genes (OB1-J to OB1-S) and one additional LRR-domain. TNLs from HapOB1 are also organized in two clusters at the distal end of chromosome 1. Gene prediction identified three TNLs (OB1-B through OB1-D) for cluster 1 and two TNLs (OB1-E and OB1-F) for cluster 2. Additionally, two TIR-domains, two NBS-LRR genes and one LRR-domain could be found within the cluster.

To determine the reasons for the unusually small number of *Rdr1*-TNLs at the two cluster positions in the HapOB1 genome, the Rd1LRR microsatellite marker from the LRR region of the gene family was analysed with DNA from haploid tissue that had been used for sequencing of the HapOB2 genome as well as DNA from the original diploid OB cultivar. Seven of the 19 genes of HapOB2 contained perfect primer binding sites and were detected on high resolution polyacrylamide gels, whereas 21 fragments were detected in DNA of the diploid OB (Figure S 3.2). The small number of *Rdr1* genes in the HapOB1 genome are likely to be an artefact, possibly due to a problem with the assembly; therefore, this sequence was not considered in further analyses.

The genomic organization of the TNLs on the chromosome in the two clusters corresponds to the two groups formed in the phylogenetic tree shown in Figure S 3.1. OB2-C through OB2-H are clustered in group 1, whereas OB2-J through OB2-S are clustered in group 2.

Analysis of the genes surrounding the clusters revealed a high level of synteny between HapOB1, HapOB2 and *F. vesca* (Table S 3.1).

The separation of the clusters in two different groups in the phylogenetic tree is further supported by a number of diagnostic sites in the derived amino acid sequences. At two positions (90 and 166), sequences of groups 1 and 2 display unique differences. At three additional positions (348, 688 and 868), one of the two groups displays unique amino acids that are replaced by two or more different sites in the other group.

In addition, the nucleotide diversity differs within each group. Though averages within the group total nucleotide differences are similar for both groups (327 for group 1 and 339 for group 2), the ratio of non-synonymous to synonymous sites is higher in group 2 (2.75) than in group 1 (1.81).

In addition to the TNLs from HapOB1 and HapOB2, the *F. vesca* genome was screened for *Rdr1* homologs. A total of 19 *Rdr1* homologs were found in *F. vesca*, of which 17 are located on chromosome 7 and two are located on chromosomes 1 and 2 (F.ve-19 and F.ve-18, respectively). The 17 TNLs on chromosome 7 are organized in clusters at the distal end of the chromosome: cluster 1 contains seven TNLs, and clusters 2 and 3 contain four TNLs (Figure 3.1). Phylogenetic analysis of the TNLs from HapOB1, HapOB2 and *F.* vesca shows that the rose genes for the two clusters from chromosome 1 are grouped with the *Fragaria* genes from the clusters on chromosome 7, whereas the genes located on other chromosomes are clearly separated from this group (Figure 3.2). Chromosome 1 of rose is syntenic with chromosome 7 of *Fragaria* [9]. Furthermore, the rose genes in cluster 2 form a group (group 2) with *Fragaria* genes in cluster 2 and 3, and each of them build a distinct single species clades within this group. In contrast, the genes from cluster 1 do not form strictly single species clades within group 1, but one clade with mixed species and two single species clades.

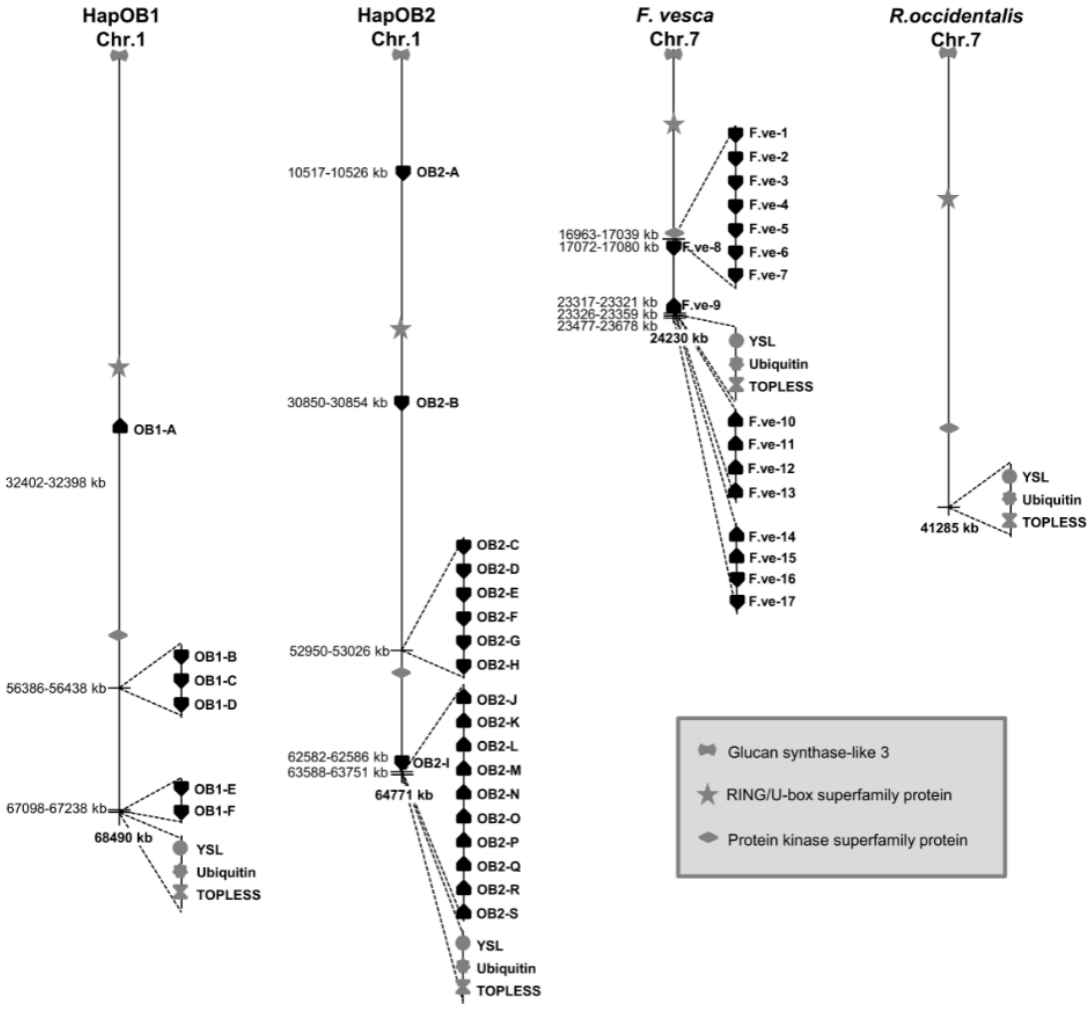

Figure 3.1: Genomic organization for *Rdr1* homologs in HapOB1, HapOB2, *Fragaria* and *Rubus*. Shown are: chromosome 1 of HapOB1 and HapOB2 with the upper cluster 1 (OB1-B through OB1-D; OB2-C through OB2-H) and the lower cluster 2 (OB1-E and OB1-F; OB2-J through OB2-S); chromosome 7 of *F. vesca* with cluster 1 (F.ve-1 through F.ve-8) and cluster 2 (F.ve-9 through F.ve-17); and chromosome 7 of *Rubus occidentalis (*no *Rdr1* homologs found). Positions of three syntenic genes (glucan synthase-like 3, RING/U-box superfamily protein, protein kinase superfamily protein) and the *Rdr1* flanking genes YSL (yellow-stripe-like protein), Ubiquitin and TOPLESS-RELATED protein are shown in grey.

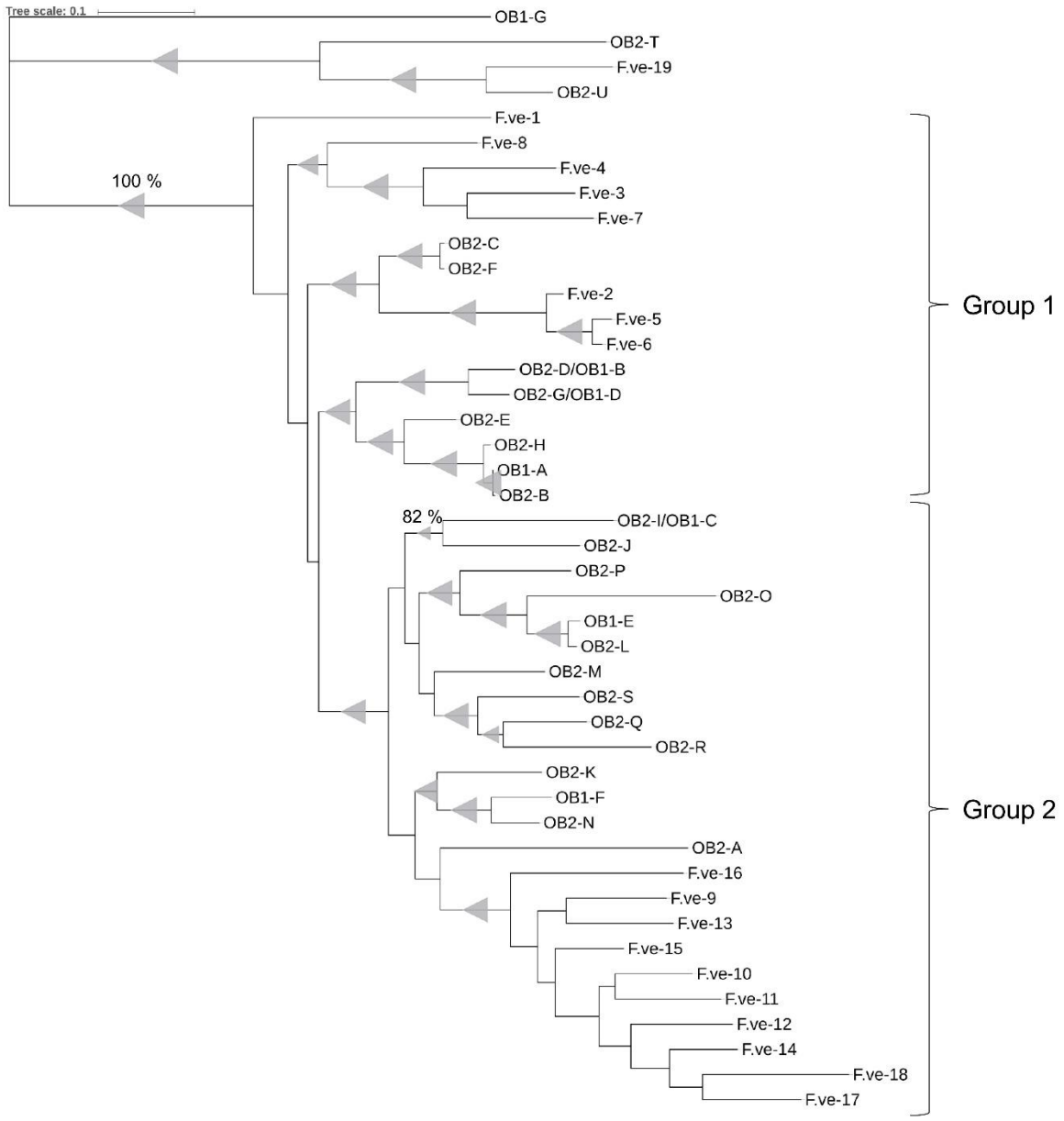

Figure 3.2: Phylogenetic analysis of the amino acid sequence of HapOB1-, HapOB2- and *F. vesca*-TNLs homologous to *Rdr1* in *R. multiflora*. The Maximum Likelihood method based on the JTT matrix-based model was used to calculate the phylogenetic tree. Test of phylogeny was performed using the bootstrap method with 500 replicates. Branches reproduced in less than 75 % of bootstrap replicates are collapsed. Bootstrap values are indicated as triangles, whereas the smallest value represents 82 % and the largest 100 %.

3.2.2 TNL structure in other Rosaceae

In a previous study [10], no *Rdr1* homologs could be observed in *P. persica* and *M. domestica* genomes at syntenic positions. Updated genome assemblies have been released since then, and these might have been corrected for assembly errors around repeat regions. We therefore analysed the genomes again for the presence of *Rdr1* homologs at syntenic positions. Rose chromosome 1 (where *Rdr1* is located) presents a good synteny with chromosome 2 in peach and chromosomes 1, 2 and 7 in apple [9]. Nevertheless, no homologous sequences for the *Rdr1* gene were found at these positions, confirming the previous results.

In addition, we also analysed syntenic positions in *Rubus occidentalis*, a species from the Rosoideae sub-family, for which a chromosome scale assembly recently became available [27]. Synteny analysis of the genes surrounding the TNL clusters revealed no *Rdr1* homologs in syntenic positions for *P. persica*, *M.* x *domestica* and *R. occidentalis* (Table S 3.1). In *Prunus* and *Malus*, more distantly related *Rdr1* homologs were only detected in non-syntenic positions (Figure S 3.3), whereas in a draft genome from *Potentilla micrantha,* another species from the Rosoideae, several contigs contained *Rdr1* homologs. The genes P.mi-12 and -13 are located on contig 1260 together with genes coding for a yellow stripe-like protein, ubiquitin and a TOPLESS-RELATED protein flanking the *Rdr1* locus in *R. multiflora* and *R. rugosa*, indicating that *Rdr1* homologs are present at syntenic positions in *P. micrantha.* Analysis for *Rdr1* homologs identified 19 for *F. vesca,* three for *R. occidentalis*, 10 for *Malus* x *domestica*, 17 for *P. persica* and 11 for *P. micrantha* (Table S 3.2).

Phylogenetic analysis showed that the non-syntenic *Rdr1* homologs from *P. persica*, *M.* x *domestica* and *R. occidentalis* are clearly separated from *Rdr1* homologs of OB and *F. vesca*, which are located on chromosome 1 (OB) and 7 (*F. vesca*) (Figure 3.3). Furthermore, some of the *P. micrantha Rdr1* homologs are grouped together with the TNLs from OB and *F. vesca*, which are located on chromosome 1 (OB) and 7 (*F. vesca*) consistent with clusters of these genes in syntenic positions for the *Rdr1* clusters.

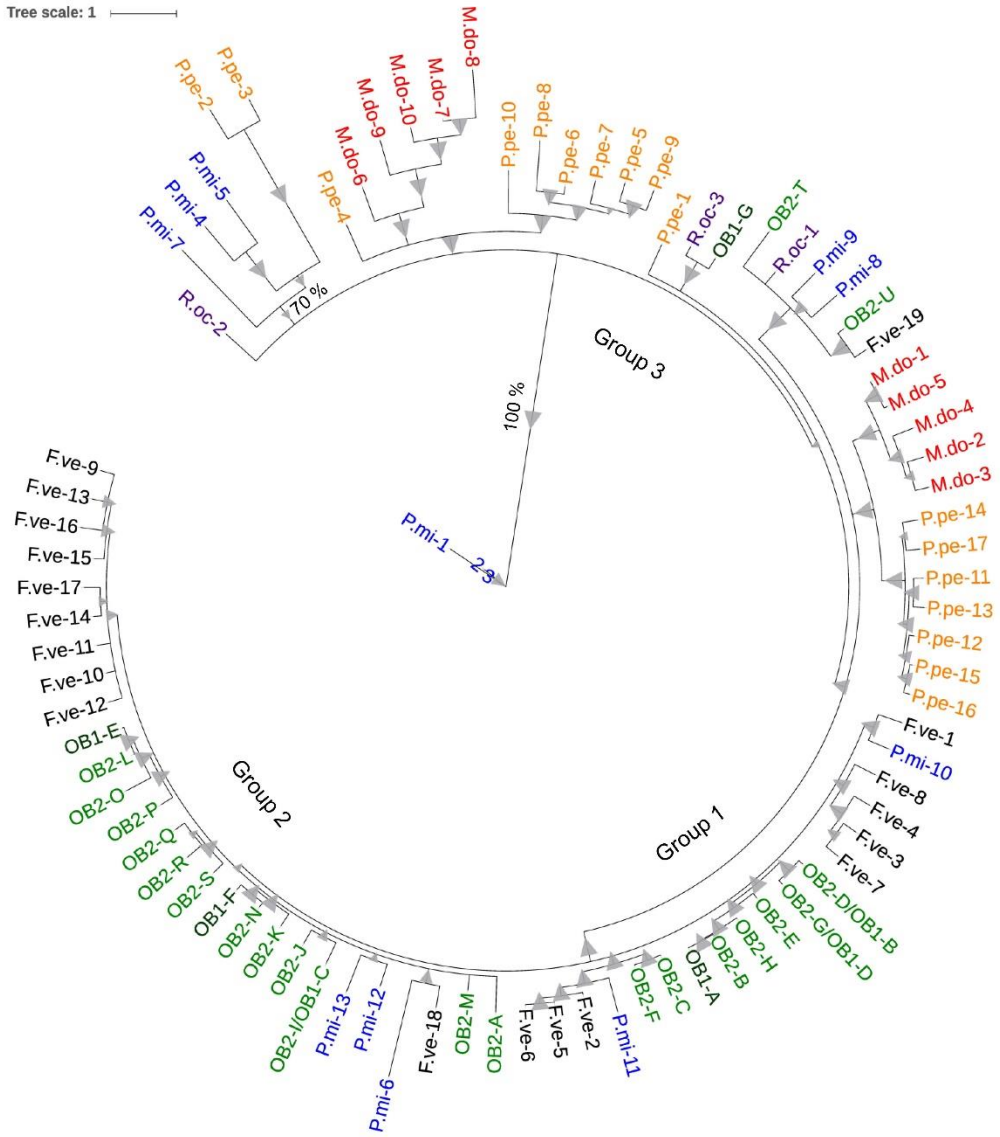

Figure 3.3. Phylogenetic analysis of the amino acid sequence of TNLs from different Rosaceae family members homologous to *Rdr1* of R. multiflora. The Maximum Likelihood method based on the JTT matrix-based model was used to calculate the phylogenetic tree. Test of phylogeny was performed using the bootstrap method with 500 replicates. Branches reproduced in less than 60 % of bootstrap replicates are collapsed. Bootstrap values are indicated as triangles, whereas the smallest value represents 70 %, and the largest value represents 100 %. For a better visualization, *Rdr1* homologs for the different Rosaceae family members are coloured as follows: HapOB1/2 (OB1+2: dark green), *M. domestica* (M.do: red), *F. vesca* (F.ve: black), *P. persica* (P.pe: orange), P. micrantha (dark blue), R. occidentalis (purple). The protein alignments are shown in Table S 3.4.

3.2.3 *Rdr1* homologs from other rose species

Analysis of seven additional recently available genome sequences [8] identified 15 *Rdr1* homologs for *R. damascena,* three for *R. persica*, eight for *R. moschata*, 13 for *R. xanthina spontanea*, 13 for *R. chinensis var. spontanea,* nine for *R. laevigata* and 12 for *R. minutifolia alba* (Table 3.1). Until recently, only highly fragmented genomes have been available for these rose species, which makes a chromosomal classification for TNLs homologs in *Rdr1* difficult.

Based on the observation that *Fragaria Rdr1* homologs from syntenic clusters form phylogenetic groups with rose homologs for *Rdr1,* we computed a phylogenetic tree to identify homologs from other rose species (Figure 3.4). For *R. multiflora* and *R. rugosa* TNLs already obtained by [25] were used. The most conspicuous group (group 3), with high bootstrap support, contains single TNLs from HapOB1/2 and *Fragaria* located on different chromosomes outside the two syntenic clusters. They are grouped together with two *R. chinensis*, two *R. minutifolia,* two *R. moschata* genes and one *R. xanthina* gene, which also most likely represent genes from outside the syntenic clusters. All *Rdr1* homologs of HapOB, *R. multiflora* [25], *R. rugosa* [10] and *Fragaria,* known to derive from cluster 2, fall into one highly supported large group (group 2) that also includes sequences from all other rose species.

Within group 2, *Rdr1* homologs from *Fragaria* form a distinct sub-group, whereas most of the other rose sequences form mixed sub-groups with no clear single species clades. In contrast, sequences clustered in group 1 do not form genus-specific sub-groups, but *Fragaria* and rose sequences form mixed sub-groups.

Table 3.1: List of *Rdr1* homologs found in different rose species.

Species	Abbreviation	TNLs
***R. multiflora* (from[25])**	R.mu (A-I)	9
***R. rugosa* (from [10])**	R.ru (A-K)	11
HapOB1	OB1 (A-G)	7
HapOB2	OB2 (A-U)	21
Rosa damascena	R.da (1-15)	15
Rosa persica	R.pe (1-3)	3
Rosa moschata	R.mo (1-8)	8
Rosa xanthina spontanea	R.xa (1-13)	13
Rosa chinensis* var. *spontanea	R.ch (1-13)	13
Rosa laevigata	R.la (1-9)	9
Rosa minutifolia alba	R.mi (1-12)	12

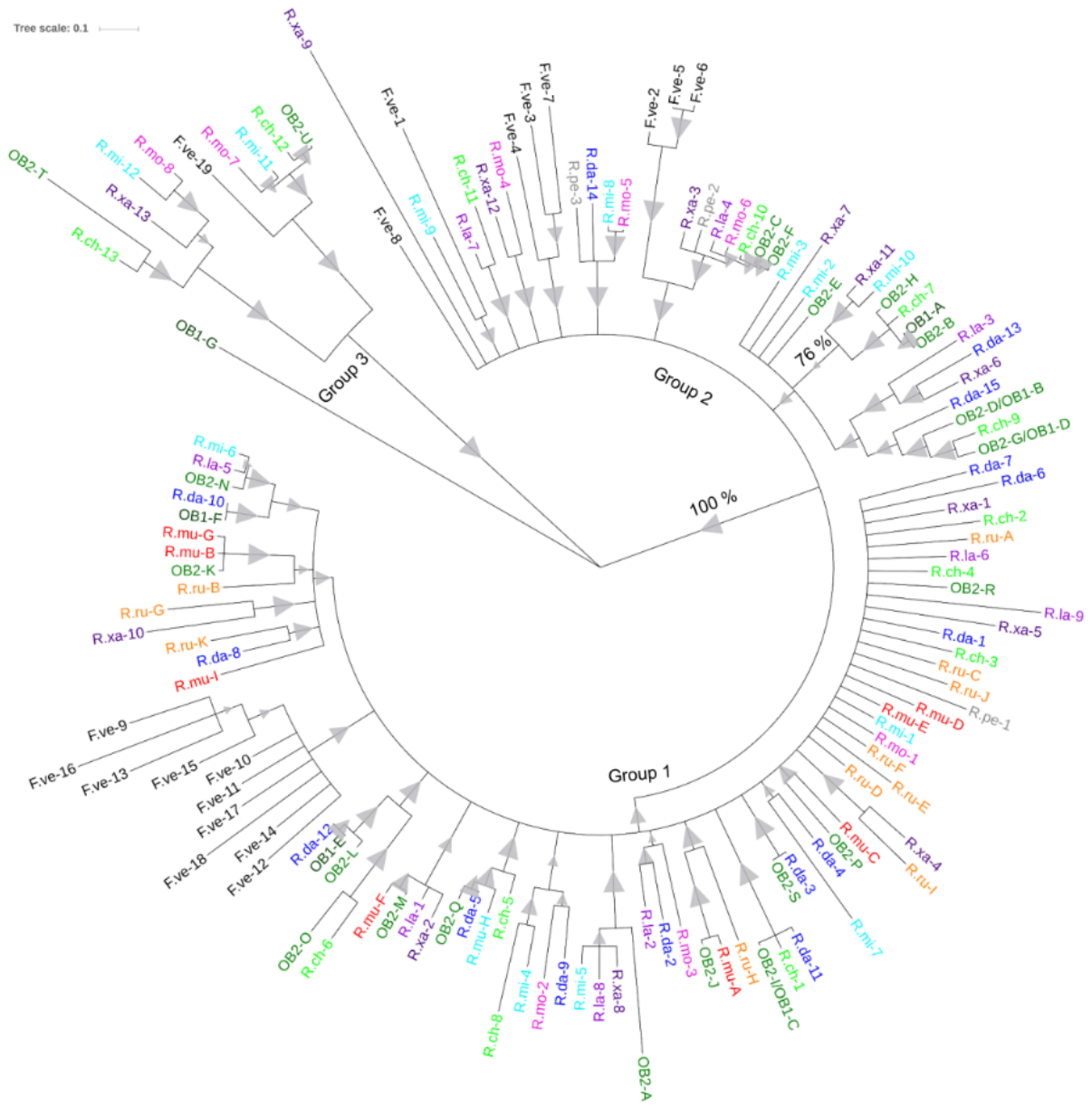

Figure 3.4: Phylogenetic analysis of the amino acid sequence of *Rdr1* homologs from different rose species and *Fragaria*. The Maximum Likelihood method based on the JTT matrix-based model was used to calculate the phylogenetic tree. Test of phylogeny was performed using the bootstrap method with 500 replicates. Branches reproduced in less than 75 % of bootstrap replicates are collapsed. Bootstrap values are indicated as triangles, whereas the smallest value represents 76 %, and the largest value represents 100 %. For better visualization, *Rdr1* homologs for the different species are coloured as follows: HapOB1/2 (OB1/2: dark green), *R. multiflora* (R.mu: red), *F. vesca* (F.ve: black), *R. rugosa* (R.ru: orange), *R. damascena* (R.da: dark blue), *R. persica* (P.pe: grey), *R. moschata* (R.mo: pink), *R. xanthina* (R.xa: dark purple), *R. chinensis* (R.ch: neon green), *R. laevigata* (R.la: purple), *R. minutifolia* (R.mi: light blue). The protein alignments are shown in Table S 3.5.

3.3 Discussion

More than 50 % of the NBS-encoding genes are organized in clusters in the genome for many species such as *Arabidopsis* (64-71 %), rice (50-74 %), potato (73 %), *Medicago* (80 %) and apple (80 %) [17,28]. Furthermore, these clusters are not evenly distributed between chromosomal positions. In *Medicago truncatula*, chromosome 6 contains approximately 34 % of all TNLs, and chromosome 3 harbours approximately 40 % of all CNLs [22]. In apple, approximately 56 % of all identified RGAs are located on six of the 17 chromosomes, with 25 % on chromosome 2 alone; whereas in grapevine, 80 % of TNLs were located on chromosomes 5, 12 and 18 [28,21]. In tomato, the majority of NBS-LRRs are located close to the telomeres, where recombination occurs frequently, while few were detected in regions called “cold spots” for recombination [29]. An accumulation of RGAs in sub-telomeric regions was also described for apple [28].

Previously, we characterized members of the *Rdr1* gene family, among which the *Rdr1* gene confers resistance to black spot [24,25] and forms a cluster of closely related genes. As no complete genome was available at that time, our analyses were constricted to the region captured by BAC contigs and previous versions of the *Fragaria* genome (and others). This research used the high-quality chromosome-scale assembly of the OB genome to analyse the structure of this gene family in more detail. Recently, two high-quality sequences at the chromosome scale from two independent haploids from the same cultivar ‘Old Blush’ were obtained [8,9]. However, even a high-quality assembly might contain assembly errors around regions of highly similar paralogues for large gene families. Evidence for this is provided by our analysis of the HapOB1 genome [8], which only predicts seven *Rdr1* paralogues at the chromosome 1 positions in contrast to the situation in the HapOB2 genome [9], where 21 TNLs were annotated. Our access to source DNA was restricted to the original ‘Old Blush’ diploid genotype and the haploid material used to generate the HapOB2 genome; therefore, we can only state that the total number of amplified copies of the *Rdr1* paralogues from the original diploid is twice as high as that from the HapOB2 genome (Figure S 3.2). Thus, the HapOB1 genome is unlikely to contain only seven paralogues; rather, assembly errors likely led to this small number. However, this remains unclear because only a fraction of the *Rdr1* paralogues can be amplified with our primer combination and we do not have access to the HapOB1 DNA.

Our analysis shows that two major clusters and two single genes are located on chromosome 1 and further relatives are located on chromosome 2 of OB. Phylogenetic analysis shows that the two major clusters form different groups, which indicates an independent development of the two clusters. Related sequences found on chromosome 2 are clearly distinct from those on chromosome 1 and are therefore not treated as members of the same family.

Re-analysis of the *Fragaria* genome reveals a similar structure with TNL clusters at syntenic positions. A phylogram of complete *Rdr1* sequences for the *Fragaria* and OB genomes show that *Fragaria* group 2 and rose group 2 are closer to each other than to *Fragaria* group 1 and rose group 1. Furthermore, genes from group 1 only form mixed groups with single species clades, whereas the genes from group 2 form single species clades. As both clusters were present before the taxa emerged, the likely cause is a faster evolution within group 2. This could be due to the known processes by which R-genes evolve (including higher rates of recombination, gene conversion and birth and death processes), which led to a concerted evolution of genes in group 1. A similar observation has been made for inbred lines of maize, in which some paralogues are organized in genotype-specific subgroups [30].

A re-analysis of the latest versions of the apple and peach genomes confirmed earlier results [10] that there are no *Rdr1*-like TNL clusters at syntenic positions in these genomes. The former conclusion remains that the emergence of the *Rdr1* clusters must have formed after the Amygdaloideae split from the Rosoideae. A high-quality genome of *R. occidentalis* recently became available; therefore, we also checked for the presence of our cluster in *Rubus*, which was not present at a syntenic position.

Genome information for *P. micrantha*, identifies a larger number of fragments, which shows that there are 5 contigs with *Rdr1* homologs.

One of these contigs (contig no. 1260) contains two *Rdr1* homologs and conserved genes flanking group 1 in roses [10]. This indicates that *Rdr1* homologs in *Potentilla* are in a putative syntenic position to the group 1 cluster in roses.

The other genes fall into groups of OB sequences that are in both clusters as well as on chromosome 2 in roses. This agrees with the Rosaceae phylogeny which places *Potentilla* and *Fragaria* into sister groups of the Potentilleae within the Rosoideae. The timeline for the evolution of the Rosaceae [26] led us to conclude that the *Rdr1* cluster

was translocated to its current position after the Rubeae split from other groups within the Rosoideae approximately 70-80 million years ago during the Cretaceous period.
A larger phylogram, including 137 sequences from ten species of *Rosa*, shows that all rose sequences form mixed clusters with few exceptions. Therefore, single species clades for the rose genes within group 1 have not been developed yet. Not all rose species can be easily differentiated taxonomically, and most are highly interfertile; this underlines a close relationship between these taxa and may be one reason for the lack of differentiation of group 1 genes.
This study is a first step in the analysis of the evolution of genes from the *Rdr1* family in roses. However, we must keep in mind that assembly processing for clustered duplicated genes can lead to assembly errors. We can then hypothesize that some genes which were studied could represent consensus sequences for several real existing genes. As shown with HapOB2, an assembly obtained from long reads should result in a high-quality chromosome scale assembly for these regions. However, the lower than expected number of *Rdr1* homologs in the HapOB1 assembly, developed from PacBio reads, shows that this is only a general principle.

3.4 Material and methods

3.4.1 Origin of sequences

For *R. multiflora* (HQ455834.1) and *R. rugosa* (JQ791545), previously published contigs spanning the *Rdr1* locus were used [10,25]. The genomes of ‘Old Blush’, HapOB1 [8] and *R. damascena* Mill. were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/), whereas the haploid genome of ‘Old Blush’, HapOB2 [9] was downloaded from a genome browser (https://iris.angers.inra.fr/obh/). The whole genomes of *F. vesca*, *Malus x domestica*, *P. persica*, *R. occidentalis* and *P. micrantha* were downloaded from the Genome Database for Rosaceae (https://www.rosaceae.org/).
Additionally, sequences of the rose species *R. persica*, *R. moschata*, *R. xanthina spontanea, R. chinensis* var. *spontanea*, *R. laevigata* and *R. minutifolia alba* were used ([9], assemblies unpublished). The origins of all used sequences are listed in Table 3.2.

Table 3.2: Origin of sequences used in this study.

Species	Reference	Information
R. multiflora	[25]	HQ455834.1
R. rugosa	[10]	JQ791545
HapOB1	[8]	NC_037088.1-NC_037094.1
HapOB2	[9]	PRJNA445774
R. damascena	Unpublished	LYNE00000000.1
F. vesca	[31]	v4.0.a1
M. x domestica	[32]	GDDH13 v1.1
P. persica	[33,34]	v2.0.a1
R. occidentalis	[27]	v3.0
P. micrantha	[35]	v1.0
R. persica	[9]	SRP143586
R. moschata	[9]	SRP143586
R. xanthina spontanea	[9]	SRP143586
R. chinensis* var. *spontanea	[9]	SRP143586
R. laevigata	[9]	SRP143586
R. minutifolia alba	[9]	SRP143586

3.4.2 Analysis of the Rd1LRR microsatellite marker in ‘Old Blush’

The *Rdr1*-TNLs in the ‘Old Blush’ genome were amplified from DNA for the haploid tissue that had been used for sequencing the HapOB2 genome as well as from DNA of the original diploid OB cultivar using the Rd1LRR microsatellite marker, presented in the coding sequences for the NBS-LRR members, and analysed on a LiCor 4300 DNA-analyser as previously described [3].

3.4.3 Gene prediction and annotation

Regions homologous to the *Rdr1* locus were identified for all species using local BLAST searches implemented in Bioedit [36]. The BLASTn method was conducted with the *muRdr1A*-sequence as a query and an E-value of 1.0E-20.

Gene prediction and annotation was performed using FGENESH and AUGUSTUS (http://www.softberry.com; http://augustus.gobics.de/). The protein domains were determined using PfamScan ([37], https://www.ebi.ac.uk/Tools/pfa/pfamscan/). Only genes with a size larger than 2 kb and coding for all three protein domains (TIR, NB-ARC, LRR) were used for further phylogenetic analyses.

3.4.4 Sequence alignment and construction of phylogenetic trees

The predicted amino acid sequences of the *Rdr1* homologs of *R. multiflora, R. rugosa*, *F. vesca*, HapOB1, HapOB2, *R. damascena*, *R. chinensis* var. *spontanea*, *R. laevigata*, *R. minutifolia alba, R. persica, R. moschata* and *R. xanthina spontanea* were aligned in MEGAX using MUSCLE (Multiple sequence comparison by log- expectation, [38]) with default options.

For the aligned *Rdr1* homologs from the different species, phylogenetic trees were constructed in MEGAX [39] using the maximum likelihood (ML) method with the Jones-Taylor-Thornton matrix-based model using a discrete gamma distribution with empirical frequencies (JTT+G+F) [40]. The best model was estimated using MEGAX. Initial trees for the heuristic search were obtained automatically. The tree topology was tested via a bootstrap analysis with 500 replicates. For a better visualization of the phylogenetic trees the software Tree Of Life (iTOL) version 4.2.3 [41] (https://itol.embl.de/) was used. Nucleotide diversity within groups of sequences was computed in MEGAX using nucleotide differences among aligned sequences.

The analysis of synonymous and non-synonymous sites was performed in MEGAX by aligning the amino acid sequences of sets of coding DNA-sequences and analysing the DNA differences with the Nei-Gobojori model [42] for 1314 positions in the final dataset.

3.4.5 Synteny analysis

For the synteny analysis of the two clusters, genes surrounding the clusters were selected based on the rose reference sequence [9]. Reciprocal BLAST were performed against the most recent available Rosaceae genomes: *Fragaria vesca* [31], *Prunus persica* [34], *Malus domestica* [32] and *Rubus occidentalis* [43]. The order of the homologous genes was checked on the genome browser of the GDR website (https://www.rosaceae.org/tools/jbrowse, [44]).

3.5 Acknowledgements

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3.7 Supplementary information

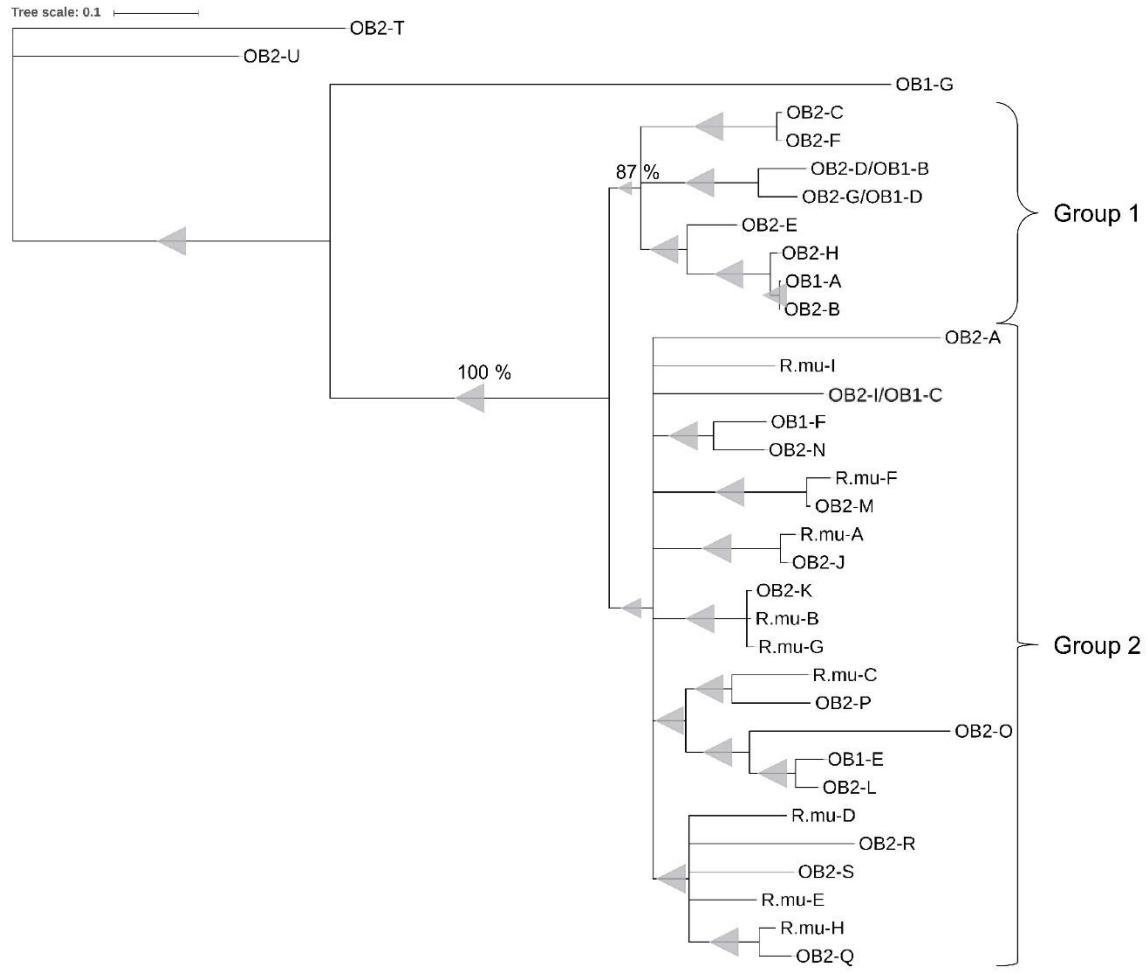
Table S 3.1.xlsx: Results of micro-synteny analysis outside the *Rdr1* family clusters.

Table S 3.2.docx: Positions and annotation of TNLs homologous to *Rdr1* on the different chromosomes of Old Blush (OB1+2), *F. vesca* (F.ve), *Prunus persica* (P.pe), *Malus domestica* (M.do), *Rubus occidentalis* (R.oc) and *Potentilla micrantha* (P.mi).

Table S 3.3.txt: Coding sequences of all used genes in this study.

Table S 3.4.txt: Muscle alignment of protein sequences used for the phylogram shown in Figure 3.3.

Table S 3.5.txt: Muscle alignment of protein sequences used for the phylogram shown in Figure 3.4.



FigureS 3.1: Phylogenetic analysis of the amino acid sequence for R. multiflora Rdr1-TNLs and homologous TNLs of HapOB1 and HapOB2. The maximum likelihood method based on the JTT matrix-based model was used to calculate the phylogenetic tree. A test of phylogeny was performed using the bootstrap method with 500 replicates. Branches reproduced in less than 75 % of bootstrap replicates are collapsed. Bootstrap values are indicated as triangles, whereas the smallest value represents 87 % and the largest 100 %.

Figure S 3.2: Results from Rd1LRR microsatellite PCR. DNA from haploid tissue that had been used for sequencing the OB2 genome as well as DNA of the original diploid OB cultivar was used in a PCR with Rd1LRR microsatellite primers (Terefe and Debener, 2011). PCR products were separated on a 6 % polyacrylamide gel.

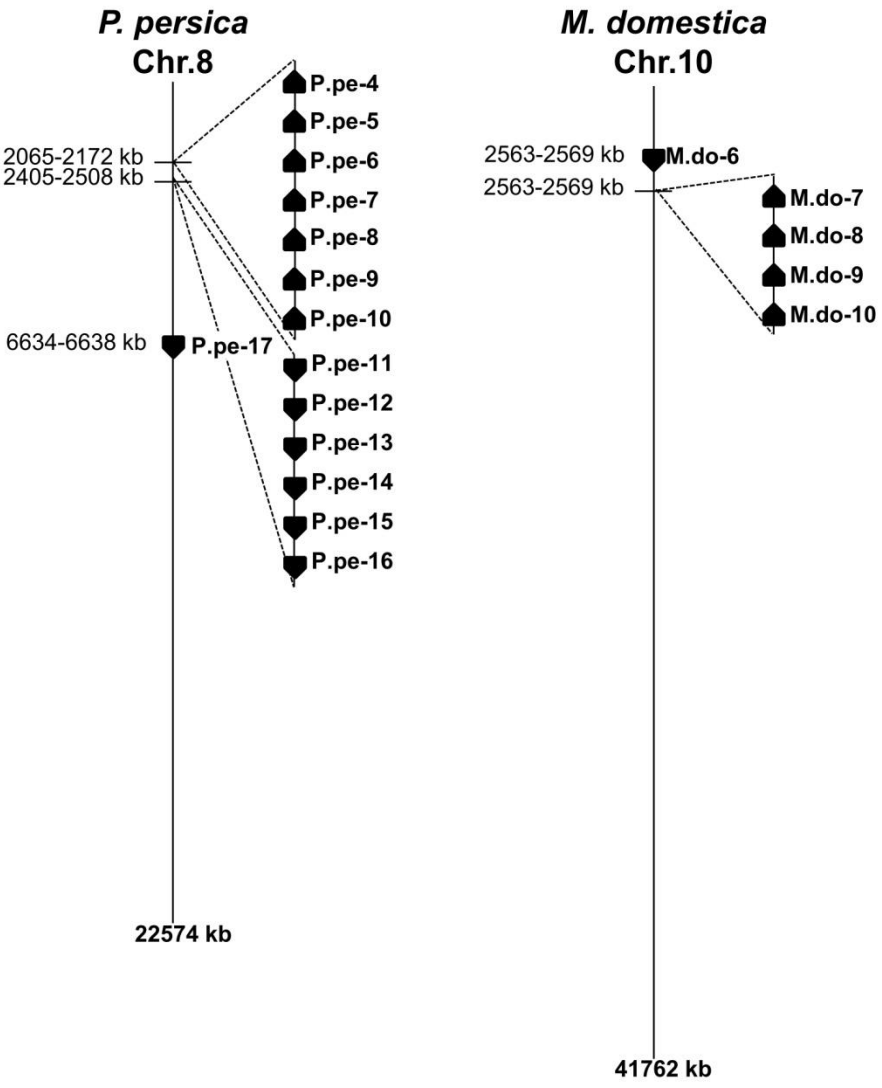


Figure S 3.3: Genomic organizations of TNLs homologous to *Rdr1*. Shown are chromosome 8 of *Prunus persica* and chromosome 10 of *Malus* x *domestica*.

4 Interaction of roses with a biotrophic and a hemibiotrophic leaf pathogen leads to differences in defense transcriptome activation

Enzo Neu[1, 2], Helena Sophia Domes[1], Ina Menz[1], Helgard Kaufmann[1], Marcus Linde[1], Thomas Debener[1]

[1]Department of Molecular Plant Breeding, Institute for Plant Genetics, Leibniz Universität Hannover, Hannover 30419, Germany
[2] KWS SAAT SE, 37574 Einbeck, Germany

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Type of article:	Research article
Contribution to the article:	Contributed substantial in performing the experiments to validate the transcriptomic data via qPCR, in analysing and interpretation of the transcriptomic data. Prepared some tables and figures, wrote parts of the manuscript and contributed in revision of the manuscript.
Contribution of the other authors:	Enzo Neu planned and performed inoculation experiments with *D. rosae* and *P. pannosa,* was involved in performing the experiments to validate the transcriptomic data via qPCR, analysed the MACE-data. Wrote majority parts of the manuscript and prepared the majority of tables and figures. Helena Sophia Domes contriburted substantial in performing the experiments to validate the transcriptomic data via qPCR, in analysing and interpretation of the transcriptomic data. Prepared some tables and figures, wrote parts of the manuscript. Helgard Kaufmann generated the samples for MACE-analysis, which were inoculated with *P. pannosa*. Thomas Debener contributed in planning of the experimental design, data analysis and writing of the manuscript. All authors contributed in revision of the manuscript.
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Interaction of roses with a biotrophic and a hemibiotrophic leaf pathogen leads to differences in defense transcriptome activation

Enzo Neu[1,2] · Helena Sophia Domes[1] · Ina Menz[1] · Helgard Kaufmann[1] · Marcus Linde[1] · Thomas Debener[1]

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Abstract

Key message **Transcriptomic analysis resulted in the upregulation of the genes related to common defense mechanisms for black spot and the downregulation of the genes related to photosynthesis and cell wall modification for powdery mildew.**

Abstract Plant pathogenic fungi successfully colonize their hosts by manipulating the host defense mechanisms, which is accompanied by major transcriptome changes in the host. To characterize compatible plant pathogen interactions at early stages of infection by the obligate biotrophic fungus *Podosphaera pannosa*, which causes powdery mildew, and the hemibiotrophic fungus *Diplocarpon rosae*, which causes black spot, we analyzed changes in the leaf transcriptome after the inoculation of detached rose leaves with each pathogen. In addition, we analyzed differences in the transcriptomic changes inflicted by both pathogens as a first step to characterize specific infection strategies. Transcriptomic changes were analyzed using next-generation sequencing based on the massive analysis of cDNA ends approach, which was validated using high-throughput qPCR. We identified a large number of differentially regulated genes. A common set of the differentially regulated genes comprised of pathogenesis-related (PR) genes, such as of PR10 homologs, chitinases and defense-related transcription factors, such as various WRKY genes, indicating a conserved but insufficient PTI [pathogen associated molecular pattern (PAMP) triggered immunity] reaction. Surprisingly, most of the differentially regulated genes were specific to the interactions with either *P. pannosa* or *D. rosae*. Specific regulation in response to *D. rosae* was detected for genes from the phenylpropanoid and flavonoid pathways and for individual PR genes, such as paralogs of PR1 and PR5, and other factors of the salicylic acid signaling pathway. Differently, inoculation with *P. pannosa* leads in addition to the general pathogen response to a downregulation of genes related to photosynthesis and cell wall modification.

Keywords Black spot · Powdery mildew · MACE analysis · High-throughput qPCR · WRKY genes · PR genes

Introduction

Fungal pathogens have developed specific strategies to obtain nutrients from their hosts depending on their lifestyle. Biotrophic fungi develop nutrient-absorbing structures, such as haustoria, to establish a long-term feeding relationship without killing their hosts. In contrast, necrotrophic fungi kill the host cells, often by secreting toxins, to feed on the dead tissue, while hemibiotrophic pathogens often change their lifestyle during development from an early biotrophic to a later necrotrophic stage (Horbach et al. 2011).

In their natural environment, roses are confronted with many different pathogens. The biotrophic fungus *Podosphaera pannosa*, which causes powdery mildew, and the hemibiotrophic *Diplocarpon rosae*, which causes black spot, are the most common and damaging pathogens in

Enzo Neu and Helena Sophia Domes have contributed equally to this work.

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✉ Thomas Debener
debener@genetik.uni-hannover.de

[1] Department of Molecular Plant Breeding, Institute for Plant Genetics, Leibniz Universität Hannover, 30419 Hannover, Germany

[2] KWS SAAT SE, 37574 Einbeck, Germany

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roses cultivated in the greenhouse and the field, respectively (Debener and Byrne 2014).

The life cycle of *D. rosae* begins on the surface of rose leaves with the germination of its conidia within 8–9 h after infection, and 3 h later, appressoria may already have formed. After an additional 3 h, subcuticular hyphae and the first haustoria may be developed (Aronescu 1934). In this biotrophic phase, all the fungal structures in the host cells are surrounded by the plasma membrane of the host. The resulting circular black spots that are typical infection symptoms are mostly surrounded by living host cells, so-called "green islands" (Gachomo et al. 2006). Few details about the infection process of *P. pannosa* in roses are available. Germ tubes may be observed 2–6 hpi (hours post inoculation), rapidly developing into mycelia that grow on the leaf surface. After approximately 1 dpi, multilobed haustoria develop under the appressoria, which are formed in regular distances (Linde and Shishkoff 2003). Under favorable conditions, asexually produced spores are released after 3–5 days, completing the life cycle on the living host tissue (Coyier 1983).

The recognition of a pathogen can either be mediated by pattern-recognition-receptors in the cell membrane, which recognize highly conserved pathogen- or microbe-associated molecular patterns (PAMP or MAMP) on the surface of a pathogen, e.g., fungal chitin or bacterial flagellin, or by R proteins that recognize the effectors secreted from the pathogen into the host cell. These two mechanisms are called PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively, and the latter is often accompanied by a so-called "hypersensitive response" (Bent and Mackey 2007; Jones and Dangl 2006).

The recognition of a pathogen by the plant cells leads to drastic changes in the transcriptome and activates a diverse set of immune responses, including the synthesis of secondary metabolites, cell wall modifications and the expression of pathogenesis-related (PR) genes (Slusarenko et al. 2000). In addition to common immune responses in both, compatible and incompatible interactions, the responses to fungal infections may involve specific reactions depending on the life style of the infecting fungus (González et al. 2013; Silvia Sebastiani et al. 2017).

Plant hormones play an important role as signaling molecules in plant defense. Salicylic acid (SA) is involved in the defense response to biotrophic and hemibiotrophic pathogens, while jasmonate (JA) and ethylene (ET) are the primary hormones involved in the response to necrotrophic pathogens. Both pathways have often been described as antagonistic. In addition, crosstalk with other phytohormones can modulate the responses to pathogens/stress (Derksen et al. 2013). In the compatible interaction between *Erysiphe necator* and *Vitis vinifera*, significant alterations in the host transcriptome were induced, essentially genes involved in signaling and secondary metabolite biosynthetic pathways (Borges et al. 2013). The analysis of the compatible interaction between *Malus*×*domestica* Borkh. and *Alternaria alternata* showed that genes associated with photosynthesis and oxidation–reduction were downregulated, while transcription factors (i.e., WRKY, MYB, NAC, and Hsf) and genes involved in cell wall modification, defense signaling, the synthesis of defense-related metabolites, including pathogenesis-related (PRs) genes and phenylpropanoid/cyanoamino acid/flavonoid biosynthesis, were activated (Zhu et al. 2017).

PR-proteins are pathogen or stress-related induced plant proteins, which are classified into 17 families based on their structural or functional similarities (van Loon et al. 2006). PR1 is one of the primary marker genes for the SA-mediated defense response, but its overall function is not yet clear. The PR2 family (endo-1,3-β-D-glucanase) and the PR5 family (thaumatin-like) are also associated with this pathway, while the other PR-protein families, such as PR4 (hevein-like protein), PR6 (proteinase inhibitor) or PR9 (peroxidase) are more closely associated with the JA-ET pathway (van Loon et al. 2006; Vidhyasekaran 2015; Derksen et al. 2013).

Among the secondary metabolites involved in plant pathogen interactions, flavonoids are widely distributed with different biological functions, such as protecting against harmful radiation and phytopathogens, binding phytotoxins and controlling auxin transport. They are synthesized through the phenylpropanoid pathway, which transforms phenylalanine into 4-coumaroyl-CoA, which finally enters the flavonoid biosynthesis pathway (Falcone Ferreyra et al. 2012; Winkel-Shirley 2002). A transcriptome analysis of *Fragaria vesca* infected with either *Colletotrichum fragariae* or *Phytophtora cactorum* showed the induction of several genes of the flavonoid biosynthetic pathway (Guidarelli et al. 2011; Toljamo et al. 2016).

In this study, we analyzed differences in the manipulation of the leaf transcriptome of roses after successful colonization of the leaves with either the hemibiotrophic fungus *D. rosae* or the biotrophic fungus *P. pannosa*.

The goal of these analyses is to gain initial insights into the changes that the two pathogens inflict on the host transcriptomes as the first step to understand host–pathogen interactions in the two systems. For this, we analyzed the transcriptomic responses against the two pathogens in the early stages of infection (0 hpi, 24 hpi and 72 hpi) using the massive analysis of cDNA ends (MACE) data from three independent inoculation experiments.

Materials and methods

Plant material

For the MACE analysis and the multiplication of the fungal isolates, the susceptible rose cultivar 'Pariser Charme' (PC)

was propagated in vitro as previously described (Davies 1980; Debener et al. 1998; Linde and Debener 2003; Dohm et al. 2001), rooted, and transplanted into fertilized substrate ("Einheitserde T", Einheitserdewerke Gebr. Patzer, Sinntal-Altengronau, Germany) in 9-cm pots and cultivated under semicontrolled conditions (12 h light/12 h darkness). The plants used to validate the MACE analysis were cultivated in climate chambers under short-day conditions (8 h light/16 h darkness) at 22 °C.

Disease assays

The *D. rosae* isolate DortE4 was used for the black spot inoculations. The single conidial isolate was maintained on detached leaves of the susceptible cultivar PC as previously described (von Malek and Debener 1998). Young unfolded leaves were infected with a suspension of 500,000 conidia/mL with a vaporizer and kept on moist tissue paper in translucent plastic boxes in an air-conditioned laboratory at 20 °C.

A multispore isolate of powdery mildew originating from the host genotype PC, which was grown in the greenhouses at Leibniz Universität Hannover (Germany), was used for inoculation. Very young, recently unfolded PC leaves were placed in an infection box covered with a 14 cm tall-100 µm nylon mesh. Infected leaves were rubbed over the mesh to cover the leaves with conidia. The conidia were allowed to settle for 30 min to ensure that they all reached the leaf surfaces. To estimate the density of the conidia, a microscope slide was placed next to the leaves, and the conidia were counted under a microscope at 100-fold magnification (20 conidia/mm^2, 25 conidia/mm^2, 60 conidia/mm^2 for the three biological replicates respectively, and the validation experiment: 16 conidia/mm^2). Infected leaves were stored in translucent plastic boxes on moist tissue paper at 20 °C. Control leaves were used and stored in conditions identical to those of the samples inoculated. The treatments and infection time points for both pathogens are listed in Table 1. Three completely independent inoculation experiments for each time point were conducted for the MACE experiment, and independent sets of three additional experiments were conducted for the qPCR experiment. These experiments were treated as biological repeat experiments.

Microscopic analysis

The control leaves and leaves infected with either *D. rosae* or *P. pannosa* were sampled at 0, 24, and 72 hpi for the microscopic analysis. Leaf pieces of approximately 1 cm^2 were fixed, stained with Alexa Fluor 488-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, USA) and examined as previously described (Menz et al. 2017).

Table 1 Overview of the treatments and sampling time points

Treatment	0 hpi[a]	24 hpi	72 hpi
P. pannosa	PC[b]+PP[c]	PC+PP	PC+PP
D. rosae	PC+DR[d]	PC+DR	PC+DR
Control	PC−Co[e]	PC−Co	PC−Co

[a] Hours post inoculation

[b] Rose genotype 'Pariser Charme'

[c] *Podosphaera pannosa*

[d] *Diplocarpon rosae*

[e] Control

MACE

Transcriptomic data was generated using the MACE technique (Kahl et al. 2012). A particular feature of this technique is that only one sequence (tag) per cDNA molecule is generated, so normalization to the length of the respective transcript/gene model is not necessary. The RNA for this analysis was extracted from the independent biological replicates using an RNeasy® Plant Mini Kit from Qiagen (Hilden, Germany) following the manufacturer's instructions with an additional DNase digestion step using an Ambion VR DNA-free™ Kit from Life Technologies (Carlsbad, CA, USA) to remove the remaining genomic DNA. The cDNA synthesis and sequencing was performed by GenXPro (Frankfurt am Main, Germany). The sequencing data were already quality- and adapter-trimmed by the provider. The raw reads of this study were placed in the NCBI Sequence Read Archive (SRA) under the accession numbers SRR6879138 to SSR6879164. Additional processing of the sequences was performed using CLC Genomic Workbench 9.0.1 (Qiagen, Hilden, Germany). The remaining pieces of the poly-A tail were removed by an additional adapter trimming step using a poly-A-adapter. Reads shorter than 35 bp were discarded. The trimmed sequences were mapped to the genomic sequence of *Rosa chinensis* var. 'Old Blush' (Hibrand Saint-Oyant et al. 2018) using the following parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.9, similarity fraction = 0.9, strand specific = both, maximum number of hits = 10 and expression value = unique counts. The expression values were normalized using the tags per million (TPM) normalization and were log2 transformed. Differential gene expression was analyzed using the EdgeR package (Robinson et al. 2010) with a false discovery rate (FDR) adjustment.

Only the genes detected in all three repeat experiments of at least one condition that had at least 1 TPM and a minimum mean fold change ± threefold, with an FDR-adjusted p value ≤ 0.05, were considered to be differentially expressed.

Additional data analysis

In addition to the annotation presented by Hibrand Saint-Oyant et al. (2018), Blast2GO 4.1.9 software (Conesa et al. 2005) was used to classify sequences with GO terms and generate an automatic functional description derived from a BLASTp search (E-Value cut-off 1E-10) against a subset of the NR database that contained only plant sequences (Ashburner et al. 2000). These GO terms were used for an enrichment analysis of the differentially expressed genes (DEGs) using a Fisher's exact test implemented in Blast2GO. The figure was designed in Microsoft Excel 2013.

To visualize of the regulated genes in heatmaps, R 3.4.0 (R Core Team) and the package "pheatmap" (Version 1.0.10; Kolde 2018) were used. The clustering default settings were used.

Based on the normalized expression values, the Spearman rank correlations between the samples and the biological repeat experiments were calculated in R 3.4.0 (R Core Team).

Validation of the MACE analysis using high-throughput qRT-PCR

To validate the MACE analysis, an independent set of three inoculations representing independent biological replicates was performed. From 20 to 30 mg of infected leaf tissue (0 hpi, 24 hpi and 72 hpi), the total RNA was isolated using a Quick-RNA™ MiniPrep Plus kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions with some minor modifications. The leaf material was frozen in liquid nitrogen and disrupted using a bead mill. Dithiothreitol (DTT) was added to the lysis buffer to a final concentration of 50 mM.

To synthesize the cDNA, 500 ng of total RNA was processed using a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems VR (Carlsbad, USA) according to the manufacturer's instructions. A set of 28 genes was chosen among the differentially regulated genes identified by the MACE analysis and subjected to BioMark high-throughput qPCR (Fluidigm Corperation, San Francisco, USA). Primers for the genes were constructed using Primer3plus (Rozen and Skaletsky 2000) and are listed in Supplementary Table 1. The primer efficiency was tested with a dilution series (1:4, 1:16, 1:64, 1:256) using a StepOnePlus™ system from Applied Biosystems (Austin, USA) as described by Menz et al. (2017). The expression of the differentially regulated genes and the three reference genes *TIP, SAND* and *UBC* (Klie and Debener 2011) were analyzed using a Fluidigm Dynamic Array IFC (96.96) (Fluidigm Corperation, San Francisco, USA) following the manufacturer's instructions. The specific target amplifications (STA) were diluted fivefold, and the qPCR conditions were as follows: 60 s at 95 °C, 30 cycles of 95 °C for 5 s and 20 s at 64 °C, and a final melting curve analysis. The data were processed using Fluidigm Real-Time PCR Analysis Software (4.3.1, Fluidigm Corporation, San Francisco, USA). A quality threshold of 0.65, a linear baseline correction and an auto global cycle threshold (Ct) were used. Ct values were used to calculate the expression ratios with using the REST 2009 software (V2.0.13, Qiagen, Hilden, Germany). The expression ratios were log2 transformed, and the coefficient of correlation (Pearson) of the significantly up- or downregulated genes in both the MACE and BioMark analysis were calculated in R 3.4.0 (R Core Team).

Results

Fungal development in the early stages of compatible interactions

To analyze the fungal development in the early stages of the rose interaction with the hemibiotrophic fungus *D. rosae* and the obligate biotrophic fungus *P. pannosa*, a microscopic analysis was performed. The development of both pathogens was similar at the beginning. At 24 hpi, the spores germinated, and the first haustoria formed (Fig. 1B, F). At 72 hpi, *D. rosae* developed long-range hyphae, and numerous haustoria were formed in the epidermis and the underlying mesophyll layer (Fig. 1C, D). However, after 72 hpi, the development progressed more in *P. pannosa* than in *D. rosae*. More fungal hyphae spread across wide parts of the leaf, and a high number of haustoria formed in the epidermis of the plant (Fig. 1G, H).

MACE sequencing results and validation

MACE sequencing was performed for the inoculated and control samples for all the time points (0, 24, 72 hpi) in the three biological repeats, each represented by an independent inoculation experiment. The sequencing resulted in 6–30 million high-quality reads per library with an average output of 12.7 million reads per library (Supplementary Table 2). Between 79.2 and 94.2% of these reads could be mapped to the genome sequence of the 'Old Blush' rose variety (Hibrand Saint-Oyant et al. 2018). The majority of the sequenced reads (76.3–92.9%) uniquely mapped to the genome (Supplementary Table 1).

To validate the results of the MACE analysis, three additional inoculation experiments were analyzed using a high-throughput RT-qPCR system. For this purpose, a set of 28 significantly up- and downregulated genes was chosen based on the MACE results. The selection primarily focused on genes that showed similar differences in expression between the infected and noninfected samples in all three biological

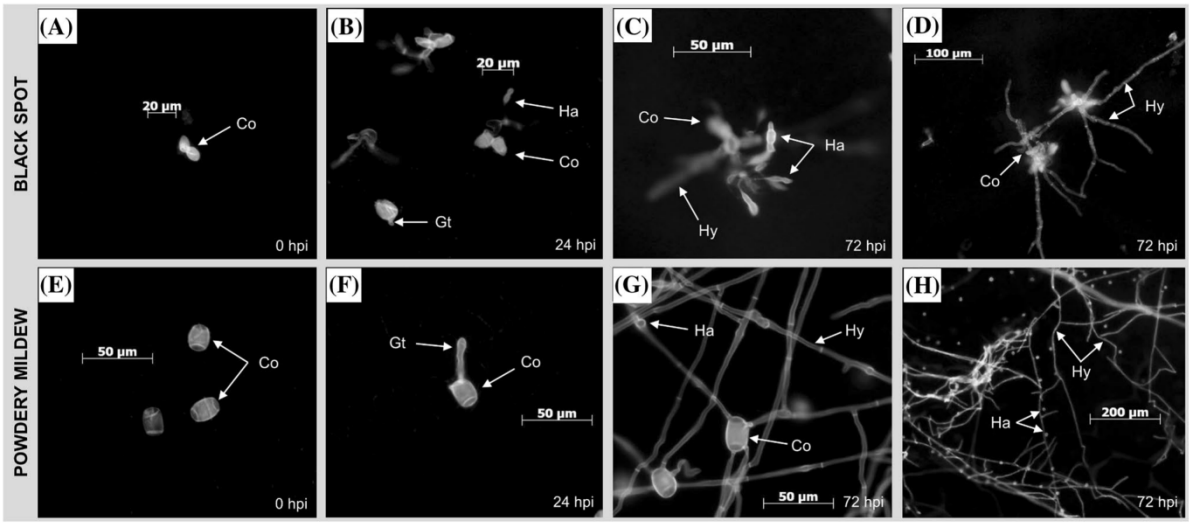

Fig. 1 Microscopic analysis of the two interaction systems. The interaction between the susceptible genotype PC and *D. rosae* (black spot, **A**–**D**) and *P. pannosa* (powdery mildew, **E**–**H**) at different time points [0 hpi (**A, E**), 24 hpi (**B, F**) and 72 hpi (**C, D, G, H**)]. *Co* conidia, *Gt* germ tube, *Ha* haustoria, *Hy* hyphae. Samples were stained with Alexa Fluor 488-conjugated wheat germ agglutinin (WGA)

replicates. A close correlation (r = 0.82) between the significant log2-fold changes in the MACE and the RT-qPCR results was detected, and except for the three data points that were downregulated in the qPCR results and upregulated in the MACE results (left upper quadrant), the same expression trends were observed (Fig. 2). The selected genes, primer sequences, PCR amplification efficiencies and expression data of the MACE and RT-qPCR analysis are listed in Supplementary Table 2.

Common responses of roses to both pathogens

To provide an overview of the functions and processes linked to the differentially expressed genes in the transcriptome, we performed a GO enrichment analysis (Fig. 3 and Supplementary Table 3) using Blast2GO with a particular interest in visualizing the pathogen-related reactions of the rose. In addition, the expression of single and typical marker genes and gene families were analyzed (Fig. 4).

The response of the susceptible rose PC to infection with *P. pannosa* and *D. rosae* leads to major changes in the leaf transcriptome (Supplementary Tables 4 and 5). The most strongly differentially regulated genes (Supplementary Table 6) comprised some defense-related genes, such as the major allergen Pru ar1, belonging to the PR10 family. In addition, some genes involved in signaling pathways, such as kinases, transcription factors and a calmodulin, as well as genes encoding cytochrome P450 proteins, were among the most highly regulated genes.

The GO terms enriched in the set of upregulated genes at 24 and 72 hpi include particular terms such as “chitinase activity”, “chitin catabolic process”, “defense response” and “response to biotic stimulus”(Fig. 3). Consistent with the drastic changes in the transcriptome, the GO term “transcription factor activity, sequence-specific DNA binding” was also overrepresented in the upregulated sequences.

In addition to these upregulated terms, other GO terms were enriched in the sets of significantly downregulated genes, such as terms related to auxin signaling, such as “auxin efflux”, “auxin-activated signaling pathway”, “cellular response to auxin stimulus” and “response to auxin”, which were enriched at 24 hpi for *D. rosae* and at 72 hpi for *P. pannosa*.

In addition, GO terms representing photosynthesis-related (“chlorophyll binding”, “photosynthesis, light harvesting”) and cell wall organization-related mechanisms (“cellular polysaccharide catabolic process”, “pectin metabolic process”) were overrepresented in the gene sets for both pathogens. In addition, walls are thin 1 (WAT1) gene with a general low expression level, which is also found in the control samples, was downregulated under both types of infections.

Pathogen-related (PR) genes

Different PR genes were strongly upregulated in rose leaves during the interactions with both pathogens (Fig. 4). In particular, many PR10 genes and major allergens (Pru av1 and Pru ar1) showed very high levels of upregulation. Of the 39

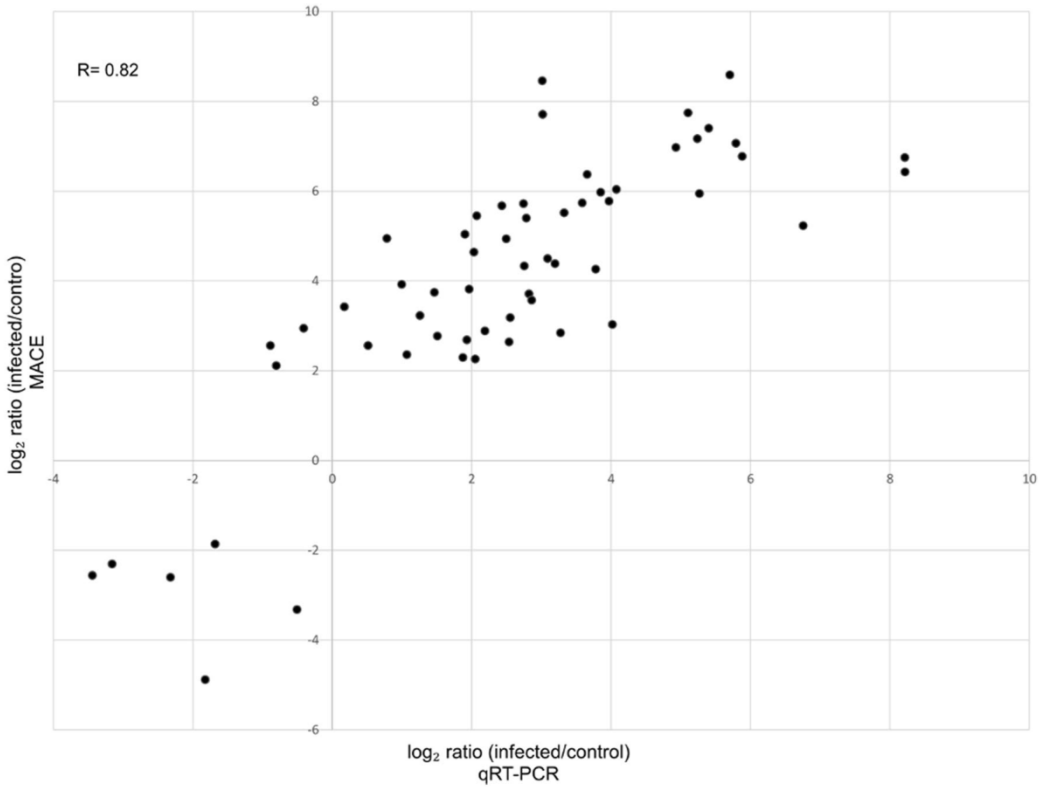

Fig. 2 Scatter plot for 28 genes analyzed with both MACE and RT-qPCR at three time points. Only the expression ratios of the significantly up- or downregulated genes from the MACE and RT-qPCR data are shown, resulting in 58 data points. The Pearson's correlation coefficient is also shown

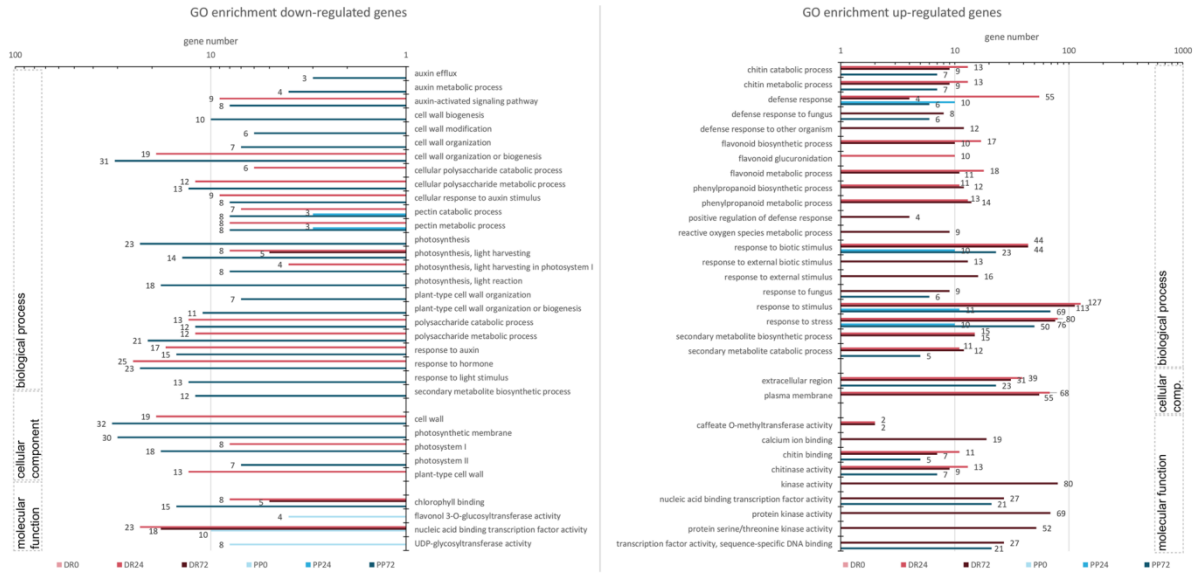

Fig. 3 Gene ontology enrichment analysis in significant down- and upregulated gene sets. This figure shows the number of genes associated with GO terms, which exhibited statistically significant enrichments in at least one of the time points or infection treatments. Interesting Go terms in the category of "biological process", "cellular component" and "molecular function" are shown

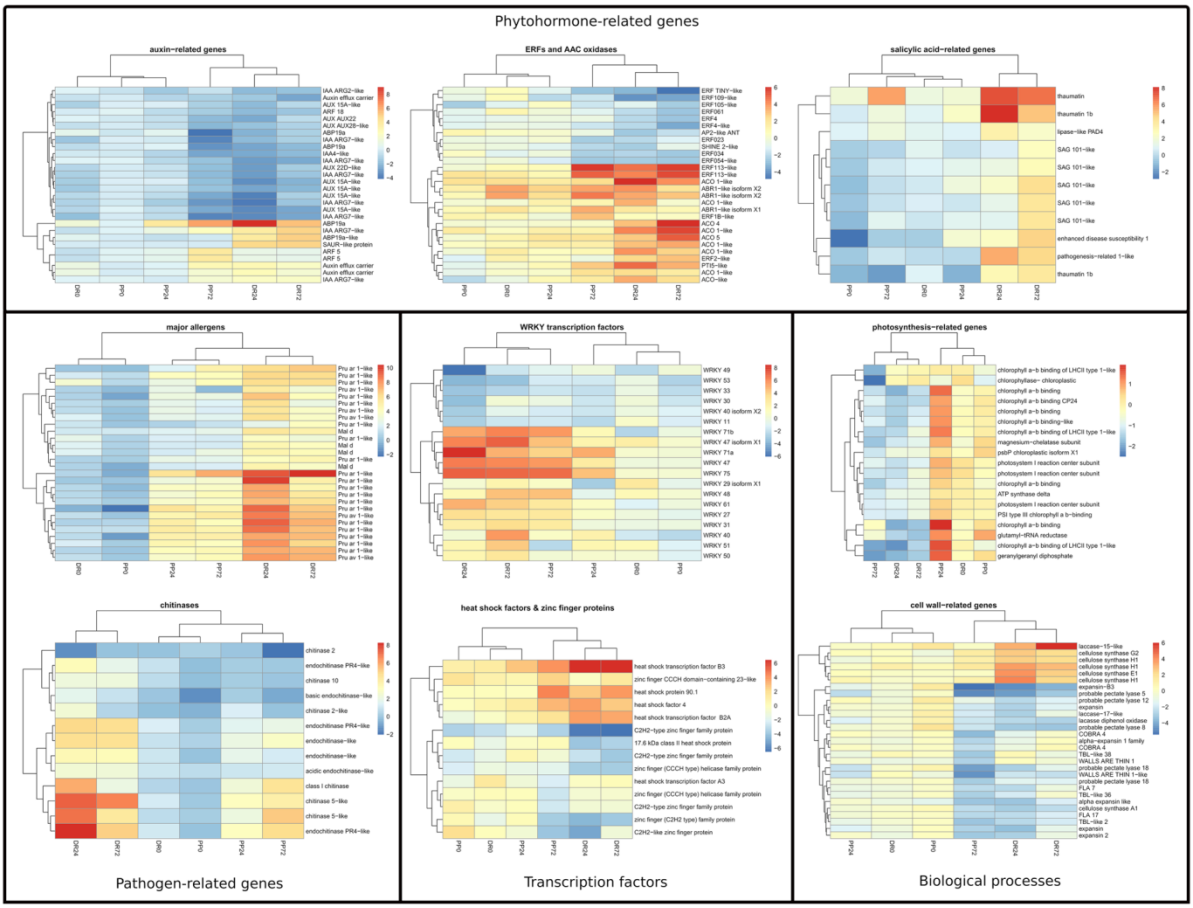

Fig. 4 Heatmaps showing the expression of genes associated with different keywords or gene families. Fold changes, compared to the control leaves, of down- (blue) and upregulation (red) are shown on a log2 scale for all the time points and infection treatments. At least in one data point, the expression is significantly different according to our criteria. "DR" stands for inoculations with *D. rosae* and "PP" for *P. pannosa* at "0", "24" and "72" hpi. The clustering default settings were used

major allergens found in the genome of 'Old Blush', 29 were highly upregulated in response to *D. rosae*, and the majority of these were already differentially expressed at 24 hpi. Of these, 15 were also significantly upregulated in response to *P. pannosa*, and 8 were already upregulated at 24 hpi. In addition to PR10, the upregulation of the chitinases, which belong to the PR3 and PR4 classes, was observed in both pathosystems. Eleven chitinases were upregulated in response to *D. rosae*, and some were more highly upregulated at 24 hpi than at 72 hpi. Six were also upregulated in response to *P. pannosa*, and four of these were already expressed at 24 hpi. In addition, six genes only annotated as "pathogenesis-related family protein" were induced in response to *D. rosae* and five in response to *P. pannosa*.

Non-race specific disease resistance protein 1 (NDR1), which acts as a downstream regulator of resistance (R) protein-derived signalling, was interestingly significantly downregulated at 24 hpi in the interaction with *D. rosae* and at 72 hpi for *P. pannosa*.

Phytohormones

Supporting the GO enrichment related to auxin signaling within the downregulated gene sets, a transcriptional downregulation of single auxin-induced/responsive genes (AUX/IAA/ARG) could be observed. In detail, 14 genes were expressed at a lower level at 24 hpi and four at 72 hpi in the leaves inoculated with *D. rosae* and nine at 72 hpi for *P. pannosa* compared to the control leaves.

In both pathosystems, ethylene-related genes also showed significant regulation; however, they exhibit both up- and down-regulations (Fig. 4). One gene encoding the ethylene-responsive transcription factor (TF) ERF109 was downregulated, while three ABR1-likes were upregulated

for interaction with *D. rosae* at 0 hpi. At 24 hpi six genes encoding for 1-aminocyclopropane-1-carboxylate oxidase 1 homologs (ACO1), and each one ACO4 and ACO5 gene were highly upregulated. In addition, one ERF2, three ABR1s and two ERF113 genes were induced. In contrast, eight ethylene-responsive TF genes (ERFs, ANT and SHINE2) and one ACO gene were expressed at a lower level than in the control plants. A similar scenario emerged after 72 h.

Rose leaves inoculated with *P. pannosa* also showed transcriptional changes in ethylene-related genes, too. Five ethylene responsive transcription factors were already downregulated at 0 hpi. At 72 hpi, five ethylene-responsive TFs were downregulated, which were different from the others downregulated at 0 hpi. In addition, similarly to an infection with *D. rosae*, six ethylene responsive TFs were upregulated, including ERF1B, ERF113 and ABR1. At 24 hpi, no ethylene-related genes were significantly differentially expressed.

Transcription factors

Inoculation with both *P. pannosa* and *D. rosae* led to a regulation of several WRKY transcription factors (Fig. 4); WRKY31, WRKY47, WRKY48, WRKY61, WRKY 71 and WRKY75 were upregulated. During infection with *P. pannosa*, upregulation could only be observed after 72 h, but in infections with *D. rosae*, the majority of the genes were upregulated at both 24 and 72 hpi.

The basic helix-loop-helix (bHLH) transcription factor MYC2 is downregulated in both interactions at 72 hpi and with *D. rosae* at 24 hpi. The downregulation is tenfold during *D. rosae* and almost fourfold during *P. pannosa* infection after 72 h. In addition, one MYC3 gene is also downregulated at 72 hpi. In contrast MYB108 is upregulated at the same time points.

The heat shock factors (Hsf) 4 (also known as HsfB1) and HsfB3 were found to be upregulated in response to *D. rosae* at 24 hpi and 72 hpi and in response to *P. pannosa* at 72 hpi.

Two C2H2-type zinc finger family proteins were downregulated in response to both pathogens. In addition, only two are downregulated in response to *P. pannosa*, and only one was downregulated in response to *D. rosae* (Fig. 4). In addition, two different zinc finger (CCCH type) helicase family proteins were downregulated in response to either *P. pannosa* or *D. rosae*.

Sugar transporters

A number of genes related to the transport of sugars were differentially expressed in roses during pathogen attack. Leaves inoculated with both pathogens upregulated a number of sugar transporters, such as ERD6. During the interaction with *D. rosae* two hexose carrier 6 (HEX6) genes, a homolog of sugar transporter 13 and one sugar phosphate/phosphate translocator were also upregulated, while other homologues of the same genes are downregulated in both leading to a contradictory scenario.

Response to *P. pannosa* compared to the response to *D. rosae*

Based on the comparison of the responses of roses to both pathogens (Fig. 5), the majority of the significantly regulated genes were specific to the reaction to one of the pathogens. Only a smaller number of the genes was regulated in response to both pathogens. One of the major differences between the two responses is the time point when the genes were regulated. During the interaction with *D. rosae*, many of the responses occurred at 24 hpi. In contrast, almost no change in the gene expression was visible in the interaction with *P. pannosa* at 24 hpi (Fig. 5). In the comparison of the later responses at 72 hpi, it is noticeable that only approximately 25% of the significantly upregulated genes and 21.6% of the significantly downregulated genes were identical between the different pathogen interaction systems. The remaining genes were exclusively regulated in response to only one pathogen.

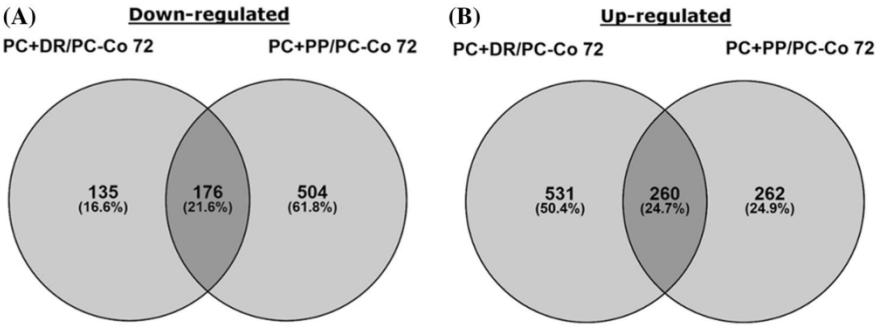

Fig. 5 Venn diagram of the quantitative comparison of the significantly regulated genes in response to inoculations with *D. rosae* and *P. pannosa*. Downregulated genes are shown in "**A**" and upregulated genes in "**B**" each for 24 and 72 hpi

Specific response of rose to *P. pannosa* infection

In total, 1450 genes (650 up- and 800 downregulated) were differentially expressed (Fig. 6). The majority of these genes, representing approximately 80% of the up- and downregulated genes, were differentially expressed at 72 hpi. The smallest number of differentially expressed genes was detected at 24 hpi. The majority of the up- or downregulated genes at 24 hpi were also regulated at 72 hpi. Directly after the inoculation (0 hpi), 131 genes were upregulated and 142 genes were downregulated in the inoculated leaves compared to the control leaves. However, only a few of those changes were also observed at the other time points. In addition, several GO terms were enriched in the gene sets at the beginning of the experiment (0 hpi), in contrast to the *D. rosae* infections.

In addition to the mentioned common GO terms related to photosynthesis, the terms "photosynthetic membrane", "photosynthesis", "photosystem II" and "response to light stimulus" were enriched in the set of downregulated genes.

The majority of the significantly downregulated genes associated with photosynthesis encode chlorophyll a/b binding proteins (*cab*), structural components of photosystem I (subunit l, K, N) and a PsbP gene of photosystem II. In addition, four genes of different steps in chlorophyll biosynthesis were downregulated, including glutamyl-tRNA reductase (HEMA1), a subunit of magnesium-chelatase (CHIH), geranylgeranyl diphosphate reductase (CHLP) and a chlorophyllase (CLH).

Also, several genes related to cell-wall organization were specifically downregulated in response to *P. pannosa*, including the genes encoding six expansins, three trichome birefringence-like proteins (TBL), two fasciclin-like arabinogalactan (FLA) proteins and two COBRA-like genes and one additional walls are thin 1-like (WAT1) gene (Fig. 4). One cellulose synthase 4 gene, three genes encoding pectin lyases, two xyloglucan endotransglucosylase genes and some genes of the lignin biosynthesis pathway were downregulated.

Furthermore, the heat shock protein (Hsp) 90.1 is specifically upregulated in response to *P. pannosa*. In contrast to the infection with *D. rosae*, no other PR genes or other genes related to a defense response specific to the interaction of roses to *P. pannosa* could be identified.

Specific response of rose to *D. rosae* infection

Almost 2000 genes were changed in their expression specifically in response to *D. rosae*, and the majority (1158) of these were upregulated with only 828 that were downregulated (Fig. 7). Less than 100 genes were considered differentially expressed directly after inoculation at 0 hpi. Approximately 80% of both the up- or downregulated genes were observed at 24 hpi. The majority of the upregulated genes and many of the downregulated genes show a similar regulation pattern at 72 hpi.

The majority of the overrepresented GO terms were the same for both 24 and 72 hpi (Fig. 3, Supp. 3). In the reaction to *D. rosae*, GO terms related to the defense response (e.g., "chitinase activity", "defense response", "response to biotic stimulus", "response to fungus") were highly enriched in the set of upregulated genes. In addition, GO terms such as "calcium ion binding", "kinase activity" or "reactive oxygen species metabolic process", which are involved in signaling processes, were also significantly overrepresented, as well as the GO terms representing secondary metabolism, especially those related to the phenylpropanoid and flavonoid pathways ("caffeate O-methyltransferase activity", "flavonoid metabolic process", "phenylpropanoid metabolic process", and "secondary metabolite biosynthetic process").

Remarkably, genes responding to the salicylic acid (SA)-mediated signaling pathway were only upregulated in the samples inoculated with *D. rosae*, including typical marker genes, such as phytoalexin deficient 4 (PAD4) and PR1 and

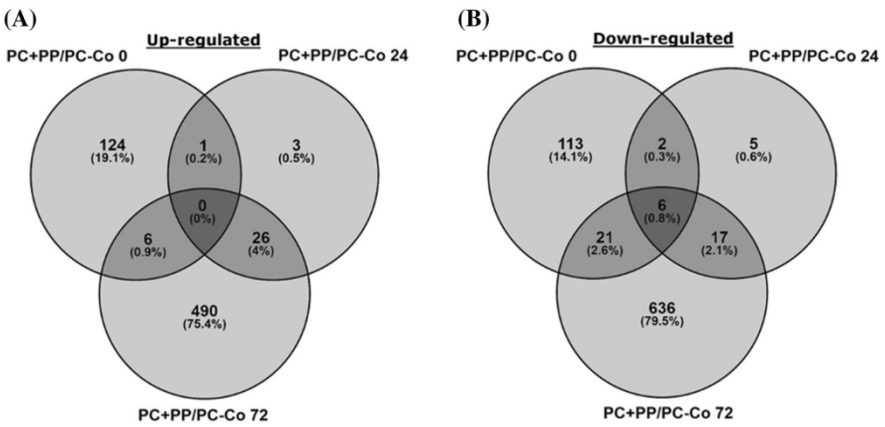

Fig. 6 Venn diagram of significantly regulated genes after *P. pannosa* inoculation. Genes that were significantly up- (**A**) and downregulated (**B**) compared to the control samples at different time points (0, 24, 72 hpi) are shown

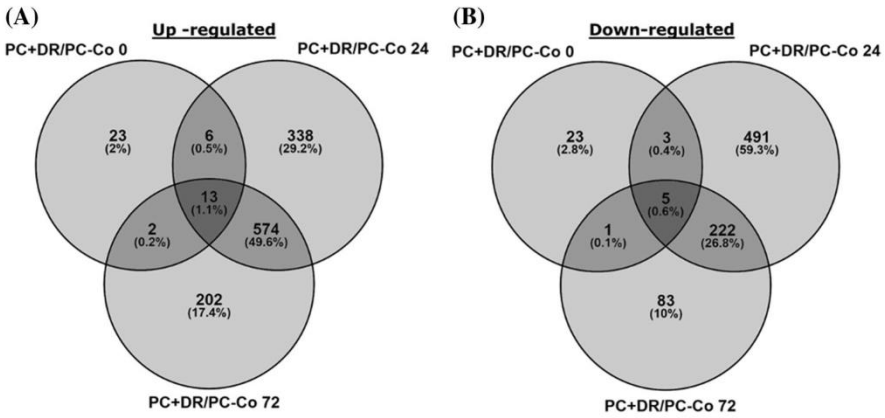

Fig. 7 Venn diagram of significantly regulated genes after *D. rosae* inoculation. Genes, that were significantly up- (**A**) and downregulated (**B**) compared to the control samples at different time points (0, 24, 72 hpi) are shown

PR5s (Derksen et al. 2013). In particular, three PR5 genes (thaumatin) were highly upregulated at 24 and 72 hpi, and one PR1 gene was among the most highly upregulated gene (Fig. 4). The upregulation of the PR1 gene was also observed in the qPCR validation. In addition, six senescence-associated carboxylesterase 101 (SAGs 101) genes were upregulated after 72 hpi.

The phenylpropanoid and the flavonoid pathways seemed to also be specifically affected. Figure 8 displays the primary steps of the flavonoid synthesis pathway and the significantly regulated genes. It can be seen that genes in almost all the steps were upregulated. In particular, the genes encoding for chalcone isomerases (CHI), dihydroflavonol 4-reductase (DFR) and flavonol synthase (FLS) were strongly upregulated at both time points, indicating that the synthesis of flavonols was induced. In addition, the pathway leading to the synthesis of proanthocyanidins (DFR, LDOX, ANR) was upregulated. The only downregulated genes in this interaction were two genes encoding UDPG-flavonoid glucosyl transferase (UFGT). This is a key enzyme in the synthesis of anthocyanins. In contrast to the reaction of PC to *D. rosae*, the reaction of PC to *P. pannosa* displayed a different gene regulation pattern. There were significantly upregulated genes, such as one of the flavanone 3-hydroxylase (F3H) genes, one FLS gene and one leucoanthocyanidin dioxygenase (LDOX) gene. In particular, enzymes acting in the first steps of the flavonoid synthesis pathway (PAL, 4CL, CHS and CHI) seemed to remain unaffected or were even downregulated. In addition, the anthocyanidin reductase (ANR) gene leading to the synthesis of proanthocyanidins was significantly downregulated.

Many genes encoding key enzymes in the phenylpropanoid pathway were upregulated in most of the cases at both time points, 24 hpi and 72 hpi, in response to *D. rosae*, with *p*-coumarate 3-hydroxylase (C3H), caffeic acid *O*-methyltransferase (COMT), caffeoyl-CoA *O*-methyltransferase (CCoAOMT) and cinnamoyl-CoA reductase

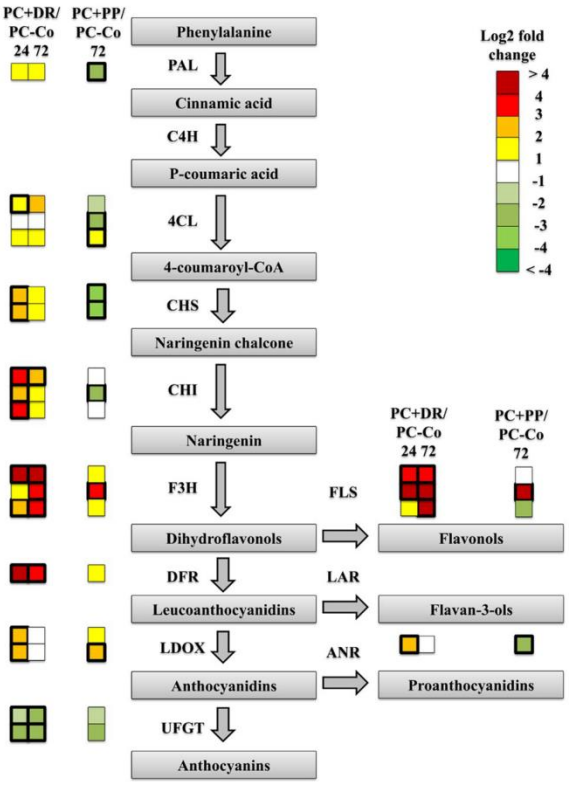

Fig. 8 Overview of the regulation of the flavonoid biosynthesis pathway. The primary steps in the flavonoid biosynthesis pathway and the regulation of genes in the pathway in response to the two pathogens are displayed. Each quadrat represents a gene. Significant changes in gene expression are indicated by thicker lines. Enzyme names are abbreviated as follows: *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaroyl-CoA-ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *DFR* dihydroflavonol 4-reductase, *LDOX* leucoanthocyanidin dioxygenase, *UFGT* UDPG-flavonoid glucosyl transferase, *FLS* flavonol synthase, *LAR* leucoanthocyanidin reductase, and *ANR* anthocyanidin reductase

(CCR) showing the strongest upregulation (Fig. 9). Of particular interest are CCoAOMT and COMT, because they are involved in the synthesis of G- and S-lignin, which are the primary components of lignin in dicots (Vanholme et al. 2010). In addition to the biosynthic genes, several peroxidases and laccases, which are involved in the cross-linking of lignin monomers, were upregulated. This pathway seemed to be less affected in response to *P. pannosa* than in response to *D. rosae*. One CCR gene and a COMT gene showed higher levels of upregulation, but some key factors were also significantly downregulated. Notably, two genes encoding cinnamyl alcohol dehydrogenase (CAD) were significantly downregulated. This enzyme catalyzes one of the later steps in the synthesis of all three types of lignin monomers.

Among the exclusively regulated transcription factors WRKY 27, 40, 48 50 and 51 that are all known to increase resistance in other pathosystems were upregulated at 72 hpi, while WRKY 53 was strongly downregulated at both 24 hpi (14.5-fold) and at 72 hpi (10.5-fold). WRKY 33 was also downregulated at both time points (9.3 and 5.6-fold), while it was only regulated 2.2-fold in the interaction with *P. pannosa* at 72 hpi.

In addition, six heat shock proteins (17.6 kDa class II heat shock protein, heat shock cognate protein 70-1, DNAJ heat shock family protein, heat shock protein 101 and two HSP20-like chaperones superfamily proteins) were only downregulated in response to *D. rosae* (Fig. 4).

Discussion

Fungal phytopathogens successfully colonize their hosts by manipulating the host defense mechanisms in different ways. To characterize the specific infection strategies based on the resulting transcriptomic changes in the early stages of infection with the hemibiotrophic *D. rosae* and the obligate biotrophic *P. pannosa*, we used next-generation sequencing based on the MACE (massive analysis of cDNA ends) approach.

A notable advantage of MACE compared to conventional RNA-Seq technology is that only one read is produced for each cDNA molecule, which allows a precise quantification, especially transcripts expressed at low levels. The sequencing (6–30 million reads per library) and mapping (76.3–92.9%) results (Supplementary Table 1)

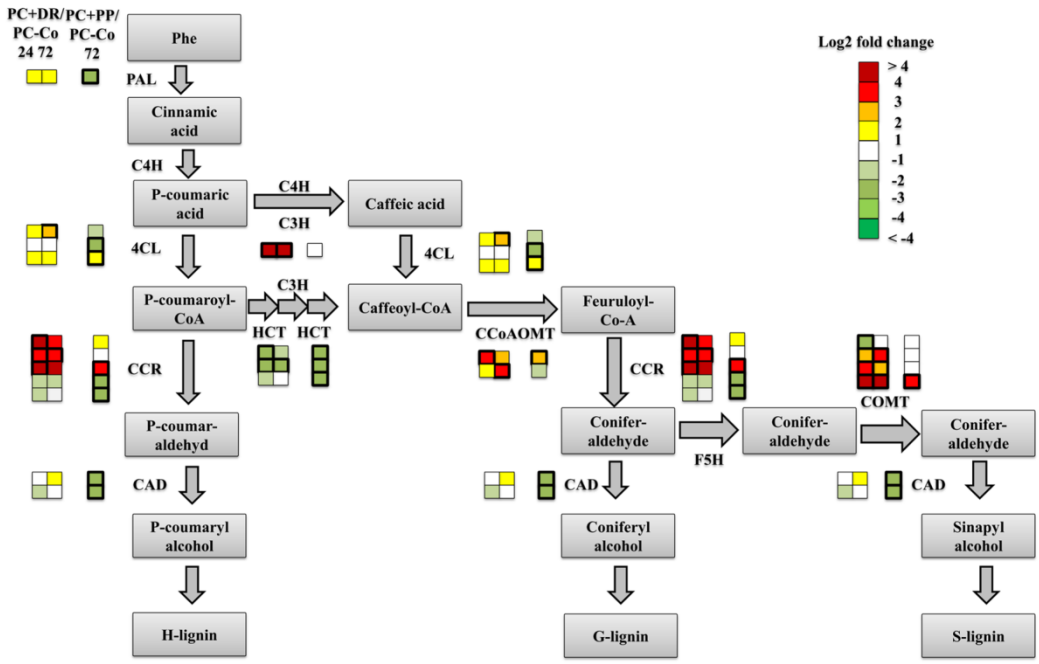

Fig. 9 Overview of the regulation of the phenylpropanoid biosynthesis pathway. The primary steps in the phenylpropanoid biosynthesis pathway and the regulation of the genes in the pathway in response to the two pathogens are displayed. Significant changes in gene expression are marked with thicker lines. Enzyme names are abbreviated as follows: *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaroyl-CoA-ligase, *HCT* hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase, *C3H* *p*-coumarate 3-hydroxylase, *CCoAOMT* caffeoyl-CoA *O*-methyltransferase, *CCR* cinnamoyl-CoA reductase, *F5H* ferulate 5-hydroxylase, *COMT* caffeic acid *O*-methyltransferase, and *CAD* cinnamyl alcohol dehydrogenase

are comparable to those of other studies that applied this technique, e.g., Hradilová et al. (2017), applied the MACE technique to pea with an output of 8 to 15 million reads per library, and 12.3– 21.7 million reads per library were generated from the RNA of apple roots (Weiß et al. 2017).

The majority of significantly differentially expressed genes indicated by the MACE technique could be validated using RT-qPCR. Factoring that all three biological repeat experiments in the MACE analysis were derived from independent inoculation experiments and that the validation included three additional independent inoculation experiments, which introduced more biological and technical variability then conventional repeats, the correlation coefficient of 0.82 is surprisingly high. However, this might be due to the stringent conditions we applied to identify the differentially expressed genes. However, the MACE data might still indicate effects, which have been caused by outliers in one biological repeat. Thus, for a meaningful interpretation of the data, all the repeats have to be considered separately, not just the mean fold changes.

The selected infection method using detached leaves offers particularly high standardization and control options. Furthermore, in past experiments the detached leaf assay was always highly correlated to experiments conducted on potted resistant and susceptible plants. However, we cannot exclude effects of the experimental procedure on the overall gene expression levels in our analyses in both controls and inoculated samples.

Common response to both pathogens

The transcriptomic changes of the susceptible rose variety PC in response to both *D. rosae* and *P. pannosa* indicate a general pathogen response, characterized by the upregulation of PR10 genes, major allergens (Pru av1, Pru ar1, and Mal d 1) that belong to this class of PR-genes (Liu and Ekramoddoullah 2006) and chitinases. In addition, the GO terms enriched in the set of upregulated genes at 24 and 72 hpi indicating that a defense response to the pathogens was in progress.

The upregulation of the PR genes is a strong indicator of pathogen recognition (Bowles 1990). In particular, PR10 genes seem to play a central role in the response of the Rosaceae. Studies of the Rosaceae model plant *Fragaria* infected by pathogens with different lifestyles, such as the necrotrophic fungus *Botrytis cinerea* (González et al. 2013), the obligate biotrophic powdery mildew fungus *Podosphaera aphanis* (Jambagi and Dunwell 2015) and the hemibiotrophic oomycete *Phytophthora cactorum* (Toljamo et al. 2016), showed a strong upregulation of PR10 genes or major allergens. Studies on apple (*Malus*) infected with *Venturia inaequalis* (apple scab) (Poupard et al. 2003; Cova et al. 2017) and treated with fungal elicitors (Pühringer et al. 2000) also showed an upregulation of the PR10 genes. The biological functions of the PR10 gene family are not completely known, but, among other activities, antifungal activity has been described (Flores et al. 2002). The role of chitinases in the defense response of plants has been analyzed extensively in different systems. They block hyphal growth, trigger other defense mechanisms through the release of elicitors and play a role in the so-called “hypersensitive response” (Grover 2012).

Consistent with the upregulation of the PR genes, the transcriptional activation of other defense related genes was observed as well. Heat shock factor (Hsf) 4 (also known as HsfB1) and HsfB3 were observed to be upregulated in response to both pathogens. Another example is the upregulation of MYB108, a transcription factor whose expression is induced through *Bortrytis* infection (Mengiste et al. 2003).

These quick and conserved responses linked to both pathosystems might indicate the involvement of a PTI reaction elicited by chitin or the penetration of the cuticle. Since we considered compatible interactions in this study, it can be concluded that the two fungi were indeed detected, but reactions initiated were not sufficient for resistance. For resistance, both the right timing and the level of defense are of paramount importance. A susceptible interaction may be caused by an inadequate response of the plant or even by direct manipulation of the defense mechanisms by the pathogen. In the next section, we discuss the present regulations against the background of a successful colonization and seek to highlight origins for the increase in susceptibility.

For example, MYC2 and MYC3 are key factors involved in defense reactions linked to the jasmonic acid pathway (Kazan and Manners 2013), and the triple mutant myc2 myc3 myc4 cannot perform several JA-mediated defense responses against bacteria and insects (Fernández-Calvo et al. 2011). Their observed downregulation at 72 hpi is consistent with an unregulated JA-mediated pathogen response. In addition, MYC2 seems to play a role during flavonoid biosynthesis and the JA pathway in the more closely related *Malus*×*domestica* (An et al. 2016).

Zinc finger proteins comprise a large and abundant family of proteins that function in many aspects of plant growth and development as well as in defense response (Yu et al. 2016; Ciftci-Yilmaz and Mittler 2008). The downregulation of the C2H2-type zinc finger family proteins and zinc finger (CCCH type) helicase family protein in response to both pathogens is consistent with the analyzed susceptible interaction. In addition, they provide starting points for factors that support a successful pathogen attack.

Plant hormones are an important part of the signaling cascade in response to biotic stress (e.g. Yang et al. 2015). A downregulated or disturbed auxin pathway may enhance susceptibility, as in the case for *Arabidopsis* mutants and their defense response to necrotrophic fungi (Llorente et al.

2008). Bouzroud et al. (2018) describe auxin responsive factors (ARFs) as part of biotic and abiotic stress signaling in *Solanum*. The role of ethylene as a defense regulator has been well established, primarily due to its function as a signaling molecule in the defense against necrotrophic pathogens in combination with jasmonic acid. Although there are major contradictions in the ethylene-related expression patterns, which could indicate a disturbed signal transmission, our observation of the upregulated ethylene responsive ABR1 genes indicates a negative impact on the abscisic acid (ABA) signaling pathway which might also contribute to susceptibility (Pandey et al. 2005).

NDR1, which interacts with RPM1 Interacting Protein4 (RIN4) to control the activation of disease resistance signaling by the CC-NB-LRR class of resistance proteins, was downregulated in both pathosystems. It was shown that NDR1 in combination with RIN4 are needed to activate a number of disease resistance pathways against bacterial and fungal pathogens in Arabidopsis (Century et al. 1995; Day et al. 2006). Two NDR1-like sequences identified in soybean (GmNDR1a, b) were also shown to be required for resistance *to Pseudomonas syringae* (Selote et al. 2013).

The network of WRKY transcription factors is involved in many stress responses, and some are key factors in the defense response (Phukan et al. 2016). The downregulated WRKY33, although only weakly downregulated in the interaction with *P. pannosa*, provides further evidence of a repression of the defense responses. This was reported several times, including such pathogens as *Botrytis* and *Alternaria* as well as *Pseudomonas syringae* in *Arabidopsis* and *Fusarium* in tomato (Birkenbihl and Somssich 2011; Buscaill and Rivas 2014; Garner et al. 2016; Rushton et al. 2010; Aamir et al. 2018). WRKY75 is a particularly interesting transcription factor, because at 72 hpi it was exclusively expressed in the inoculated samples. It is notable that this transcription factor was initially described only in the context of phosphate stress (Devaiah et al. 2007). However, descriptions connected with defense reaction could also be found in recent years (Encinas-Villarejo et al. 2009; Jiang et al. 2014).

Changes in the transcriptome specific to the interaction with *P. pannosa*

In contrast to the *D. rosae* infection the upregulation of PR10 and chitinase genes, and thus the reaction to *P. pannosa* occurred only at 72 hpi instead of 24 hpi. In addition, the expression levels were often lower and no specific group of genes related to common defense functions was found to be upregulated exclusively in the reaction to *P. pannosa*. One of the reasons for the low number of significantly regulated genes at 24 hpi and the decreased expression levels could be that the inoculation density was lower for *P. pannosa* than for *D. rosae*. In addition, the infection zone was restricted to the epidermis with fewer penetration sites. Either fewer transcriptional changes were initiated as response or genes with low expression remained below our stringent criteria to select the DEGs. In addition to differences in the upregulation of the PR genes a gene for a heat shock protein (Hsp) 90.1 is upregulated specifically in response to *P. pannosa*. In wheat, Hsp90 genes were found to be essential for resistance to the stripe rust fungus (Wang et al. 2011, 2015).

A specific reaction to *P. pannosa* infection is that the genes involved in the light reaction of photosynthesis were downregulated more strongly than those in response to *D. rosae*, which is surprising, considering that *P. pannosa* only infects epidermal cells, which are not photosynthetically active, while *D. rosae* infects also infects the cell layers below the epidermis. However, the downregulation of the photosynthic genes is often observed in plant-pathogen interactions (e.g., Cremer et al. 2013; Milli et al. 2012; Balan et al. 2018). This reaction may be explained by a pathogen-induced source-sink transition of infected leaf tissues (Berger et al. 2007). In addition to their role to nourish pathogens, sugar transporters and sugar signaling have a large impact on the defense response of plants as described by Bezrutczyk et al. (2018). Interestingly, Morkunas and Ratajczak (2014) propose an increased resistance due to the high sugar levels in different pathosystems. For rice, it is well described that defective OsSWEET13 or 14 genes, bidirectional sugar transporters, lead to the resistance of rice against *Xanthomonas oryzae pv. oryzae* (e.g., Zhou et al. 2015; Blanvillain-Baufumé et al. 2017). In rose leaves infected with *P. pannosa* sugar transporters were upregulated, a first indication of the manipulation of the host metabolism by the fungus. However, further studies are needed to determine the influence of *P. pannosa* on photosynthesis and sugar metabolism.

Another process strongly regulated in response to *P. pannosa* and contributing to susceptibility was the cell wall modification process. Different factors, such as expansins, TBLs or FLAs, were downregulated in response to the pathogen. They are known to be involved in modification processes such as cell wall loosening, plant cell expansion and secondary cell wall formation (Cosgrove 2000; Schindelman et al. 2001; Johnson et al. 2003; Van Sandt et al. 2007; Bischoff et al. 2010). The WAT1 genes might be of particular interest. Two WAT1 gene homologs were expressed in our samples, and one was downregulated with a generally low expression level also in the control leaves, while the other, which had a much higher expression level, was exclusively downregulated in response to *P. pannosa* at 72 hpi. WAT1 was identified as an essential factor for secondary cell wall formation by loss-of-function mutations in *Arabidopsis* (Ranocha et al. 2010). In addition, *wat1* mutants showed a dwarf phenotype and downregulation of almost all the genes

in the lignin biosynthetic pathway. The downregulation of the highly expressed WAT1 gene exclusively observed in response to *P. pannosa* might be an important factor leading to the observed differences in cell wall formation and the lignin pathway. Since it is not regulated in the interaction with *D. rosae* it constitutes one of the differences in the suppression of the host response that contrasts with the infection strategy of the two pathogens. Interestingly, *wat1* mutants also showed increased tissue-specific resistance to vascular pathogens (Denancé et al. 2013).

Many obligate biotrophs and hemibiotrophs induce host responses via the SA pathway in which one of the key features is the transcriptional activation of the PR1 genes. We observed the downregulation of a PR1 gene after *P. pannosa* infection at both time points (Supplementary Table 4) and together with a lack of regulation of other the SA related genes, such as EDS1 and PAD4 homologs, this points to a suppression of part of the SA-based defense in the *P. pannosa*-rose pathosystem (van Loon et al. 2006; Derksen et al. 2013).

Surprisingly, we observed a large number of DEGs at the starting point at 0 hpi. This might be a temporary effect due to our inoculation method. After 24 h, the number of DEGs dropped to a similar level at the start point of the *D. rosae* inoculation before dramatically rising again. Of the total of 131 upregulated genes only one (a putative RNA polymerase) was still upregulated at 24 hpi and of the 142 downregulated, 16 still showed a significantly lower expression. During *P. pannosa* inoculation (see "Material and Methods") the detached rose leaves lay for 30–60 min in an open inoculation box, which may have caused a generalized stress response and, in particular, osmotic stress. For example, the two aquaporin genes TIP1-2 and PIP2-1 were upregulated compared to the control, and PIP2-1 was upregulated in both the control and *D. rosae*-inoculated leaves.

Changes in the transcriptome specific to the interaction with *D. rosae*

The transcriptomic changes specific to the interaction with *D. rosae* include the upregulation of many genes encoding enzymes in the lignin biosynthetic and flavonoid pathways. The synthesis of lignin or lignin-like phenolic polymers is a common phenomenon in response to pathogens. For instance, Chinese cabbage plants infected with the necrotrophic bacterium *Erwinia carotovora* accumulated high levels of lignin monomers, and 12 genes involved in lignin biosynthesis were upregulated (Zhang et al. 2007). Infection with the fungus *Sclerotinia sclerotium* led to the lignification of *Camelina sativa* cells and was correlated with upregulation of the CCR genes (Eynck et al. 2012). In addition, the defense response of the model plant *Arabidopsis thaliana* infected with *Pseudomonas syringae* was accompanied by the upregulation of the CAD genes. Loss-of-function mutations of these genes resulted in plants with a reduced resistance to the pathogen (Tronchet et al. 2010). Lignin has different functions in plant defense. As part of the secondary cell wall, it provides a physical barrier against the entry of the pathogen. Simultaneously, it prevents the spread of pathogen toxins and enzymes into the neighboring cells and the transfer of water and nutrients from the host to the pathogen. In addition, cell wall components can have signaling functions in defense (Miedes et al. 2014).

In addition, to the lignin biosynthetic pathway, genes encoding almost all enzymes of the flavonoid pathway were upregulated specifically in response to *D. rosae*. Products of the flavonoid pathway have various functions in plants. For example, they act as chemical messengers that interact with insects and microbes and function as pigments to attract pollinators or protect against UV light. More importantly, many phytoalexins are synthesized by this pathway (Piasecka et al. 2015; Falcone Ferreyra et al. 2012). In *Fragaria*, catechin and catechin-derived proanthocyanidins have been shown to be involved in the defense response to *Alternaria alternata* and *B. cinerea* in infected leaves and fruits, respectively (Yamamoto et al. 2000; Puhl and Treutter 2008). In addition, a recent transcriptomic study of roots infected with *P. cactorum* showed an upregulation of the flavonoid pathway genes leading to these products (Toljamo et al. 2016). Interestingly, in our data, FLS genes were highly upregulated, indicating that the synthesis of flavonols is more important in the response to *D. rosae* than in the response to *P. pannosa*. In plants, flavonols function as antioxidants during high light conditions and as detoxifying agents against reactive oxygen species (ROS), which might explain their role in plant defense (Pollastri and Tattini 2011). Metabolic analyses focusing on this group of metabolites might be needed to confirm the induction of this pathway in the rose–*D. rosae* interaction.

Different SA response genes were exclusively upregulated in response to *D. rosae*. PAD4, EDS1 and SAG101 cooperate to stimulate the production of SA and are essential for SA-mediated pathogen responses (Zhou et al. 1998; Feys et al. 2005; Rietz et al. 2011), which are typically characterized by the upregulation of the PR1, PR2 and PR5 genes. PR1 and different thaumatins (PR5) were also exclusively upregulated in response to *D. rosae*, except for one PR5 paralog that was also upregulated in response to *P. pannosa*. This could be an indication that the additional *D. rosae*-specific stress responses are regulated by the SA-mediated signaling pathway, which is either suppressed during the interaction with *P. pannosa* or not upregulated in the *P. pannosa* pathosystem because *D. rosae* is recognized differently and signaling and desired resistance is attempted via other pathways. Interestingly, it is described, e.g. for tomato (Rahman et al. 2012), that the SA pathways may promote

necrotrophic disease development, possibly by antagonizing JA. That could also be helpful for the later stages of the *D. rosae* infection. In addition, Cui et al. (2018) proposed that EDS1/PAD4 suppresses MYC2 and therefore the JA pathway, which could explain the stronger downregulation of the leaves infected with *D. rosae* that was observed.

All of these reactions could be based on a partial ETI reaction, in which specific effectors of *D. rosae* are detected. We know from previous experiments that there are more than 20 genes in the rose genome that are similar to the resistance-mediating *muRdr1A* gene of the Rdr1-locus (Terefe-Ayana et al. 2011) but do not mediate a full resistance. However, they might function to activate a partial ETI reaction, leading to the differences in the reaction of roses to the pathogens observed.

An indication of the specific interference of black spot with host resistance is the strong downregulation of WRKY33 and 53 known to have a function in resistance (Birkenbihl and Somssich 2011). In particular the downregulation of WRKY 33 and WRKY 53 transcription factors related to resistance and the upregulation of WRKYs 27, 40, 50 and 51 as repressors of resistance is interesting, since it indicates major differences in how both pathogens interfere with the SA-inducible host resistance response.

In addition, the specific downregulation of six heat shock proteins in response to *D. rosae* (17.6 kDa class II heat shock protein, heat shock cognate protein 70-1, DNAJ heat shock family protein, heat shock protein 101 and two HSP20-like chaperones superfamily proteins) can be reconciled with the current susceptible interaction, since their regulation might may decrease the strength or the timing of the defense response (Park and Seo 2015; Lee et al. 2012).

Conclusions

With this first analysis of the rose defense transcriptome, we showed contrasting responses of the host to two fungal pathogens, the hemibiotrophic *D. rosae* and the biotrophic *P. pannosa* displaying different lifestyles. In addition to a common response to both pathogens, characterized by an upregulation of the PR10 genes and chitinases, processes such as photosynthesis and cell wall modification were primarily downregulated in response to *P. pannosa*, while the secondary metabolism in form of the phenylpropanoid and flavonoid pathway was primarily upregulated in response to *D. rosae*. Surprisingly, PR1 and components of the SA-pathway were exclusively upregulated in response to the hemibiotrophic *D. rosae* and not, as often observed in other biotrophic systems, also in the interaction with *P. pannosa*. In contrast, the transcriptional regulation of some factors known to interfere with host resistances was distinct for both pathogens. This information is an important first step to understand the response to both rose pathogens and revealed many processes, which merit analysis in more detail.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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5 A comparison of transcriptomic changes during incompatible and compatible interactions between roses and *D. rosae*

5.1 Introduction

R-genes, like *Rdr1*, are known to activate defense responses such as transcriptional induction of pathogenesis-related (*PR*) genes, production of reactive oxygen species, fortification of the cell wall, synthesis of antimicrobial compounds and hypersensitive response (HR) (Dangl and Jones, 2001; Hammond-Kosack and Jones, 1997; Kombrink and Somssich, 1997; Nürnberger *et al.* 2004; Xiao *et al.* 2008). In rice, transcriptomic analysis of the incompatible interaction with the hemibiotrophic fungus *Magnaporthe oryzae* resulted in an induction of genes related with the cell wall, beta-glucanase, proteolysis, receptor-like kinases, PR genes, WRKY genes, several MAPK and Myb TFs, as well as signalling-related genes. Genes related to redox state, peroxidase and glutathione-S-transferase also showed an up-regulation in infected tissues (Wang *et al.* 2014). In the incompatible interaction of chickpea and *Fusarium oxysporum f.* sp. *ciceris*, defense-related genes, such as PR10, PR4, LRR protein kinase, cinnamate 4 hydroxylase, proline-rich cell wall, cysteine proteinase, superoxide dismutase and squalene monooxygenase, were significantly higher expressed compared to the compatible interaction (Saabale *et al.* 2018). In wheat, genes coding for a caffeoyl-CoA O-methyltransferase, PR-5-like protein, protein kinase, ethylene-responsive RNA helicase, peroxidase and peroxisomal membrane protein were induced in the incompatible interaction with *Puccinia striiformis* f. sp. *Tritici* (Wang *et al.* 2010).

In chapter 4 (Neu *et al.* 2019), MACE analysis were used to analyse differences in the manipulation of the rose leaf transcriptome in the early infection with either the hemibiotrophic fungus *D. rosae* or the biotrophic fungus *P. pannosa*. Here, the existing MACE, RNA-Seq and high-throughput RT-qPCR data generated by Neu (2018) and Neu *et al.* (2019) were used as a starting point for the analysis of transcriptomic changes during the incompatible interaction between roses and *D. rosae* caused by the *R*-gene *Rdr1*. The identification of *muRdr1A* as the functional *R*-gene against a broad spectrum of *D. rosae* was followed by the transformation of *muRdr1A* in the susceptible genotype PC (Menz *et al.* 2018, chapter 2). The use of transgenic roses harbouring *muRdr1A* allows the simultaneous analysis of the resistant compared to the susceptible genotype in the same genetic background.

5.2 Material and Methods

5.2.1 Plant material

For the multiplication of the fungal isolates as well as for the RT-qPCR analysis, the rose genotypes ‘Pariser Charme’ (PC), ‘Arthur Bell’ (AB), 14/29-9 and PC::muRdr1A-38 were propagated *in vitro* as previously described (Davies, 1980; Debener *et al.* 1998), rooted, transplanted into fertilized Einheitserde T substrate (Einheitserdewerke Gebr. Patzer, Sinntal-Altengronau, Germany) in 9 cm pots and cultivated in climate chambers under short-day conditions (8 h light/16 h darkness) at 22 °C. PC and AB are susceptible genotypes not carrying the *Rdr1* locus and the progeny 14/29-9 resulted from a cross of AB x PC::muRdr1A-58 (Menz *et al.* 2018, chapter 2).

5.2.2 Infection with *D. rosae*

The single conidial isolate DortE4 (Malek and Debener, 1998) was used to infect young unfolded leaves of the genotypes ‘Pariser Charme’ (PC), ‘Arthur Bell’ (AB), PC::muRdr1A-38 and 14/29-9. For the infection, a suspension of 500,000 conidia/mL was applied to the leaves using a vaporizer. Leaves were kept on moist tissue paper in translucent plastic boxes in an air-conditioned laboratory at 20 °C. Samples were taken from three biological replicates of inoculated and mock-inoculated leaves after 0 h, 24 h and 72 h. Successful infection was tested via microscopic analysis. *D. rosae* inoculated and mock-inoculated leaves were stained with Alexa Fluor 488 conjugated wheat germ agglutinin (Invitrogen, Carlsbad, USA) and examined as previously described by Menz *et al.* (2018) (chapter 2).

5.2.3 MACE data and high-throughput RT-qPCR

In addition to the transcriptomic data presented in chapter 4 (Neu *et al.* 2019), MACE data were generated from the incompatible interaction between *D. rosae* and the resistant genotype 91/100-5, as well as between *D. rosae* and the transgenic PC genotype carrying the *muRdr1A* gene of the *Rdr1*-locus (PC::muRdr1A-43) (Neu, 2018). For the genotype PC::muRdr1A-43, only one biological replicate was used to generate MACE data. Additionally, the same RNAs of PC and 91/100-5 inoculated with *D. rosae* (72hpi) used for the MACE approach were used for the application of conventional RNAseq (Neu, 2018) .To validate the results of the MACE analysis, three additional inoculation experiments, including the transgenic genotype PC::muRdr1A-

57, were conducted with subsequent analysis by a high-throughput RT-qPCR system. The samples used for MACE, RNASeq and high-throughput RT-qPCR are shown in Table 5.1.

For the validation, a set of significantly up- and down-regulated genes was chosen based on the MACE results (Neu *et al.* 2019). Additionally, genes known to have a function in the defense response of plants were chosen (Table S 5.2).

Table 5.1: Overview of the samples used for MACE, RNASeq and high-throughput RT-qPCR (Neu, 2018).

Genotype	Treatment	Time point	Method	Biological repetitions
PC	Mock-inoculation	0 hpi, 24 hpi, 72 hpi	MACE	3
PC	*D. rosae*	0 hpi, 24 hpi, 72 hpi	MACE	3
PC	No treatment	0 hpi	MACE	3
91/100-5	*D. rosae*	24 hpi, 72 hpi	MACE	3
PC::muRdr1A-43	*D. rosae*	24 hpi, 72 hpi	MACE	1
PC	*D. rosae*	72 hpi	RNA-Seq	3
91/100-5	*D. rosae*	72 hpi	RNA-Seq	3
PC	Mock-inoculation	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3
PC	*D. rosae*	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3
91/100-5	Mock-inoculation	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3
91/100-5	*D. rosae*	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3
PC::muRdr1A-57	Mock-inoculation	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3
PC::muRdr1A-57	*D. rosae*	0 hpi, 24 hpi, 48 hpi, 72 hpi	High-throughput RT-qPCR	3

5.2.4 Expression analysis using RT-qPCR

Genes showing an up-regulation in the MACE analysis during the incompatible interaction between 91/100-5 and *D. rosae* as well as in the incompatible interaction between PC::muRdr1A-43 and *D. rosae* (Neu, 2018) were selected for the RT-qPCR analysis. Specific primers for each gene were designed with Primer3plus (Rozen and Skaletsky, 2000) and are listed in Supplementary Table S 5.1. As internal control, specific primers for the gene encoding the ubiquitin conjugating protein (*UBC*) (JN399227.1) and the SAND-family protein (*SAND*) (JN399228.1) (Klie and Debener, 2011) were used.

Total RNA was isolated using the *Quick*-RNA™ Miniprep Plus Kit (Zymo Research, Irvine, USA) according to the manufacturer's protocol. For cDNA synthesis, 500 ng of

total RNA was processed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems VR, Carlsbad, USA) according to the manufacturer's instructions. The amplification was performed in a 10 µL volume with 2.5 µL cDNA (1:8 diluted) using qPCRBIO SyGreen Mix Lo-ROX (Nippon Genetics Europe GmbH, Dueren, Germany) according to the manufacturer's instructions. The cycling conditions were: 95 °C for 2 min, 40 cycles of 95 °C for 5 s, and 30 s at 60 °C, which was performed in a StepOnePlus™ System (Applied Biosystems, Austin, USA). Baseline correction was performed using the StepOne™ Software, and a common threshold of 0.5 was set for the quantification cycle (Cq). The primer efficiencies were estimated for each reaction using LinRegPCR 11.1 (Ruijter *et al.* 2009), and Ct-values were used to calculate the relative expression quantities (RQs) using the REST 2009 software v2.0.13 (Qiagen, Hilden, Germany) according to Pfaffl (2001).

Due to the high number of genes, only biological replicates without technical replicates were tested in the first round of gene expression analysis. Genes with differences in expression higher than twofold between the compatible (PC+DortE4) and incompatible interaction (PC::muRdr1A-38+DortE4) were further tested with three technical replicates and in a different genetic background (AB and 14/29-9).

5.3 Results

For the analysis of transcriptomic changes during the incompatible interaction between roses and *D. rosae*, existing MACE, RNASeq and high-throughput RT-qPCR data were used. An overview of the number of used genes and the results of the expression analysis via RT-qPCR and high-throughput RT-qPCR is given in Figure 5.1.

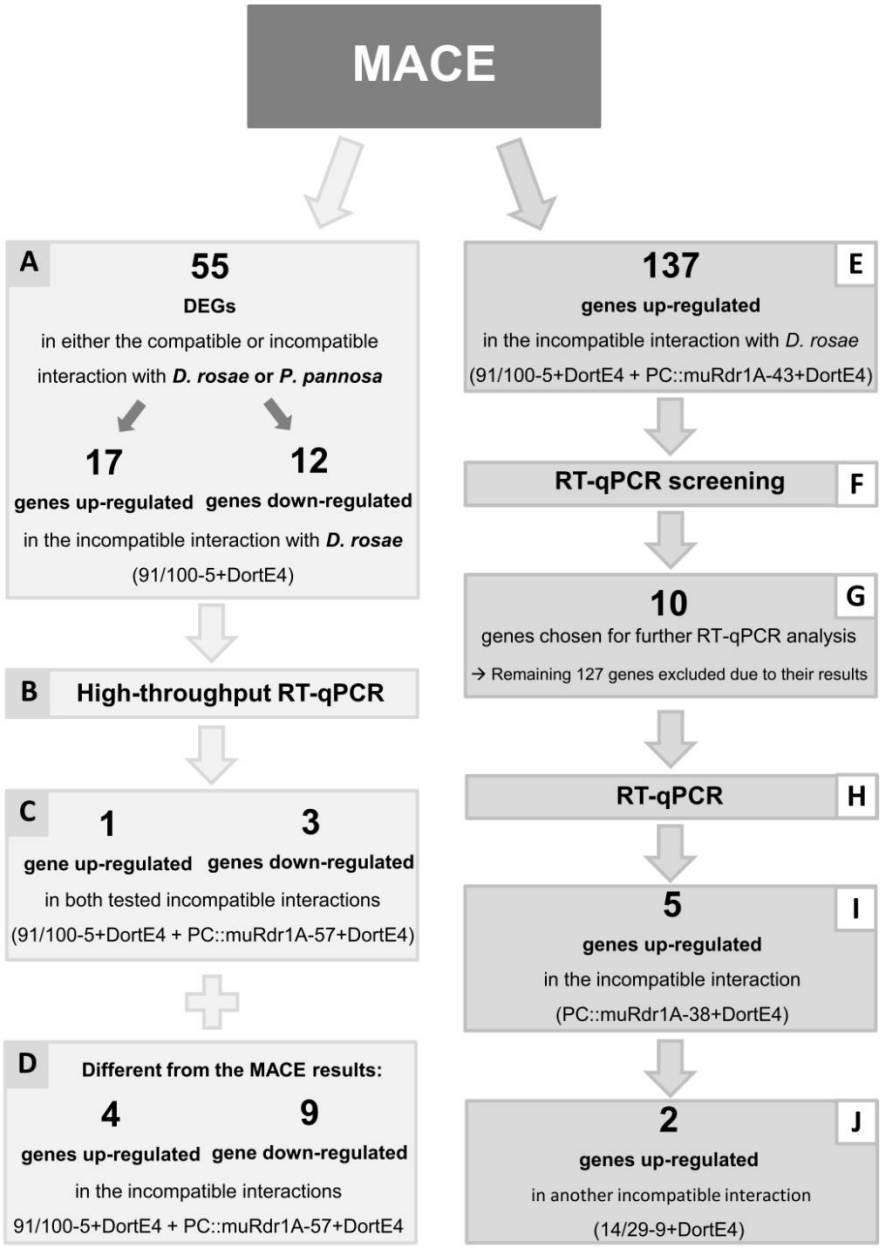

Figure 5.1: Overview of the expression analysis via high-throughput RT-qPCR and conventional RT-qPCR. MACE data were used to select differentially expressed genes (DEGs) in the incompatible interaction compared to the compatible interaction with *D. rosae* (or *P. pannosa*). DEGs were either tested via high-throughput RT-qPCR (**A+B**) or via conventional RT-qPCR (**E-H**). Results are shown for high-throughput RT-qPCR in **C+D** and for conventional RT-qPCR in **I+J**. The genotype 91/100-5 carrying the *Rdr1* locus is resistant against the *D. rosae* isolate DortE4. 'Pariser Charme' (PC), not carrying the *Rdr1* locus, was transformed with the *Rdr1* family member *muRdr1A*, resulting in clones PC::muRdr1A-38, PC::muRdr1A-43 and PC::muRdr1A-57.

To validate the results of the MACE analysis, a set of 55 differently expressed genes (DEGs) in either the compatible or incompatible interaction of roses with *D. rosae* or *P. pannosa* was selected by Neu *et al.* (2019) (Chapter 4). The expression of the selected genes during the infection of roses with either *D. rosae* or *P. pannosa* were analysed by high-throughput RT-qPCR (Supporting Information, Table S 5.2). In the following, only the data related to *D. rosae* resistance are interpreted (Figure 5.1-A+B). From the chosen 55 genes, 17 genes were up-regulated and 12 genes down-regulated in the incompatible interaction with *D. rosae* (91/100-5+DortE4 *vs* PC+DortE4). Only one of the chosen up-regulated genes (6G96) and three of the down-regulated genes (4G16, 4G79, 4G89) in the incompatible interaction with *D. rosae* and the transgenic rose (PC::muRdr1A-57) showed a positive correlation with the MACE results (Figure 5.1-C, Table 5.2). The expressions (Log2FC) of the four genes during the incompatible and compatible interaction are shown in Figure 5.2. Similar to the MACE results, 6G96 was significant up-regulated (3.8-fold) at 24 hpi in the incompatible interaction PC::muRdr1A-57+DortE4 compared to the compatible interaction PC+DortE4 (Table 5.2). At 0 hpi, a significantly 2.9-fold up-regulation was observed in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4. In the mock-inoculated PC::muRdr1A-57, 6G96 was also significantly up-regulated compared to PC (mock-inoculated) at all time points.

At 24 hpi, 4G79 was significantly down-regulated (15.6-fold) in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4, whereas the interaction PC+DortE4 compared to the mock-inoculation was 62-fold up-regulated. At 48 hpi, 4G79 was significantly up-regulated (7.5-fold) in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4. A significant up-regulation was also observed in the interaction PC::muRdr1A-57+DortE4 compared to mock-inoculation and PC+DortE4 compared to mock-inoculation at 48 hpi and 72 hpi .

4G89 was significantly down-regulated (5.6-fold) at 24 hpi in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4, but significantly up-regulated (4.3-fold) at 48 hpi.

4G16 was significantly down-regulated (4-fold) at 48 hpi and 72 hpi in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4, but also significantly down-regulated in the mock-inoculated PC::muRdr1A-57 compared to the mock-inoculated PC at all time points.

The regulations of all 55 DEGs in the incompatible interaction and compatible interaction in MACE analysis and high-throughput RT-qPCR are listed in Table 5.2.
Unlike the MACE results four genes were significantly up-regulated and nine genes were significantly down-regulated in both incompatible interactions with *D. rosae* (91/100-5+DortE4 and PC::muRdr1A-57+DortE4) (Figure 5.1-D). For example, 7G046 encoding for gibberellin-regulated 6 was significantly up-regulated in both incompatible interactions with *D. rosae* at 24 hpi and 72 hpi. In contrast, no up-regulation could be observed at 48 hpi and the MACE data showed a 5-fold down-regulation at 72 hpi. Gene 4G88, coding for a probable polygalacturonase non-catalytic subunit JP650, is 3-fold up-regulated in both incompatible interactions with *D. rosae* at 24 hpi. Gene 7G12, coding for caffeic acid 3-O-methyltransferase, was 3-fold down-regulated in both incompatible interactions with *D. rosae* at 24 hpi and 72 hpi. Gene 7G68, coding for disease resistance response 206-like, was more than 10-fold down-regulated in both incompatible interactions with *D. rosae* at 72 hpi.
1G40, coding for laccase-15-like isoform X1, was 10-fold down-regulated in both incompatible interactions with *D. rosae* at 24 hpi, but 7-fold up-regulated at 48 hpi in the incompatible interaction (PC::muRdr1A-57+DortE4) compared to PC+DortE4.
For 2G17 (encoding a lactoylgutathione lyase glyoxylase I family protein), new primers were designed due to unspecific amplification in the qPCR in one genotype and tested in RT-qPCR (see below, Figure 5.3).
The results of the other genes, including those known to have functions in the defense response of plants, e. g. pathogen or related stress induced plant proteins (PR-proteins) or transcription factors are shown in Table 5.2. A large number of these genes showed high variations in their regulation between the time points and the different methods used. Further, high standard deviations could be also observed between the biological replicates for many of the genes.

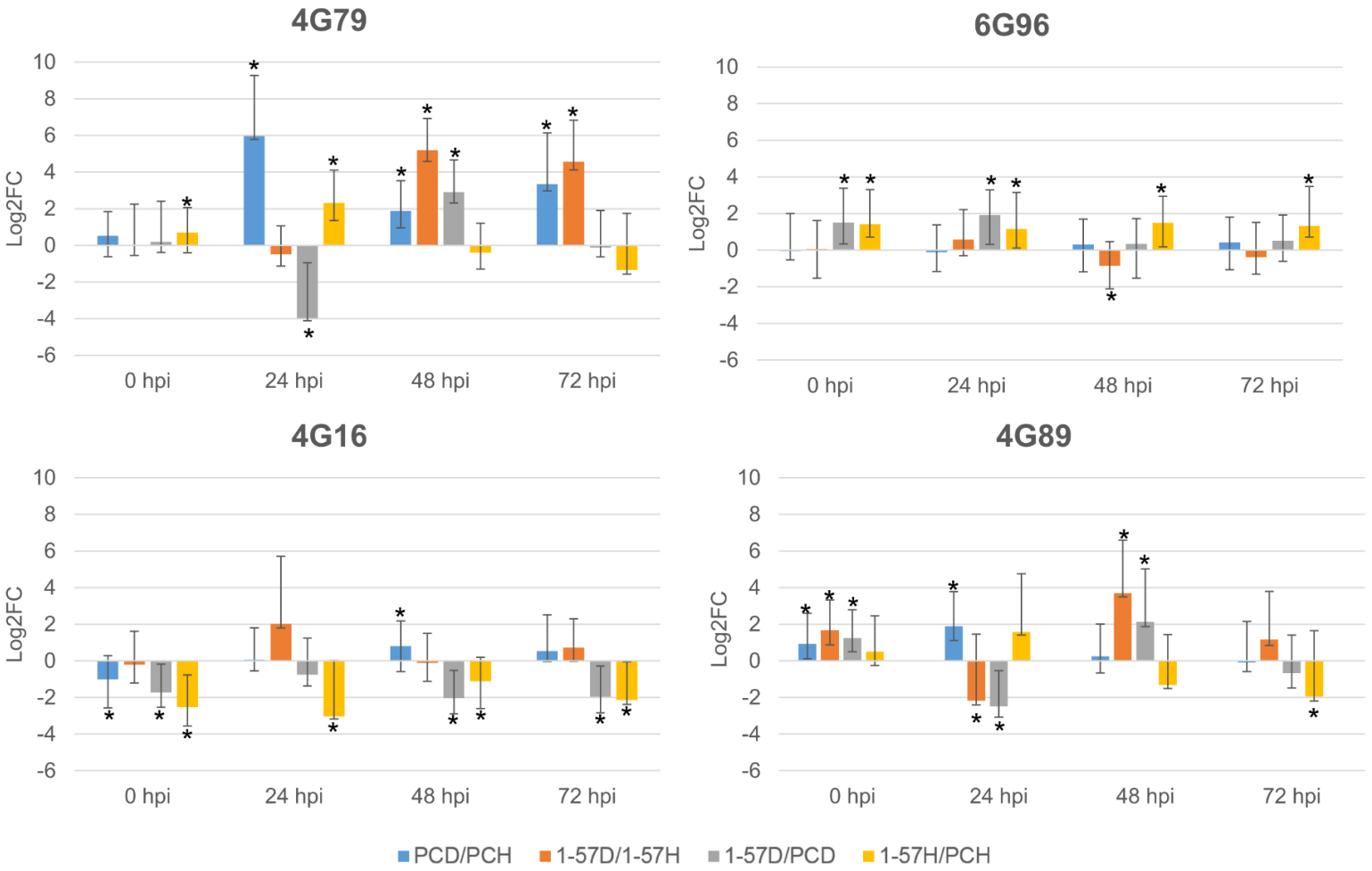

Figure 5.2: High-throughput RT-qPCR expression analysis of four genes in an incompatible and compatible interaction at different time points. 'Pariser Charme' (PC), not carrying the *Rdr1* locus, was transformed with the *Rdr1* family member *muRdr1A*, resulting in clone PC::muRdr1A-57 (1-57). Both genotypes were inoculated with the *D. rosae* isolate DortE4 (D) and mock-inoculated with water (H). Samples were taken after 0 hpi, 24 hpi, 48 hpi and 72 hpi. For better visualisation, the logarithm base 2 of the fold-change (Log2FC) was used (Fold changes are listed in Table 5.2 or Supplementary Table 2). Log2FC are averages of three independent biological replicates with three technical replicates. The reference genes *TIP*, *UBC* and *SAND* were used for normalization. Standard errors are indicated by error bars and significant expression differences (*p-value* <0.005) are indicated by an asterisk.

Table 5.2:Comparison of the regulation of 55 DEGs in the incompatible interaction and compatible interaction in MACE analysis and high-throughput RT-qPCR. The resistant genotype 91/100-5 (91), the susceptible genotype 'Pariser Charme' (PC) and the resistant clone PC::muRdr1A-57 (1-57) were inoculated with the *D. rosae* isolate DortE4 (D). Samples were taken after 0 hpi, 24 hpi, 48 hpi and 72 hpi. The fold-changes (FC) are averages of three independent biological replicates with three technical replicates. The reference genes *TIP*, *UBC* and *SAND* were used for normalization in high-throughput RT-qPCR. Significant expression differences (*p-value* <0.005) are indicated by an asterisk. HT-qPCR: High-throughput RT-qPCR. Genes mentioned in Text are highlighted in red.

			0 hpi		24 hpi			48 hpi		72 hpi		
Name	**Contig**	**Sequence Description**	**HT-qPCR 91D/ PCD** [FC]	**HT-qPCR 1-57D/ PCD** [FC]	**MACE 91D/ PCD** [FC]	**HT-qPCR 91D/ PCD** [FC]	**HT-qPCR 1-57D/ PCD** [FC]	**HT-qPCR 91D/ PCD** [FC]	**HT-qPCR 1-57D/ PCD** [FC]	**MACE 91D/ PCD** [FC]	**HT-qPCR 91D/ PCD** [FC]	**HT-qPCR 1-57D/ PCD** [FC]
0G07	RC0G0060700	Inhibitor of trypsin and hageman factor	**0.11***	1.77	1.74	**0.25***	**2.10***	**0.35***	**2.74***	0.30	**0.06***	**0.27***
0G070	RC0G0107000	pathogenesis-related PR-1	0.97	1.14	1.39	1.85	0.55	0.51	1.67	0.18	2.45	1.05
0G45	RC0G0034500	IAA-amino acid hydrolase ILR1-like 4	1.16	**2.00***	3.26*	1.30	1.16	**2.68***	1.73	2.93	**1.36***	**0.65***
0G670	RC0G0067000	4-coumarate-- ligase 2-like	**0.37***	1.34	**4.08***	**0.32***	1.36	**0.28***	**0.34***	0.50	**0.17***	**0.40***
1G01	RC1G0100100	transcription factor TGA2-like	**0.54***	0.90	1.45	**0.45***	1.01	**0.45***	**0.52***	0.67	**0.36***	**0.60***
1G21	RC1G0272100	---NA---	**3.22***	**4.42***	**22.64***	**5.72***	1.97	**2.90***	1.65	**0.12***	1.21	0.83
1G40	RC1G0594000	laccase-15-like isoform X1	0.84	4.07	0.58	**0.13***	**0.14***	1.26	**7.08***	1.00	0.88	0.50
1G63	RC1G0586300	caffeic acid 3-O-methyltransferase	**1.46***	0.87	0.95	0.62	0.90	**0.50***	0.29	0.35	**0.44***	**0.60***
2G15	RC2G0141500	cytochrome P450 84A1-like	1.10	0.72	**4.02***	1.23	1.36	**2.04***	0.56	1.99	**2.26***	**1.40***
2G17	RC2G0441700	Lactoylglutathione lyase glyoxalase I family	**0.17***	0.79	0.67	**2.28***	**3.89***	**1.87***	1.39	2.26	0.43	0.77
2G19	RC2G0071900	inositol oxygenase 2-like	0.69	**6.29***	1.23	2.26	1.60	0.68	**2.70***	**5.84***	0.46	0.64
2G33	RC2G0413300	cinnamoyl- reductase	0.63	1.00	1.40	0.81	1.20	**0.57***	**0.44***	0.68	**0.47***	**0.61***
2G47	RC2G0334700	sigma factor binding chloroplastic	0.68	**0.33***	**0.15***	1.28	**0.38***	**0.43***	**0.35***	**0.17***	**0.30***	0.41
2G51	RC2G0585100	PREDICTED: uncharacterized protein LOC105352727	**0.04***	0.34	**0.32***	2.38	3.65	0.74	**0.42***	**0.20***	**0.13***	0.38
2G91	RC2G0389100	peroxidase P7-like	**7.08***	1.65	**18.82***	0.64	1.08	1.08	1.86	3.11	0.62	0.97
3G02	RC3G0390200	Pathogenesis-related P2	0.45	2.65	0.47	0.23	**0.17***	**0.43***	**3.44***	1.22	**0.14***	**0.57***
3G11	RC3G0391100	Pathogenesis-related P2	0.93	2.57	**0.10***	0.24	**0.15***	**0.45***	**3.82***	0.86	**0.14***	**0.61***
3G26	RC3G0282600	probable anion transporter chloroplastic	1.27	**2.80***	**4.94***	0.33	0.76	**0.63***	**1.93***	0.82	0.96	1.04
3G27	RC3G0212700	phenylalanine ammonia lyase	**0.34***	0.41	1.67	**0.29***	**0.55***	**0.34***	**0.30***	0.28	0.79	**0.32***
3G36	RC3G0223600	thaumatin	**2.39***	**20.59***	**0.13***	0.21	**0.04***	**0.16***	1.57	1.32	**0.11***	1.12
3G63	RC3G0376300	serine threonine- kinase EDR1	**0.59***	1.10	1.29	**0.73***	0.92	**0.83***	0.58	0.83	**0.70***	1.00
4G16	RC4G0481600	probable serine threonine- kinase At1g18390	**0.38***	**0.30***	**0.21***	**0.36***	0.59	**0.29***	**0.24***	**0.13***	**0.47***	**0.26***
4G21	RC4G0292100	pathogenesis related PR10	1.26	3.48	1.22	**1.55***	**1.36***	1.13	0.89	1.75	1.05	**1.22***
4G28	RC4G0292800	major allergen Pru av 1-like	0.79	3.20	1.03	**0.61***	0.90	0.76	**1.42***	0.58	**0.28***	**0.53***
4G30	RC4G0473000	MLP 423	**0.08***	**0.08***	0.77	1.35	**5.11***	**0.38***	0.47	0.49	0.70	**2.60***
4G40	RC4G0344000	probable WRKY transcription factor 75	0.51	1.07	1.72	0.76	**0.35***	**3.26***	**3.42***	0.64	**0.50***	**0.41***
4G51	RC4G0045100	Phosphate transporter 1,7 isoform 1	1.80	**10.39***	0.71	**2.87***	**2.36***	1.66	1.72	0.66	**0.20***	1.08
4G79	RC4G0137900	chitinase 5-like	**1.57***	1.14	**0.17***	**0.17***	**0.06***	1.44	**7.50***	1.49	**0.25***	0.91

Continuation Table 5.2: Comparison of the regulation of 55 DEGs in the incompatible interaction and compatible interaction in MACE analysis and high-throughput RT-qPCR. The resistant genotype 91/100-5 (91), the susceptible genotype 'Pariser Charme' (PC) and the resistant clone PC::muRdr1A-57 (1-57) were inoculated with the *D. rosae* isolate DortE4 (D). Samples were taken after 0 hpi, 24 hpi, 48 hpi and 72 hpi. The fold-changes (FC) are averages of three independent biological replicates with three technical replicates. The reference genes *TIP*, *UBC* and *SAND* were used for normalization in high-throughput RT-qPCR. Significant expression differences (*p-value* <0.005) are indicated by an asterisk. HT-qPCR: High-throughput RT-qPCR. Genes mentioned in Text are highlighted in red.

			0 hpi		24 hpi			48 hpi		72 hpi		
Name	**Contig**	**Sequence Description**	**HT-qPCR 91D/ PCD [FC]**	**HT-qPCR 1-57D/ PCD [FC]**	**MACE 91D/ PCD [FC]**	**HT-qPCR 91D/ PCD [FC]**	**HT-qPCR 1-57D/ PCD [FC]**	**HT-qPCR 91D/ PCD [FC]**	**HT-qPCR 1-57D/ PCD [FC]**	**MACE 91D/ PCD [FC]**	**HT-qPCR 91D/ PCD [FC]**	**HT-qPCR 1-57D/ PCD [FC]**
4G88	RC4G0468800	probable polygalacturonase non-catalytic subunit JP650	0.57	**0.16***	1.14	**3.02***	**2.90***	1.00	**0.60***	0.78	**1.95***	0.83
4G89	RC4G0128900	cinnamoyl- reductase-related family	**0.51***	**2.36***	**0.24***	**0.25***	0.18	0.73	**4.37***	1.36	0.59	0.63
4G99	RC4G0469900	probable xyloglucan endotransglucosylase hydrolase 33	**5.03***	2.04	**47.11***	**3.40***	2.27	1.10	0.59	**145.94***	1.08	0.52
5G10	RC5G0421000	probable receptor kinase At5g24010	**3.18***	1.38	n.a.	**4.13***	1.06	**4.26***	1.05	20.39*	**3.04***	**0.71***
5G12	RC5G0381200	---NA---	0.56	**4.51***	**0.36***	**0.36***	0.79	0.54	1.26	0.47	**0.08***	1.08
5G35	RC5G0063500	---NA---	**2.95***	0.76	**0.16***	**1.72***	**0.44***	1.32	0.49	**0.16***	1.49	**0.29***
5G58	RC5G0005800	heavy metal transport detoxification superfamily	1.29	1.10	0.85	**1.78***	**2.99***	0.83	**0.32***	0.61	0.79	1.69
5G59	RC5G0235900	G-type lectin S-receptor-like serine threonine- kinase	0.85	**4.92***	n.a.	n.a	n.a	0.73	n.a	**10.26***	0.28	**0.20***
6G062	RC6G0106200	caffeoyl- O-methyltransferase	0.76	0.79	**3.09***	0.73	1.10	**0.34***	**0.25***	0.38	**0.21***	**0.30***
6G09	RC6G0380900	inorganic phosphate transporter 1-4	1.39	**3.18***	1.26	**2.12***	1.57	**2.04***	**1.68***	0.72	**0.51***	1.10
6G158	RC6G0415800	sugar transporter ERD6-like 7	0.94	**5.95***	1.52	**2.62***	1.62	**3.97***	0.93	3.83	1.07	1.22
6G51	RC6G0405100	TIME FOR COFFEE-like isoform X2	**4.73***	**4.35***	2.17	0.74	**0.57***	**2.68***	1.45	0.90	1.06	1.17
6G53	RC6G0055300	pathogenesis-related 1-like	1.10	1.35	**0.34***	0.35	**0.08***	**0.57***	1.07	0.86	**0.37***	0.77
6G558	RC6G0055800	pathogenesis-related leaf 6-like	1.22	**9.04***	1.25	0.31	0.21	0.52	**2.67***	0.69	**0.41***	**0.38***
6G562	RC6G0256200	peroxidase 12-like	0.98	**1.83***	**0.31***	0.76	0.78	1.14	0.97	1.07	**1.34***	**2.16***
6G76	RC6G0197600	MATE efflux family chloroplastic	**0.44***	**0.20***	3.78	1.48	**1.59***	**4.06***	1.25	2.79	1.71	0.93
6G96	RC6G0269600	transcription elongation factor SPT6	**5.80***	**2.86***	**5.00***	**4.88***	**3.80***	**4.59***	1.26	**4.67***	**4.73***	1.43
7G09	RC7G0550900	phosphate-responsive 1 family	0.82	1.25	**3.07***	**0.42***	0.96	**0.54***	**0.62***	1.32	0.85	**0.54***
7G12	RC7G0101200	caffeic acid 3-O-methyltransferase	**0.38***	**3.07***	1.15	**0.32***	**0.35***	1.25	1.60	0.35	**0.13***	**0.35***
7G16	RC7G0031600	lipase-like PAD4	0.77	1.02	1.17	0.49	0.57	**0.43***	**0.41***	0.39	**0.21***	**0.42***
7G20	RC7G0182000	probable WRKY transcription factor 29 isoform X1	**4.62***	**11.33***	**3.15***	0.82	1.28	**3.16***	**4.69***	1.79	1.02	0.85
7G22	RC7G0352200	probable inorganic phosphate transporter 1-9	1.99	**3.80***	1.41	**0.60***	0.74	**0.41***	0.58	0.56	**0.37***	**0.50***
7G23	RC7G0012300	SPX and EXS domain-containing 1-like isoform X1	**2.87***	**3.28***	**6.62***	**4.82***	1.17	**2.96***	1.36	**6.64***	1.43	0.79
7G046	RC7G0046000	gibberellin-regulated 6	3.85	0.63	1.18	**13.88***	**6.70***	3.36	0.68	**0.18***	**79.85***	**11.44***
7G68	RC7G0176800	disease resistance response 206-like	**0.23***	**1.98***	**3.68***	0.47	1.14	**0.57***	0.81	0.37	**0.04***	**0.10***
7G98	RC7G0109800	ethylene-responsive transcription factor ERF113-like	1.34	**4.39***	2.56	0.60	1.03	2.11	**3.17***	2.68	0.86	1.18
7G99	RC7G0109900	ethylene-responsive transcription factor ERF113-like	0.77	2.21	**4.07***	0.39	1.61	1.42	**2.73***	2.96	0.63	0.84

In addition to the gene set analysed by high-throughput RT-qPCR, a set of 137 genes significantly up-regulated in the incompatible interaction between roses and *D. rosae* (91/100-5+DortE4 and PC::muRdr1A-43+DortE4) was selected based on the MACE and RNASeq results for gene expression analysis using standard RT-qPCR (Figure 5.1-E).

Out of 137 genes, ten were chosen for further gene expression analysis. The remaining 127 genes were excluded due to unspecific amplifications in the qPCR, high deviations between the biological replicates or contradicting results (Figure 5.1-G). Further gene expression analysis revealed five genes showing stable expression differences higher than two-fold between the compatible (PC+DortE4) and incompatible interaction (PC::muRdr1A-38+DortE4) at 24 hpi and 72 hpi, respectively (Figure 5.1-G-I, Table 5.3). The results between the incompatible interaction (PC::muRdr1A-38+DortE4 *vs* mock-inoculation) compared to the compatible one (PC+DortE4 *vs* mock-inoculation) at 24 hpi are shown in Figure 5.3. Gene 0G56 was up-regulated in both compatible and incompatible interactions, with the incompatible interaction showing a 1,5-fold higher up-regulation. Gene 2G17 was 3,5-fold up-regulated during the incompatible interaction, whereas during the compatible interaction no differential regulation was observed. For 5G51, a significant up-regulation was observed only during the compatible interaction. No regulation or a down-regulation was observed for the genes 1G80 and 4G01. Further, no up-regulation of the genes 0G56 and 2G17 was observed in another incompatible system (14/29-9+DortE4 *vs.* mock-inoculation) (data not shown).

Table 5.3: List of ten genes further analysed by RT-qPCR.

Name	Contig	Annotation
0G56	RC0G0085600	cytochrome P450
1G80	RC1G0508000	NA
2G17	RC2G0441700	Lactoylglutathione lyase / glyoxalase I family protein
3G44	RC3G0304400	Peroxidase superfamily protein
4G01	RC4G0450100	MLP-like protein 31
4G21	RC4G0432100	Glucose-methanol-choline (GMC) oxidoreductase family protein
5G51	RC5G0265100	Leucine-rich repeat transmembrane protein kinase
5G73	RC5G0257300	Kunitz family trypsin and protease inhibitor protein
7G23	RC7G0092300	Pyruvate kinase family protein
7G46	RC7G0454600	serine-type endopeptidase inhibitors

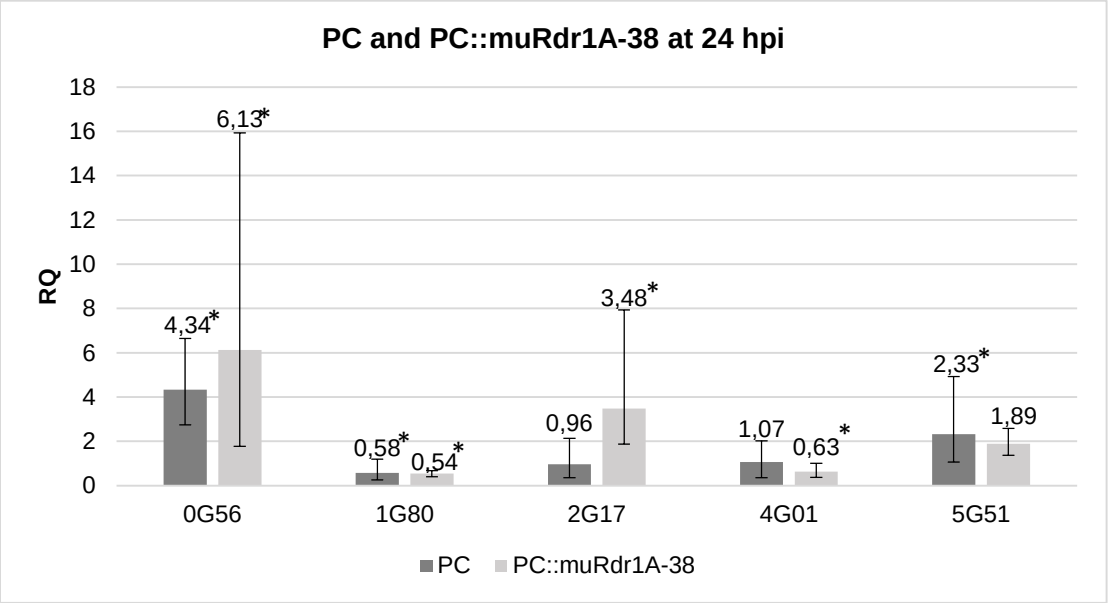

Figure 5.3: RT-qPCR expression analysis of selected genes for PC and PC::muRdr1A-38 at 24 hpi. 'Pariser Charme' (PC), not carrying the *Rdr1* locus, was transformed with the *Rdr1* family member *muRdr1A*, resulting in clone PC::muRdr1A-38. Both genotypes were inoculated with the *D. rosae* isolate DortE4 and mock-inoculated with water. The relative expression values (RQ) of each gene (0G56, 1G80, 2G17, 4G01, 5G51) are averages of three independent biological replicates with three technical replicates after inoculation compared to mock-inoculation. The reference genes *UBC* and *SAND* were used for normalization. RQ_{min} and RQ_{max} (95**%** confidence interval) are indicated by error bars and significant expression differences (*p-value* <0.05) are indicated by an asterisk.

At 72 hpi, four of the five genes were significantly up-regulated with a minimum of eightfold in the incompatible interaction (PC::muRdr1A-38+DortE4 *vs* mock-inoculation) (Figure 5.4). Comparing another incompatible interaction (14/29-9+DortE4 *vs* mock-inoculation) with another compatible interaction ('Arthur Bell'+DortE4 *vs* mock-inoculation) (Figure 5.5), gene 4G21 was up-regulated in both interactions, and the genes 7G23 and 7G46 showed no differential regulation. However, an up-regulation of the 3G44 and 5G73 genes was also observed in the incompatible interaction (14/29-9+DortE4) compared to the compatible interaction ('Arthur Bell'+DortE4 *vs* mock-inoculation). Thus, an up-regulation of two genes during the infection of *D. rosae* (72 hpi) could be detected in two different resistant genotypes compared to the susceptible ones (Figure 5.1-J).

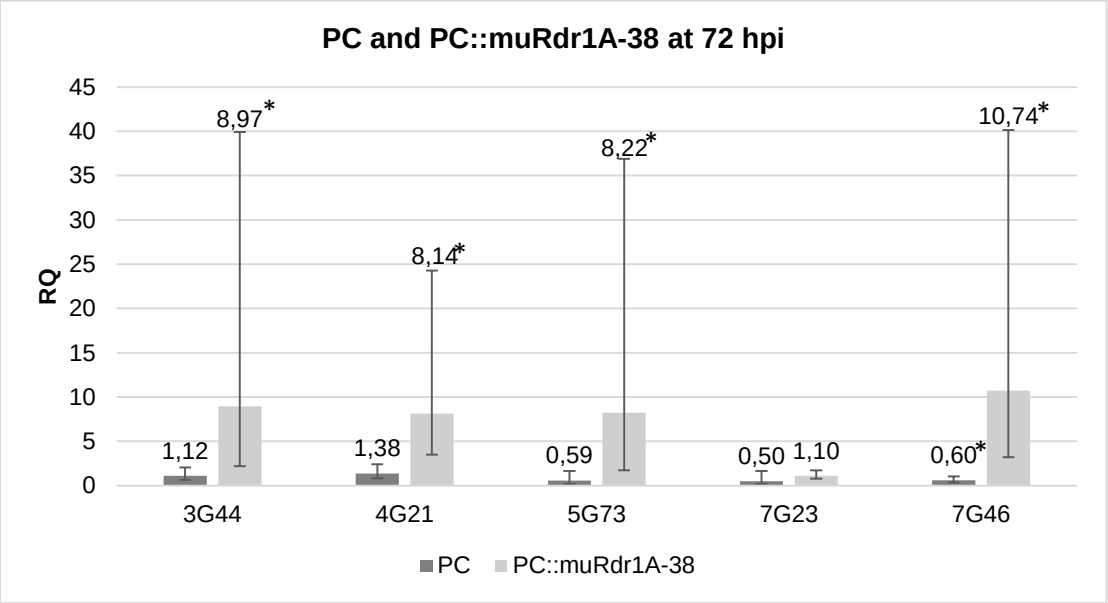

Figure 5.4: RT-qPCR expression analysis of selected genes for PC and PC::muRdr1A-38 at 72 hpi. 'Pariser Charme' (PC), not carrying the *Rdr1* locus, was transformed with the *Rdr1* family member *muRdr1A*, resulting in clone PC::muRdr1A-38. Both genotypes were inoculated with the *D. rosae* isolate DortE4 and mock-inoculated with water. The relative expression values (RQ) of each gene (3G44, 4G21, 5G73, 7G23, 7G46) are averages of three independent biological replicates with three technical replicates after inoculation compared to mock-inoculation. The reference genes *UBC* and *SAND* were used for normalization. RQ_{min} and RQ_{max} (95**%** confidence interval) are indicated by error bars and significant expression differences (*p-value* <0.05) are indicated by an asterisk.

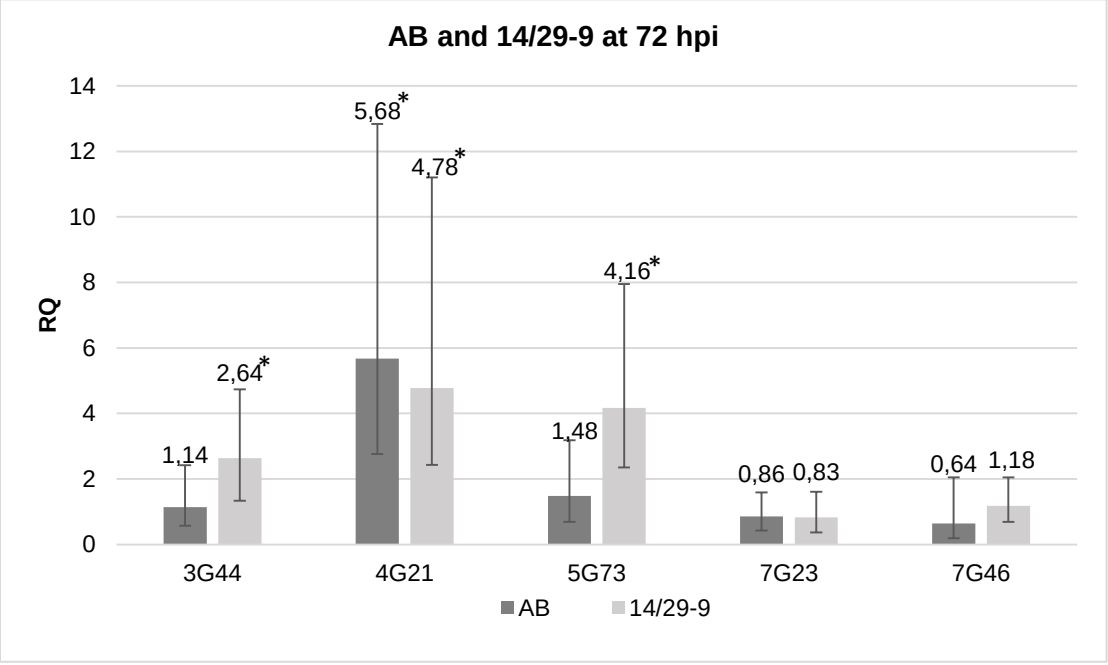

Figure 5.5: RT-qPCR expression analysis of selected genes for AB and 14/29-9 at 72 hpi. 'Arthur Bell' (AB), a susceptible genotype not carrying the *Rdr1* locus, and a progeny (14/29-9), resulting from a cross of AB x PC::muRdr1A-58, were inoculated with the *D. rosae* isolate DortE4 and mock-inoculated with water. The relative expression values (RQ) of each gene (3G44, 4G21, 5G73, 7G23, 7G46) are averages of three independent biological replicates with three technical replicates after inoculation compared to mock-inoculation. The reference genes *UBC* and *SAND* were used for normalization. RQ_{min} and RQ_{max} (95**%** confidence interval) are indicated by error bars and significant expression differences (*p-value* <0.05) are indicated by an asterisk.

5.4 Discussion

For the MACE analysis, in addition to the susceptible genotype PC (Neu *et al.* 2019, chapter 4) the resistant genotype 91/100-5 without mock-inoculation and one biological replicate of the transgenic genotype PC::muRdr1A-43 without mock-inoculation were used (Neu, 2018). These MACE data, in combination with existing RNASeq data generated by Neu (2018) and high-throughput RT-qPCR data were used as the starting point for the analysis of transcriptomic changes during the incompatible interaction compared to the compatible interaction between roses and *D. rosae*.

5.4.1 High-throughput RT-qPCR

In the high-throughput RT-qPCR analysis, only four genes showed a positive correlation with the MACE results in the resistant genotypes PC::muRdr1A-57 and 91/100-5. The transcript 6G96, coding for the transcription elongation factor SPT6, showed a significant up-regulation at 24 hpi in the incompatible interaction PC::muRdr1A-57+DortE4 compared to the compatible interaction PC+DortE4. However, a similar up-regulation at 0 hpi as well as in the mock-inoculated PC::muRdr1A-57 compared to PC (mock-inoculated) at all time points was observed, leading to the assumption, that 6G96 is not induced by *D. rosae* infection, but may have an effect on the resistance against *D. rosae* due to an altered basic expression level effected by *Rdr1.* The transcript 4G16, coding for probable serine threonine-kinase At1g18390, showed a significant down-regulation at 48 hpi and 72 hpi in the interaction PC::muRdr1A-57+DortE4 compared to PC+DortE4, but also at 0 hpi. Therefore, expression of 4G16 is not effected by *D. rosae* infection, but a role in the resistance against *D. rosae* due to an altered basic expression level effected by *Rdr1* is possible. Nevertheless, expression of *Stpk-V*, a putative serine and threonine protein kinase gene, led to high and broad-spectrum powdery mildew resistance after transformation into a susceptible wheat variety (Cao *et al.* 2011).

The fact that 4G79, coding for chitinase 5-like, showed a significant up-regulation at 24 hpi, 48 hpi and 72 hpi in the interaction PC+DortE4 compared to the mock-inoculation indicates a general defense response. The up-regulation of 4G79 only after 48 hpi in the interaction PC::muRdr1A-57+DortE4 compared to mock-inoculation could be due to a slower infection process. The selected infection method offers a high

standardization, but effects of abiotic factors (e. g. temperature and humidity) that could slow down the infection process cannot be excluded.

In addition, due to the unstable regulation of 4G89, coding for a cinnamoyl- reductase-related family, it is unclear whether this gene plays a role in the resistance against *D. rosae* or not.

In contrast to the MACE results, the significant up- and down-regulation of some genes in both incompatible interactions with *D. rosae* (91/100-5+DortE4 and PC::muRdr1A-57+DortE4 compared PC+DortE4) makes them to interesting candidate genes. For example, 7G046, encoding a gibberellin-regulated 6, showed a significant 5-fold down-regulation at 72 hpi in the MACE data, but was significantly up-regulated in both incompatible interactions with *D. rosae* at 24 hpi and 72 hpi. For both genotypes, three independent inoculations were performed and tested in high-throughput RT-qPCR with three technical replicates and three reference genes. Therefore, these results are probably more reliable than the MACE results. Gibberellins (GAs) are known as key determinants in plant-pathogen interactions (Vleesschauwer *et al.* 2013). However, inconsistent results concerning the role of gibberellins in the resistance against fungi have been obtained in previous studies: In rice, GA was shown to enhance the resistance against the necrotrophic root pathogen *Pythium graminicola* (Vleesschauwer *et al.* 2012) and induce susceptibility to hemibiotrophic pathogens like *Xanthomonas oryzae pv. oyrzae* and *Magnaporthe oryzae* (Yang *et al.* 2008). In contrast, in *Arabidopsis*, GAs promote susceptibility to virulent biotrophs and resistance to necrotrophs (Navarro *et al.* 2008; Vleesschauwer *et al.* 2014). Nevertheless, these results indicate that the induction of 7G046, encoding a gibberellin-regulated 6, could be related to the *Rdr1*-mediated resistance against *D. rosae*.

Transcript 1G40, coding for laccase-15-like isoform X1, was 10-fold down-regulated in both incompatible interactions with *D. rosae* at 24 hpi, but 7-fold up-regulated at 48 hpi in the incompatible interaction. Due to this unstable regulation, it is also unclear whether this gene plays a role in the resistance against *D. rosae* or not.

In wheat, the transcriptional level of a caffeic acid 3-O-methyltransferase (*TaCOMT-3D*) in sharp eyespot-resistant lines was higher when compared to the susceptible ones and significantly increased after inoculation with *Rhizoctonia cerealis* (Wang *et al.* 2018). This is in contrast to the results of transcript 7G12, also coding for caffeic

acid 3-O-methyltransferase, which was 3-fold down-regulated in both incompatible interactions with *D. rosae* at 24 hpi and 72 hpi. However, 7G12 is up-regulated in both, the compatible and incompatible interaction compared to the respective mock-inoculation (Electronical appendix, Table S 5.2). Caffeic acid 3-O-methyltransferases (COMT) are involved in the synthesis of primary components of lignin in dicots and lignin is thought to play a role as physical barrier against the entry of the pathogen (Miedes *et al.* 2014; Vanholme *et al.* 2010). This leads to the assumption, that the up-regulation of 7G12 during the infection with *D. rosae* compared to the mock-inoculation could be part of an early defense response, which is not related to the *Rdr1* mediated resistance.

The 10-fold down-regulation of 7G68, coding for disease resistance response 206-like, in both incompatible interactions with *D. rosae* at 72 hpi is also in contrast to other studies where the pea gene DRR206 (Disease Resistance Response-206) was shown to be induced to high, sustained levels very early in the successful resistance response of pea to *F. solani* f. sp. *phaseoli* (Culley *et al.* 1995) and constitutive expression of DRR206 conferring resistance to *Leptosphaeria maculans* in *Brassica napus* (Wang et al. 1999). However, 7G68 showed a 7-fold (48 hpi) and 18-fold (72 hpi) up-regulation in the compatible interaction and a 19-fold (48 hpi) and 6-fold (72 hpi) up-regulation in the incompatible interaction compared to the respective mock-inoculation (Electronical appendix, Table S 5.2). The up-regulation of 7G68 in the incompatible interaction at an earlier time point could be related to the *Rdr1* resistance.

Transcript 4G88, coding for a probable polygalacturonase non-catalytic subunit JP650 is up-regulated in both incompatible interactions with *D. rosae* at 24 hpi. This is in contrast to the results observed in tomatoes, where reduced polygalacturonase (PG) levels increased resistance to the fungal pathogens *Geotrichum candidum* and *Rhizopus stolonifer* (Kramer *et al.* 1992). The contradictory results to other studies and the inconsistent results between MACE and high-throughput RT-qPCR make a statement about a putative role in the *Rdr1*-mediated resistance difficult.

In other studies, the analysis of transcriptional changes during the incompatible interaction of plants with a hemibiotrophic fungal pathogen resulted in the up-regulation of receptor-like kinases, PR genes, WRKY genes, several MAPK and Myb TFs, caffeoyl-CoA O-methyltransferases, peroxidases and glutathione-S-transferases (Wang *et al.* 2010; Wang *et al.* 2014). In high-throughput RT-qPCR, several genes with

known functions in defence response, such as *PAD4* (*phytoalexin-deficient 4*), WRKY 75, PR 10, peroxidases, a caffeoyl- O-methyltransferase, receptor-like kinases were tested. However, no correlation to *D. rosae* resistance was observed. In most of studies, it is not clear if the data arrived from three independent biological replicates. This is of great importance, considering only one biological replicate can lead to contradictory results. For example, considering only biological replicate 2 for 7G046, encoding a gibberellin-regulated 6, would result in a down-regulation at 24 hpi instead of an up-regulation (data not shown).

5.4.2 Standard RT-qPCR

To expand the gene set for the analysis of transcriptomic changes caused by *Rdr1*, 137 genes were selected for standard RT-qPCR due to a significant up-regulation in the incompatible interactions 91/100-5+DortE4 and PC::muRdr1A-43+DortE4 compared to the compatible interaction PC+DortE4 in the MACE analysis and in the interaction 91/100-5+DortE4 compared to PC+DortE4 in the RNASeq analysis. From this gene set, only ten genes were chosen for further gene expression analysis due to their stable expression differences (> 2-fold) between the compatible and incompatible interaction in the first round of gene expression analysis.

At 24 hpi, only two of the five tested genes (0G56 and 2G17) showed a significant up-regulation (> 2-fold) in an incompatible interaction compared to a compatible interaction (PC+DortE4 and PC::muRdr1A-38+DortE4). However, four of the five tested genes showed a significant up-regulation higher than eight-fold at 72 hpi. The up-regulation of the genes 0G56, 2G17, 4G21, 7G23 and 7G46 seems to be genotype-specific, since no up-regulation was observed in the incompatible interaction of a progeny of ‘Arthur Bell’ and PC::muRdr1A-58 (14/29-9) inoculated with DortE4.

Nonetheless, the up-regulation of the genes 3G44 and 5G73 was also observed in the incompatible interaction (14/29-9+DortE4), leading to the assumption that they might play a role in the resistance reaction against *D. rosae*. The gene 3G44 (RC3G0304400) encodes a peroxidase superfamily protein and 5G73 (RC5G0257300) a Kunitz family trypsin and protease inhibitor protein. Therefore, both belong to the class of PR-genes. Peroxidase superfamily proteins and protease inhibitor proteins are known to be involved in plant defence responses to pathogen attacks. In *Arabidopsis*, resistance to *B. cinerea* was observed in transformed plants overexpressing class III peroxidase

(PER) family proteins and protease inhibitor (PI) family proteins (Chassot *et al.* 2007). Furthermore, peroxidase activity was increased as a disease resistance response more rapidly in resistant (*Hibiscus trionum*) compared to susceptible (*Althea armeniaca*) species in the family Malvaceae (Golubenko *et al.* 2007). Enhanced resistance against the fungus *R. solani* was also observed in transgenic tobacco overexpressing *NtKTI1*, a member of the Kunitz plant proteinase inhibitor family (Huang *et al.* 2010). Furthermore, genes related to redox state, peroxidase and glutathione-S-transferase showed a higher up-regulation at 48 hpi compared to 12 hpi, indicating an increase of ROS in infected tissues (Wang *et al.* 2014). In the incompatible interaction of roses with *D. rosae*, the up-regulation of gene 3G44, coding for a peroxidase superfamily protein, was also observed at the later time point (72 hpi). In summary, out of 137 tested genes only two genes (3G44 and 5G73) were identified to play a role in the *D. rosae* resistance in roses, independently of their genetic background. This small number might be due to the incomplete set of MACE data for the resistant genotypes (transgenic genotype PC::muRdr1A-43 and 91/100-5) used to select the genes. Both MACE and RNASeq data were only generated for three biological replicates of genotype 91/100-5 inoculated with DortE4 and not for a mock-inoculation. The finding of mainly genotype-specific regulations was probably due to the fact that the resistant genotype could only be compared with the susceptible one for the selection of the resistance related DEGs. Although a transgenic genotype (PC::muRdr1A-43) was also used for the selection, it was only based on one biological replicate. These results show that the identification of resistance related DEGs on the basis of the comparison between a resistant and a susceptible genotype can be challenging. In most of the studies, a resistant cultivar is compared to a susceptible cultivar; this can lead to artefacts as the transcriptomic differences may be caused by genotypic differences rather than by fungal infection. For this reason, it is extremely important to confirm the results in other genotypes. For future experiments, a comparison with a mock-inoculation, or preferentially an analysis in the same genetic background, would help to identify *D. rosae* resistance related DEGs.

6 General Discussion

Previous studies on black spot resistance in roses have characterised *Rdr1* as a single dominant resistance gene belonging to a cluster of nine highly similar TNL-genes (Biber *et al.* 2010; Kaufmann *et al.* 2003; Kaufmann *et al.* 2010; Malek and Debener, 1998; Terefe-Ayana *et al.* 2011). Earlier experiments to identify the functional *Rdr1* gene resulted in either *muRdr1H* or *muRdr1A* as the most likely candidate for *Rdr1* (Terefe-Ayana *et al.* 2011; Yasmin, 2011).

Experiments with the Rd1LRR microsatellite marker, present in the coding sequences of most NBS-LRR members (Terefe and Debener, 2011), showed that *Rdr1* cluster genes do not segregate into progeny. To analyse the function of the *muRdr1A* and *muRdr1H* single *Rdr1* family members, stable transgenic roses harbouring these genes were generated. Additionally, transgenic roses harbouring the functional *Rdr1* gene allow the simultaneous analysis of the resistant genotype compared to the susceptible one in the same genetic background. Therefore, the first part of the thesis was focused on the analysis of the stable transgenic plants harbouring *muRdr1A* and *muRdr1H* for their expression level, transgene copy number and their resistance against a broad spectrum of *D. rosae* isolates in order to identify the active *Rdr1* gene.

6.1 Identification of the active *Rdr1* gene

muRdr1A and *muRdr1H* were introduced on somatic embryos of the susceptible genotype PC through *Agrobacterium tumefaciens*-mediated gene transformation. The number of regenerated transgenic shoots obtained was very low. Out of 4,926 (*muRdr1A*) and 6,295 (*muRdr1H*) embryo clusters used for transformation, only 22 (*muRdr1A*) and 14 (*muRdr1H*) regenerated shoots were positive for the integration of the transgene. Similar low transformation frequencies (around 3%) were observed for the same genotype (Dohm, 2003), where the process is highly dependent on the *in vitro* regeneration system (Dohm *et al.* 2001). Southern blot analysis of the transgenic plants revealed that they were derived from a single event per gene. Due to the time-consuming generation of transgenic roses with a minimum of 9 months (Dohm, 2003), crossings were performed, instead of making new transformations, to analyse independent integration events. The use of another susceptible genotype (‘Arthur Bell’)

as crossing partner also offered the advantage of analysing the *Rdr1* function in a different genetic background.

In chapter 2 (Menz *et al.* 2018), *muRdr1A*, acting as a single TNL gene, was identified to be the functional *Rdr1* gene. As it remained unclear how the *Rdr1* resistance works, the spectrum of the *Rdr1* mediated resistance was analysed. Inoculations performed with 15 single conidial isolates belonging to six different races (classified by Whitaker *et al.* (2010b)) showed that *Rdr1* confers a broad spectrum resistance against 13 of the tested isolates. The isolates were collected in several countries in Europe as well as in Australia and South Africa.

According to McDonald and Linde (2002), a major part of the durability of resistance genes is due to the nature of the pathogen population rather than to the nature of the resistance gene. The risk of pathogens to overcome a resistance is dependent on their mutation rate, population size, gene flow and reproduction system. Pathogens producing airborne asexual spores, e. g. *Phytophthora infestans*, have a high potential for gene flow, whereas pathogens distributed mainly by splash water, e. g. *Diplocarpon rosae*, exhibit limited gene flow and thus a lower risk for plants. Furthermore, outcrossing pathogens create more new genotypes and therefore pose a greater risk than inbreeding pathogens. The propagation of *D. rosae* is mainly due to asexually produced conidia and a global analysis of black spot populations for their gene diversity indicated a slow evolution of new alleles in the *D. rosae*/rose pathosystem (Horst *et al.* 2007; Münnekhoff *et al.* 2017). Thus, the risk of *D. rosae* to overcome resistance is rather low and rose cultivars with broad-spectrum resistance against *D. rosae* could be successfully used over several years in different countries throughout the world (Lühmann *et al.* 2010; Münnekhoff *et al.* 2017).

For almost complete resistance against *D. rosae* in roses, the *Rdr1* resistance can be stacked with only a few race-specific *R*-genes against pathogenic races overcoming the *Rdr1* mediated resistance, such as R6 and AB13. In rice, broad-spectrum and durable resistance can be achieved by *R*-gene pyramiding (Cho *et al.* 2013; Ellur *et al.* 2016). In potato, the transfer of three *R*-genes from wild potato species provided complete resistance against late blight in the field over several seasons (Ghislain *et al.* 2018). Moreover, functional stacking of three broad-spectrum potato *R*-genes (*Rpi*) led to a resistance spectrum corresponding to the sum of the spectra of the three individual *Rpi* genes (Zhu *et al.* 2012).

In addition to ‘Arthur Bell’, the genotypes ‘Ausmas’ (15/1), ‘Papageno’ (15/2), ‘König Stanislaus’ (15/3) and ‘George Vancouver’ (14/33) were used as crossings partners for PC::muRdr1A-58. ‘Papageno’, ‘Ausmas’ and ‘König Stanislaus’ were susceptible against all tested isolates, whereas ‘George Vancouver’ was resistant (including R6 and AB13). All four progeny of 14/33 carried *Rdr1* and showed the *Rdr1* mediated resistance, but were susceptible against R6 and AB13 (data not shown). Nevertheless, this shows the potential of *Rdr1* for resistance breeding in roses. The resistance mediated by *Rdr1* can easily be detected in rose varieties by standard PCR. Rose varieties carring *Rdr1* can then be used as crossings partners with varieties carring R6 resistance. Such combinations of *Rdr1* with *R*-genes providing resistance to R6 are currently underway. However, this is a time consuming process, because the resistance to R6 has to be tested in an elaborate disease assay. The identification of the *R*-gene providing resistance to R6 would help to facilitate the gene pyramiding with *Rdr1*. However, gene pyramiding does not always lead to improvement of the resistance spectrum (Wu *et al.* 2019). Xiao *et al.* (2016) showed that after pyramiding of *two* resistance genes, the resistance level against rice blast was lower than that of the monogenic lines.

Furthermore, the secretome of the *D. rosae* published by Neu and Debener (2019) could help to identify the effector/*Avr* gene that is recognised by *Rdr1* and to identify new *R*-genes.

Due to the fact that the single TNL gene *muRdr1A* confers a broad spectrum resistance against *D. rosae*, it is also an interesting resistance gene regarding other rose species and other Rosaceae, since the genus *Diplocarpon* also contains pathogens infecting other members of the Rosaceae family, such as *Malus* (*D. mali*) or *Fragaria* (*D. earlianum*) (Ainsworth, 2008). Therefore, the next parts of the thesis were focused on the identification, genomic organisation and evolution of *Rdr1* family members in roses, as well as in other Rosaceae.

6.2 Identification, genomic organisation and evolution of *Rdr1*-family members

A locus homologous to *Rdr1* has been already identified at syntenic positions in *R. rugosa* and *Fragaria* (Terefe-Ayana *et al.* 2012). However, the analysis of the *Rdr1* family in roses (*R. multiflora* and *R. rugosa*) were restricted to the region captured by BAC contigs (Biber *et al.* 2010; Kaufmann *et al.* 2010; Terefe-Ayana *et al.* 2012). The recently published chromosome scale assemblies of the *R. chinensis* 'Old Blush' genomes HapOB1 (Raymond *et al.* 2018) and HapOB2 (Hibrand Saint-Oyant *et al.* 2018) allowed the identification and analysis of more *Rdr1* family members. The number of identified *Rdr1* homologues varied from 7 (HapOB1) to 21 (HapOB2). Along with the 'Old Blush' genome, several sequences from rose species from different subgenera were also published (Hibrand Saint-Oyant *et al.* 2018). In nine analysed rose species, the number of identified *Rdr1* homologues varied from 3 (*R. persica*) to 15 (*R. damascena*). The variation of identified *Rdr1* homologues could be due to the quality of the assemblies since the assembly of clustered duplicated regions is error-prone. However, the number of TNLs varies between and within genera (van Eck and Bradeen, 2018). In potato, large variations in the number of *R1* homologues within a sub-cluster were found among three haplotypes. In haplotype B, six homologues were found, whereas, in haplotype A and C, only one and two homologues were observed, respectively (Kuang *et al.* 2005).

The use of the high-quality chromosome scale assemblies of the 'Old Blush' genomes (Hibrand Saint-Oyant *et al.* 2018; Raymond *et al.* 2018) to analyse the structure of the *Rdr1* gene family resulted in a genomic organisation in two major clusters at the distal end of chromosome 1. In *Fragaria*, NBS-LRR genes were shown to occur as single loci and in clusters with an unequal distribution across the genome, often at the distal ends of chromosomes (van Eck and Bradeen, 2018). A similar structure of *Rdr1* homologues in roses was found in an updated version of the *Fragaria* genome at syntenic positions, indicating a presence of the clusters prior to the split of *Fragaria* and *Rosa*.

Phylogenetic analysis of *Rdr1* homologues identified for 'Old Blush' led to the assumption that the two clusters developed independently. With the addition of *Rdr1* homologues found in *Fragaria vesca*, genes from cluster 1 formed mixed groups, while the genes from cluster 2 (including *muRdr1A*) formed species-specific subgroups, indicating a faster evolution within cluster 2. It has been shown that the evolution of

NBS-LRR-encoding genes can be rapid or slow, depending on genetic mechanisms such as unequal crossing-over, insertions/deletions, gene conversion, point mutations, illegitimate recombination and birth and death processes (Kuang *et al.* 2004; McHale *et al.* 2006; Wicker *et al.* 2007). In lettuce, rapid (type I) and slow (type II) evolving genes belong to the major cluster of NBS-LRR encoding genes (RGC2) (Kuang *et al.* 2004). The *R1* resistance-gene cluster in *Solanum demissum* contains three groups of independently evolving type I *R*-genes with >90% nucleotide identity within each group (Kuang *et al.* 2005).

For the identification of *Rdr1* homologues in other Rosaceae genomes, *Malus* and *Prunus* showed no *Rdr1* homologues at syntenic positions (confirming earlier results of (Terefe-Ayana *et al.* 2012)). Thus, the emergence of the *Rdr1* clusters must have been after the Amygdaloideae split from the Rosoideae. Furthermore, the *Rdr1* clusters were not present at syntenic positions in *Rubus,* indicating an insertion to their current positions probably after the Rubeae split from other groups, like Roseae or Potentilleae, within the Rosoideae (after Xiang *et al.* (2016)).

The genus *Rosa* is subdivided in four sub-genera, with the subgenus *Rosa* comprising most of the species, including the ones analysed in this study (*R. xanthina*, *R. rugosa*, *R. damascena*, *R. moschata*, *R. multiflora*, *R. chinensis* and *R. laevigata*). In addition, *R. minutifolia* belongs to the subgenus *Hesperhodos* and *R. persica* belongs to the monotypic subgenus *Hulthemia* (Wissemann, 2003). A cluster analysis of the *Rdr1* family members from the ten species belonging to different subgenera showed mixed clusters for all rose sequences, with a few exceptions, indicating a close relationship between these species.

The sequence information and genomic organisation of the *Rdr1* family members described in this study may be used as a valuable source to analyse the role of related genes concerning their disease resistance in other species within Roseae, Colurieae, Potentilleae or Agrimonieae. The sequence identity of the *Rdr1* homologues could be used in gene-silencing approaches to investigate if any member of the *Rdr1* family encodes for other resistance traits. *R*-genes from one cluster were previously found to confer resistance to different pathogens (e.g. virus and nematode) as well as against different isolates from a fungus (Botella *et al.* 1998; Ellis, 1999; Kuang *et al.* 2005; van der Vossen *et al.* 2000).

6.3 Response of roses to *D. rosae* infection

Transcriptomic analysis of the compatible interaction of *D. rosae* and roses with the MACE approach showed an up-regulation of genes related to common defence mechanisms, while leaves inoculated with *P. pannosa* showed a down-regulation of genes related to photosynthesis and cell wall modification (Neu *et al.* (2019), chapter 4). A general response to both pathogens (*D. rosae* and *P. pannosa*) was mainly characterized by the up-regulation of PR10 genes, major allergens (Pru av1, Pru ar1, and Mal d 1) and chitinases, indicating a PTI reaction caused by fungal chitin or the penetration of the cuticle.

D. rosae, as a hemibiotrophic fungus, employs both biotrophic and necrotrophic infection strategies. An initial biotrophic stage is followed by the transition from biotrophy to necrotrophy and a later necrotrophic state, which is characterised by cell death. The transcriptional changes during the infection stages of hemibiotrophic fungi have been already analysed in tomato (Jupe *et al.* 2013; Zuluaga *et al.* 2016). In the compatible interaction between tomato and the hemibiotrophic fungus *Phytophthora capsici*, two major transcriptional switches associated with early infection and the biotrophy to necrotrophy transition were observed (Jupe *et al.* 2013).

With the analysis of the transcriptomic changes from 0-72 hpi, only the early stages of infection, and thus the biotrophic stage and probably also the transition stage, of *D. rosae* were analysed. At 24 hpi, the spores germinated and the first haustoria were formed; after 72 hpi, long-range hyphae and numerous haustoria were formed. Only about six days after the infection, necrotrophic intracellular hyphae were formed followed by a fungal reproductive stage in which acervuli with new conidia are formed (Blechert and Debener, 2005; Drewes-Alvarez, 2003; Gachomo *et al.* 2006; Gachomo and Kotchoni, 2007). For a precise characterisation of the stages, further analyses are necessary to determine the exact time point of the respective phase. Additionally to microscopic and macroscopic analysis of the fungus development, Zuluaga *et al.* (2016) performed an expression analysis of *P. infestans* genes used as markers for biotrophy and necrotrophy.

The majority of responses during the interaction with *D. rosae* occurred at 24 hpi and included the up-regulation of many genes encoding enzymes in the lignin biosynthetic and flavonoid pathways. The synthesis of lignin or lignin-like phenolic polymers was previously observed in response to other pathogens (Eynck *et al.* 2012; Zhang *et al.*

2007). Similar to the early response to *D. rosae*, WRKY-type transcription-factor families were also found to be induced upon the infection with *P. capsici* in tomato (Jupe *et al.* 2013). Additionally, Jupe *et al.* (2013) identified many down-regulated receptor-like kinases (RLKs) in the biotrophic stage, which may enhance the virulence of the fungus. During the transition stage in the interaction of *Phytophthora infestans* and tomato, genes including an endo-β-1,3-glucanase (GH-17), lipoxygenase, chitinase (GH-19) and *PR1* showed the highest transcript abundance (Zuluaga *et al.* 2016). Similar results were found in the interaction of *D. rosae* and roses, where PR1 was among the most highly up-regulated genes. The transcriptional changes found during the early stages of infection with the hemibiotrophic fungi in tomato are thought to be due to an initial PAMP or an effector induced-response, which is either insufficient, avoided or suppressed by the fungi (Jupe *et al.* 2013; Zuluaga *et al.* 2016). Therefore, a similar conclusion can be made for the early infection with *D. rosae*. The genotype PC also contains a number of *Rdr1* homologous with unknown function (unpublished results). Thus, an initial response could be triggered by other *Rdr1* family members, which are broken by the *D. rosae* isolate DortE4. Stable transgenic RNAi-plants of the susceptible genotype PC, where the *Rdr1* family is down-regulated, were already generated (data not shown). These plants might be used for further transcriptomic experiments in order to prove if the early response to *D. rosae* is partially mediated by *Rdr1* family members.

6.4 *Rdr1* initiated transcriptomic changes during incompatible interactions between roses and *D. rosae*

In addition to the common immune responses of roses to *D. rosae* infection, specific responses caused by *Rdr1* in the incompatible interaction between roses and *D. rosae* were analysed. The presence of stable transgenic roses harbouring *Rdr1* (Menz *et al.* (2018), chapter 2) allowed a transcriptomic analysis of an incompatible compared to a compatible interaction in the same genetic background. As transcriptomic differences can be genotype-specific, crosses with the stable transgenic roses harbouring *Rdr1* allowed an additional analysis in a different genetic background. However, for the MACE analysis, the resistant genotype 91/100-5 was compared to the susceptible genotype ‘Pariser Charme’. The use of a resistant cultivar compared to a susceptible cultivar for transcriptomic analysis during an incompatible interaction like in other

studies, e. g. in rice (Zhang *et al.* 2017) or chickpea (Saabale *et al.* 2018), can lead to artefacts as the transcriptomic differences may be caused by genotypic differences rather than by fungal infection. The use of stable transgenic plants rule out possible artefacts caused by genotypic differences, but the transformation process and the long *in vitro* culture can also introduce genetic mutations that can lead to artefacts (Arnold, 2008; Latham *et al.* 2006).

Another approach for transcriptomic analysis during the interaction of roses and *D. rosae* could be the use of one rose genotype harbouring *Rdr1* (e. g. 91/100-5 or one of the stable transgenics harbouring *Rdr1*) and two different isolates of *D. rosae*, one for a incompatible (e.g. DortE4) and one for the compatible interaction (e.g. R6). A similar approach was used for the analysis of the interaction of rice with the hemibiotrophic fungus *M. oryzae* (Wang *et al.* 2014).

In this study, MACE data were used to select genes with a potential role in the defence response of roses against *D. rosae*. From a set of 55 differently expressed genes analysed in a high-throughput RT-qPCR system and 137 genes analysed by standard RT-qPCR, two genes showed significantly higher expression in the incompatible interaction compared to the compatible interaction, independently of the genetic background. Both genes, one encoding a peroxidase superfamily protein and the other encoding a Kunitz family trypsin and protease inhibitor protein, belong to the class of PR-genes and are known to be involved in plant defence responses (Chassot *et al.* 2007; Golubenko *et al.* 2007; Huang *et al.* 2010). This low number of genes significantly up- or down-regulated in the incompatible interaction could be due to the selection of genotypic differences from MACE analysis rather than transcriptomic differences caused by *Rdr1*. Additionally, because of high variations between the biological replicates, many genes had to be excluded for further analysis. However, this also shows how challenging it is to analyse the complex interaction system of plant and pathogen.

In contrast to the MACE results, some genes were significantly up- or down-regulated in both incompatible interactions with *D. rosae* (91/100-5+DortE4 and PC::muRdr1A-57+DortE4 compared to PC+DortE4) in high-throughput RT-qPCR. The results of both, MACE analysis and high-throughput RT-qPCR are highly dependent on several factors, such as RNA quality, reference sequences/genes or bioinformatic challenges. In chapter 4 (Neu *et al.* 2019), the MACE technique could be validated using high-

throughput RT-qPCR with a correlation coefficient of 0.82. However, the variability between MACE and high-throughput RT-qPCR results seems to be higher than expected. Therefore, the high-throughput RT-qPCR results are probably more reliable than the MACE results, because three independent inoculations with three technical replicates and three reference genes were tested for all genotypes. In addition, the quality of the MACE results is dependent on the quality of the sequence assemblies. For complex genomes, like the tetraploid rose genotypes PC and 91/100-5, highly redundant sequences can lead to false positive results and it is more difficult to distinguish isoforms or splice variants. Furthermore, sequenced 3' ends of cDNAs, which include larger parts of the untranslated region (UTR) cannot be annotated (Kahl *et al.* 2012; Wang *et al.* 2009). However, the results of the genes coding for a gibberellin-regulated 6, a probable polygalacturonase non-catalytic subunit, a caffeic acid 3-O-methyltransferase, a disease resistance response 206-like and a laccase-15-like are mostly in contrast to other studies, thus it remains unclear whether these genes play a role in the resistance against *D. rosae* or not.

Further, the analysis of transcriptomic changes during a plant-pathogen interaction is dependent on the successful infection process. Abiotic factors (e. g. temperature and humidity) can slow down the infection process and lead to a time-shift in gene expression when two interaction systems are compared.

In rice, among the up-regulated genes in the incompatible interaction with the hemibiotrophic fungus *M. oryzae*, many genes showed increased expression at 12 hpi (Wang *et al.* 2014). In our study, the first time point was 24 hpi; thus, the use of an earlier time point (e.g. 12 hpi) could help to identify more DEGs related to *D. rosae* resistance in roses.

In contrast to other studies in which genes such as PR-proteins (Saabale *et al.* 2018; Wang *et al.* 2010; Wang *et al.* 2014), transcription factors (Buscaill and Rivas, 2014; Erpen *et al.* 2018; Nakashima *et al.* 2009), WRKYs (Wang *et al.* 2014), receptor-like kinases (Wang *et al.* 2014) were shown to have a function in the resistance to fungal pathogens, no correlation to *D. rosae* resistance was observed in this study. This could be due to the set of selected genes. In further experiments, the gene set could be extended to more genes known to have a function in the resistance to hemibiotrophic fungi. However, transcriptomic differences caused by *D. rosae* may be completely different from other hemibiotrophic fungi. Further, in most of the studies, it is not clear

if the data arrived from three independent biological replicates and the inconsistent results obtained in this study show that in some cases three biological replicates may be insufficient.

6.5 Conclusion

In this study, *muRdr1A*, a member of a cluster with nine highly similar TNL-genes, was identified to be the functional *Rdr1* resistance gene against *D. rosae*. In a disease assay, *muRdr1A* showed to confer a broad-spectrum resistance against *D. rosae* independently of the genetic background. Thus, *Rdr1* can be used as a tool for the breeding of rose varieties with a durable broad-spectrum resistance against *D. rosae*. The analysis of *Rdr1* family members in other rose species, as well as in other Rosaceae, revealed a genomic organisation of the family in two major clusters with different ancient origins. This information and the sequence information provided in this study can be used as a valuable source to analyse the role of *Rdr1* homologs concerning their disease resistance in other species.

The transcriptomic analysis during the compatible interaction of roses and *D. rosae* indicated an initial PTI reaction elicited by chitin or the penetration of the cuticle, which is either insufficient, avoided or suppressed by *D. rosae*.

This study also provides first insights in transcriptional changes during the incompatible interaction of roses and *D. rosae* caused by *Rdr1*. However, due to the selection of candidate genes based on the MACE results, most of the observed DEGs were probably due to genotypic differences and not by *D. rosae* infection. In addition, the high variability between the biological replicates indicates, that three replicates are insufficient to analyse transcriptomic changes during plant-pathogen interactions.

Nonetheless, the transgenic roses harbouring *Rdr1* provide a tool for analysis of incompatible and compatible interactions of roses and *D. rosae* in the same genetic background and, thus, should be used for further transcriptomic approaches to analyse the *Rdr1* mediated defence response. Furthermore, the transgenic roses harbouring *Rdr1* and their progeny offer a tool for basic research on the function of TNL genes.

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Electronical appendix

Chapter 3

Table S 3.1: Results of micro-synteny analysis outside the *Rdr1* family clusters (XLSX)

Table S 3.2: Positions and annotation of TNLs homologous to *Rdr1* on the different chromosomes of Old Blush (OB1+2), *F. vesca* (F.ve), *Prunus persica* (P.pe), *Malus domestica* (M.do), *Rubus occidentalis* (R.oc) and *Potentilla micrantha* (P.mi). (DOCX)

Table S 3.3: Coding sequences of all used genes in this study. (TXT)

Table S 3.4: Muscle alignment of protein sequences used for the phylogram shown in Figure 3.3. (TXT)

Table S 3.5: Muscle alignment of protein sequences used for the phylogram shown in Figure 3.4. (TXT)

Chapter 4

Supplementary Table 1: Summary of MACE sequencing and read mapping (XLSX)

Supplementary Table 2: MACE validation (XLSX)

Supplementary Table 3: GO Enrichment (XLSX)

Supplementary Table 4: MACE data, PC+PP vs. PC-Co (XLSX)

Supplementary Table 5: MACE data, PC+DR vs. PC-Co (XLSX)

Supplementary Table 6: Example of most highly up- and downregulated genes (XLSX)

Chapter 5

Table S 5.1: Primer sequences and expression ratios of the 137 genes used for standard RT-qPCR. (XLSX)

Table S 5.2: MACE data, primer sequences and expression ratios (FC and Log2FC) of the 55 genes used for high-troughput RT-qPCR (Chip 1 and 2). (XLSX)

Lebenslauf

Persönliche Daten

Ina Menz
Voltastraße 8
30165 Hannover
Tel: +49 1776749286
E-Mail: ina.menz@gmx.de

Geboren am 20.12.1988 in Friedberg (Hessen)

Ausbildung

Seit 01/07/2014	**Doktorandin zur Promotion** (Fachgebiet Biologie) Institut für Pflanzengenetik, Abteilung Molekulare Pflanzenzüchtung Leibniz Universität Hannover Thema der Promotion: Rolle der *Rdr1*- Familie bei der qualitativen und quantitativen Resistenz gegen Sternrußtau bei Rosen
24/03/2014	**Abschluss Master of Science** Thema der Masterarbeit: Genetische Untersuchung zur Vererbung der Blütengröße in Chrysanthemen (*Chrysanthemum*)
01/10/2011 – 24/03/2014	**Masterstudium Pflanzenbiotechnologie** an der Leibniz Universität Hannover
29/09/2011	**Abschluss Bachelor of Science** Thema der Bachelorarbeit: Diversitätsanalyse an Chrysanthemen mittels molekularer Marker
01/10/2008 – 29/09/2011	**Bachelorstudium Pflanzenbiotechnologie** an der Leibniz Universität Hannover
16/08/1999 – 06/06/2008	**Allgemeine Hochschulreife** Ernst-Ludwig-Schule Bad Nauheim
21/08/1995 – 01/07/1999	**Grundschule Ober-Rosbach**

Berufliche Erfahrungen

15/2/2010 – 31/7/2013	**Studentische Hilfskraft Institut für Erdmessung** Leibniz Universität Hannover Literaturpflege und Verwaltung der Institutsbibliothek
15/09/2010 – 16/10/2010	**Studentische Hilfskraft Institut für Pflanzenernährung** Leibniz Universität Hannover Organisation und Vorbereitung einer Tagung
01/07/2008 – 31/07/2008	**Praktikum** bei der KWS SAAT AG und der PLANTA Angewandte Pflanzengenetik und Biotechnologie GmbH

Publikationen

2019	Neu, E. Domes, H.S. **Menz, I.** Kaufmann, H. Linde, M. and Debener, T. (2019) Interaction of roses with a biotrophic and a hemibiotrophic leaf pathogen leads to differences in defense transcriptome activation. *Plant molecular biology*, **99**, 299–316.
2017	**Menz, I.**, Straube, J., Linde, M., Debener, T. (2017): The TNL gene Rdr1 confers broad-spectrum resistance to Diplocarpon rosae. In: Molecular Plant Pathology.
2016	Klie, M., **Menz, I.**, Linde, M.; Debener, T. (2016): Strigolactone pathway genes and plant architecture: association analysis and QTL detection for horticultural traits in chrysanthemum. In: *Mol Genet Genomics* 291: 957.
2013	Klie, M.; **Menz, I.**; Linde, M.; Debener, T. (2013): Lack of structure in the gene pool of the highly polyploid ornamental chrysanthemum. In: *Mol Breeding* 32 (2), S. 339–348.

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