Identification and Molecular Characterization of the *Rdr1* Resistance Gene from Roses

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Summary

The current investigation was focused on the identification and characterization of *Rdr1* resistance locus that was introgressed from the wild rose species *Rosa multiflora*. The functional characterization of *Rdr1* resistance locus led to the identification of two resistance genes active against black spot of roses; RGA1 and RGA8 later named as *Rdr1-1* and *Rdr1-8*. However *Rdr1-8* displayed more consistent and profound protection (41%) against black spot infection as compared to *Rdr1-1* (26%). *Rdr1* is the first monogenic dominant resistance gene described in the genus *Rosa* that confers resistance to black spot of roses caused by *Diplocarpon rosae*, a facultative biotrophic parasitic ascomycete. According to the previous studies the *Rdr1* resistance locus carries nine copies of resistance gene analouges (RGA) of the Toll-interleukin 1 receptor (TIR), nucleotide binding site (NBS), leucine rich repeat (LRR) type within an interval of 220Kb of DNA. In the current study all nine RGAs are named as RGA1, RGA2 to RGA9. Available sequence analysis of the Rdr1 locus revealed RGA4 as a pseudogene, disrupted by a transposon insertion of about 7 kb in its first intron emphasizing its possible inactivation due to the large size of the insertion. On the basis of sequence analysis the number of Rdr1 candidates was reduced to 8 (RGA1- 3 and RGA5- 9).

The putative 8 candidates for *Rdr1* gene were isolated by enzymatic digestion of the corresponding BACs harbouring *Rdr1* locus (155F3, 29O3, 94G8 and 20F5; Genebank of *Rosa multiflora*). The first step of the characterization of candidate genes (CGs) was the expression analysis. Initially the heterologous system of *N. benthamiana* was used to demonstrate their expression and activity against Dort E 4 (race 5 of *D. rosae*). Although all RGAs were found to be expressed suggesting the presence of required regulatory elements for the expression of CGs they did not responded to Dort E 4 inoculations and further analysis revealed the fact that the fungus cannot be propagated in tobacco. Expression profiles of RGAs in different tissues (leaves, petals and roots) of homologous system (resistant rose genotypes: 91/ 100-5 and *Rosa multiflora*) reduced the number of candidates to five; RGA 1, 3, 7, 8 and 9. Two of the remaining five

candidates, RGA8 and RGA1 were able to restrict the growth of Dort E4 significantly when used to complement the susceptible rose genotype transiently. *Agrobacterium* mediated transient homologous disease assay proved RGA8 and RGA1 as the major resistance genes that confers resistance to black spot isolate Dort E 4 in roses. The race specificity of these genes was demonstrated by observing their activity against race 6 of *D. rosae* in the transient disease assay and these were found to be inactive against race 6. The functionality of *Rdr1* locus against race 5 was also shown by reverse genetics approach. *Rdr1* locus of resistance rose genotype 91/ 100-5 was transiently knockout followed by fungal colonization that was not possible before. As a part of this study *Agrobacterium* mediated stable transformations were carried out to genetically complement a susceptible rose genotype and *Arabidopsis* that are ready for follow up studies, due to time limitations further results could not be included here.

The 5' and 3' RACE were determined for RGA8 that revealed the presence of a full length cDNA of 3369 bp encoding a predicted polypeptide of 1122 amino acids (aa) with an average molecular weight of 123.4 kDa. The deduced aa sequence show homology to TIR, NBS and LRR domains. The full length cDNA sequence for RGA3 was already available whereas for RGA7 it was determined during this investigation. The comparison of three proteins (RGA 8, 3 and 7) revealed high similarity ranging between 58-80% and presence of 119 unique amino acids in RGA8 protein. The overall trend of selection operating on the three paralogs of *Rdr1* gene family is sequence conservation however; the N-terminal halves of the genes suggest more sequence conservation when compared to C-terminal region of genes.

Key words: *Rosa*, Black spot, *Rdr1*, *Agrobacterium* mediated transient disease assay

Identifizierung und Molekulare Charakterisierung des Resistenzgens *Rdr1* aus Rosen

Aneela Yasmin

Zusammenfassung

Das Ziel der vorliegenden Arbeit war die Identifizierung des Resistenzgens *Rdr1* welches aus *Rosa multiflora* in Kulturrosen eingekreuzt worden war. Die funktionelle Charakterisierung von Kandidatengenen am *Rdr1*-Lokus führte zur Identifizierung von zwei Genen, RGA1 und RGA8, die jeweils Resistenz gegenüber Sternrußtau vermitteln können. Dabei zeigte RGA8 mit 41% eine konsistenteren und grundlegenderen Schutz gegen die pilzliche Infektion als RGA1 (26%). Der *Rdr1*-Lokus umfasst das erste genetisch charakterisierte Resistenzgen gegen Sternrußtau an Rosen, hervorgerufen durch *Diplocarpon rosae*, einen parasitischen, hemibiotrophen Askomyceten. Vorhergehende Untersuchungen hatten gezeigt, dass in einem physikalischen Intervall von 220 kb um den *Rdr1*-Lokus neun Kopien einer Genfamilie mit hoch signifikanter Ähnlichkeit zu Toll-interleukin1 receptor (TIR), nucleotide binding site (NBS), leucine rich repeat (LRR) Genen vorkommen und dass diese Gene (RGA1-RGA9) die wahrscheinlichsten Kandidatengene für *Rdr1* darstellen. Nach Analyse der Sequenzen konnte RGA4 als Kandidatengen ausgeschlossen werden, da eine 7 kb lange Insertion zur Inaktivierung führte und es daher als Pseudogen anzusehen ist. Für die verbleibenden acht Kandidatengene wurden Subklone von den vier BAC Klonen, die das DNA-Intervall des *Rdr1*-Lokus überspannen, hergestellt.

Als erster Schritt wurde die Funktionalität der potentiellen Promoterbereiche der Kandidatensequenzen durch transiente heterologe Expression in *Nicotiana benthaminiana* getestet. Alle acht Kandidatengene werden im heterologen System exprimiert, jedoch konnten so noch keine Komplementationsexperimente durchgeführt werden, da sich *Nicotiana* als Nichtwirt für Diplocarpon herausstellte und damit keine Reaktion auf die Isolate der Rasse 5 provoziert werden konnten. Die Analyse der Expressionsprofile der Genfamilie in Rosengeweben reduzierte die Anzahl echter Kandidaten auf fünf (RGA 1, 3, 7, 8 und 9). In einem transienten Komplementationssystem welches im Rahmen dieser Arbeit für Rosenblätter entwickelt wurde, konnten nur RGA1 und RGA8 das Wachstum des Isolats Dort E4 signifikant reduzieren und damit die *Rdr1* Funktion auf diese beiden Kandidaten eingrenzen. Mit

diesem Assay konnte keine Reduktion des Wachstums der Rasse 6 erreicht werden, gegen die *Rdr1* nicht wirksam ist. Die Bestätigung, dass die *Rdr1* Funktion von dieser Genfamilien bedingt wird, konnte durch einen zweiten „reverse-genetics“ Ansatz erhalten werden. Hier wurde ein RNAi-Konstrukt, welches gegen einen konservierten Bereich aller Mitglieder der Genfamilie gerichtet ist im transienten Assay getestet und erhöhte im resistenten Genotyp 91/100-5 im Vergleich zu einem *GUS*-Kontrollkonstrukt signifikant die Anfälligkeit des ansonsten hochresistenten Genotyps. Die im Rahmen dieser Arbeit begonnene stabile Transformation von Rosen sowie *Arabidopsis* mit einzelnen Kandidatengenen konnte leider aus Zeitgründen nicht abgeschlossen werden.

Für RGA8 wurden die 3´und 5´ Bereiche des Transkriptes bestimmt und die Gesamtlänge der cDNA mit 3369 Basenpaaren bestimmt, die für ein daraus abgeleitetes Protein von 1122 Aminosäuren mit einem ungefähren Molekulargewicht von 123,4 kDa kodieren. Die abgeleitete Aminosäuresequenz weist signifikante Ähnlichkeiten zu TIR, NBS und LRR Domänen bekannter TNL Gene. Neben der bereits bekannten Volllängensequenz von RGA3 wurde in der vorliegenden Arbeit auch die cDNA-Sequenz von RGA 7 bestimmt und alle drei Sequenzen miteinander verglichen. Die Ähnlichkeit auf der Ebene der abgeleiteten Aminosäuresequenz liegt zwischen 58 und 80% über das gesamte Protein mit insgesamt 119 RGA8 typischen Polymorphismen. Insgesamt ergibt sich eine höhere Konservierung des N-Terminus im Vergleich zum C-Terminus der drei Gene.

Schlagwörter: *Rosa*, Sternrußtau, *Rdr1*, *Agrobacterium* transienten Komplementationssystem

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosin triphosphate
Avr	Avirulence
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
bp	base pair
°C	Celsius grade/ centigrade
cDNA	complementary DNA
CAPS	Cleaved Amplified Polymorphic Sequence
CC	Coiled-Coil
CDD	Conserved Domain Database
CG	Candidate Gene
CNL	CC-NBS-LRR
cM	centiMorgan
DNA	deoxyribonucleic acid
dNTP	2´-deoxynucleoside 5'triphosphate
et al.	et alii (alitar)
ETI	Effector-Triggered Immunity
HR	Hypersensitive response
IRD	Infrared dye
kb	kilo base
kDa	kilo dalton
l	liter
lacZ	β-Galactosidase
LRR	Leucine-Rich-Repeat
LZ	Leucine Zipper
MAMP	Microbe-Associated Molecular Pattern
min	minute
ml	milliliter
mM	milli molar
NBS	Nucleotide-Binding-Site
NCBI	National Center for Biotechnology Information
OD	Optical Density

PAGE	Polyacrylamide Gel Electrophoresis
ORF	Open Reading Frame
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
QTL	Quantitative Trait Locus
qRT	*quantitative* Real Time PCR
PAMP	Pathogen-Associated Molecular Pattern
PC	Pariser Charme
PCR	Polymerase Chain Reaction
PR	Pathogenesis-Related
R	*Rosa*
RACE	Rapid Amplification of cDNA Ends
Rdr1	Resistance gene against *D. rosae*
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance Gene Analogue
R-gene	Resistance gene
RH	Relative humidity
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP	Resistance Proteins
rpm	rotations per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
SNP	Single Nucleotide Polymorphism
s	seconds
SSCP	Single Strand Conformation Polymorphism
SSR	Simple Sequence Repeat
Taq	*Thermus aquaticus*
T-DNA	Transfer DNA
TIR	Toll-Interleukin 1 –Receptor
TNL	TIR-NBS-LRR

1. General Introduction

Among ornamental plants, rose is the most popular and adored flowering plant. The rationale for the popularity of rose flower is thought to be due to its color, size, fragrance and other physical attributes. The rose has been considered as a symbol of love, beauty, even war and politics from way back in time. The variety, color and even number of roses carry symbolic meanings. Therefore, they have been the most popular choice of flowers for the purpose of gifting across the world. Moreover, this woody perennial shrub is used as garden plants, cut flowers and pot flowers. They are native to the northern hemisphere i.e. Europe, North America, Asia and the Middle East (Joyaux, 2003). The world's leading exporters of the cut flowers are the Netherlands, Denmark, the United States of America (USA), Columbia and Kenya (Döpper and Unterlercher, 2007). In 2008, about 365.9 million pieces of rose cut flowers and 38.4 millions of rose pot plants were sold in Switzerland (Blumenboerse, Zurich), Germany (Landgard) and the Netherlands (Dutch Auctions) alone. According to the provisional data for 2008, Germany has spent 236.512 million Euro for rose cut flower imports only (AIPH/ Union Fleurs, 2009). The broad expansion and competition in the international trade of floricultural products demand blemish free ornamentals with eye catching visual quality. However, disease susceptibly, lack of proper soil management and bad sanitation pose major challenges to produce and maintain quality roses for such markets. In addition to that the use of roses in landscaping, demands low-maintenance roses that can survive without major protection measures and pruning.

Rose diseases caused by different pathogens are one of the major causes of lower yields, weak and compromised growth, poor visual quality, and in extreme cases the death of stressed plants. The economically important pests of roses are mites, aphids, thrips, whiteflies, scale insects, weevils, caterpillar, nematodes and beetles. Whereas, the main diseases of roses include, powdery mildew (*Podosphaera pannosa),* black spot (*Diplocarpon rosae* Wolf), botrytis or grey mold (*Botrytis cinerea*), downy mildew (*Peronospora sparsa* Berk), rust (*Phragmidium mucronatum, Phragmidium tuberculatum*), and crown-gall (*Agrobacterium tumefaciens*) (Linde, 2003; Dreves-Alvarez, 2003; Gleason, 2003; Xu, 2003; Shattock, 2003). Currently, these diseases are controlled by intensive spraying of agro-chemicals that has many reservations in terms of financial costs, unfavorable environmental consequences, health hazards and legal restrictions. The most devastating fungal diseases of roses are powdery mildew, black

spot and downy mildew. Powdery mildew usually infects roses grown in greenhouses. Whereas the black spot is a problem for field and garden roses grown in humid and moist conditions throughout the world (Horst, 1983) and is controlled by continuous spraying of fungicides during spring and summer. Although the safest option for the sustainable environment is the use of resistant varieties with durable genetic resistance, most of the cultivated roses lack natural resistance against black spot. Economic importance of rose as an ornamental plant, general disease susceptibility of cultivated roses combined with concerns over the use of pesticides portraits a scenario which favors breeding for resistant rose varieties as an important goal for many rose breeding programs. Numerous wild roses, resistant to black spot are reported. These include *R. bella, R. californica* v. *plena, R. majalis, R. nanothamnus, R. multiflora, R. rugosa, R. wichuraiana, R. roxburghii, R. virginiana, R. carolina and R. laevigata* (Schulz *et al.,* 2009; Drewes-Alvarez, 2003). The introgression of the natural genetic resistance to modern roses is very suitable strategy for creating resistant cultivars.

So far the conventional and molecular methods of breeding possess some limitations to manipulate rose, owing to high heterozygosity, polyploidy and limited knowledge of the genetic make-up of roses. Meanwhile, the exploitation of natural genetic resistance also requires understanding of the resistance genes in terms of diversity, genomic organization and functionality. In this regard, our research group has previously characterized a single monogenic dominant resistance gene locus (*Rdr1*) against black spot (*Diplocarpon rosae*) through phytopathological methods in tetraploid roses (Debener *et al.,* 1998; Von Malek and Debener, 1998). Additionally, *Rdr1* was mapped to a telomeric position of linkage group 1 in the diploid population 94/1 (Debener and Mattiesch, 1999; Von Malek and Debener, 2000). The construction of two large insert BAC libraries (Kaufmann *et al*., 2003; Biber *et al.,* 2009) and recent sequencing of BAC clones identified the location of *Rdr1* gene within a 220kb region (Biber *et al.,* 2009). The 220 kb region contains 9 copies of "resistance-gene-analogues" sequences (RGA) of the T1R-NBS-LRR type of resistance genes (Terefe *et al.,* 2010). As the only known function of this class of genes is resistance and no other resistance like sequence could be detected within the region there is a chance that one of the 9 genes is the functional gene.

The current research was aimed to functionally characterize the *Rdr1* resistance gene family of roses and to identify the key single gene that confers resistance to black spot. Concomitantly, on one hand plant pathogen interaction studies with particular reference to functional genomics will provide the basis for conventional and molecular resistance breeding and on the other hand they will help to identify and isolate the functional rose resistance genes.

2. Review of Literature

2.1. Rose

2.1.1. Taxonomy and classification:

The genus *Rosa* comprises of about 200 different species (Wissemann, 2003a) with a basic chromosome number of 7. The ploidy levels for wild species range between diploid to octoploid (Gudin, 2000), however, most of the modern cultivars are tetraploid. Cultivated roses comprise a huge genetic variability with more than 20000 varieties as they are heterozygous outcrossers. Nevertheless, only eight to ten wild species had a major contribution in rose cultivation during the last 2000 years (Chandler and Lu, 2005; Gudin, 2000).

The genome complexity in terms of various modes of reproduction and character inheritance make the infrageneric taxonomy of the genus *Rosa* very difficult (Wissemann and Ritz, 2005; and 2007). The most useful classification in use is the one defined by Alfred Rehder (1960) with some modifications. According to this classification, the genus *Rosa* is divided into four sub genera i.e. *Hulthemia, Hesperrhodos, Platyrhodon* and *Rosa*. The subgenus *Rosa* is further divided into 10 sections (Wissemann, 2003) (Table 1.1). On the contrary, the most popular classification of cultivated roses is the one formatted by the American Rose Society and the World Federation of Roses (Cairns, 2003). Consistent with that classification, roses can be classified into three groups: the species; Old Garden Roses; and Modern Roses. Wild roses bloom once a year with a flower of four to five petals. In the varieties, all roses recognized before 1867 are classified as Old Garden Roses. They usually bloom fragrant flowers once per season as summer starts and some of their varieties include China, Tea, Moss, Damask, Bourbon, Hybrid Perpetual, and Noisette roses. All roses recognized after 1867 are grouped as Modern Roses. They produce flowers many times a year and some of their important varieties include hybrid Tea, Floribunda, and Grandiflora (Cairns, 2003).

Table 1.1: Subdivisions* of the subgenera *Rosa.*

Section	Species No.	Origin	Chromosome No.	Subdivisions
1. *Pimpinellifoliae* (DC.) Ser. 1825	15	Asia, Europe	2n=2x, 4x=14, 28, balanced	uncertain
2. *Rosa* (=sect. *Gallicanae* (DC.) Ser. 1825)	1	Asia, Europe	2n=4x=28	Many taxa given species rank
3. *Caninae* (DC.) Ser. 1825	50	Europe	Unbalanced heterogamous fully sexual reproduction 2n=4x, 2n=5x, 2n=6x	Six sub sections
4. *Carolinae* Crep. 1891	5(?)	North America	2n=4x=28 2n=2x=14	NA
5. *Cinnamomeae* (DC.) Ser. 1825	Uncertain (80)	Asia, Europe, North America	2x, 4x, 6x, 8x	NA
6. *Synstylae* DC. 1813 (diploid climbers)	25	Asia, Europe, North America	2n=2x=14, 3x=14, 21	NA
7. *Indicae* Thory 1820	3	China	2n=2x, 3x, 4x= 14,21,28	NA
8. *Banksianae* Lindl. 1820	2(?)	China	2n=2x=14	NA
9. *Laevigatae* Thory 1820	1	China	2n=2x=14	NA
10. *Bracteatae* Thory 1820	1or 2	South East Asia	2n=2x=14	NA

* This division is based on different physical features of wild roses as stipules, sepals, blooms, styles, leaves, thorns, etc. (Cairns 2003; Wissemann 2003).

2.1.2. Genetics of rose traits

Plant geneticists and molecular biologists have paid very little attention to this thorny shrub may be due to the problems related to its polyploid nature, germination, reproduction and/or fertility. However, during the last two decades there are continuous attempts to generate valuable genomic resources for these inspiring ornamentals (reviewed by Byrne, 2009; Table 1.2). Dugo and colleagues (2005) mapped 13 QTLs in total controlling simple flowers with five petals to double flowers, powdery mildew resistance, leaf size, flowering time and size of the flowers. The gene *t4* was identified as a QTL controlling the number of prickles on the stems (Crespel *et al.*, 2002). In addition to that four marker maps are also constructed, 3 in diploid (Debener and Mattiesch, 1999; Crespel *et al.*, 2002; Dugo *et al.*, 2005; Yan *et al.*, 2005) and one in tetraploid rose population (Rajapakse *et al.*, 2001).

Table 1.2: Some gene loci mapped in roses (Debener and Linde, 2009)

Gene	Function	References
Blfo; d6	Transition of stamens to petals, double flowers	Debener and Mattiesch (1999) Crespel *et al.*, (2002) Dugo *et al.*, (2005)
Blfa	Pink flower color	Debener and Mattiesch (1999)
*Rdr1** *Rdr3*	Resistance to rose black spot	Von Malek *et al.*, (2000), Kaufmann *et a*l., (2003) Whitaker *et al.*, (2010)
Rpp1	Resistance to rose powdery mildew	Linde *et al.*, (2004)
prickles	Prickles on the petioles	Rajapakse *et al.*, (2001)
r4	Recurrent blooming	Crespel *et al.*, (2002)

*Rdr1** is the first single dominant resistance gene described in the genus *Rosa*. It confers resistance against black spot race 5 (Dort. E4). The resistance due to *Rdr1* is assumed to follow the gene-for-gene model (Von Malek and Debener, 1998).

2.2. Black spot disease in roses

Black spot of roses was first reported in 1815 by Fries in Sweden (Drewes-Alvarez, 2003). The causative agent of this foliar disease of roses is *Diplocarpon rosae*, a plant pathogenic ascomycete that belongs to the family of Dermateaceae (Nauta & Spooner, 2000). The conidial or imperfect stage of the fungus is known as *Marssonina rosae* (Lib.) Lind (Baker, 1948). *Diplocarpon rosae* flourishes in humid and wet conditions of spring. During infection it produces ascospores, and conidia that are dispersed by rain

and direct contact through arthropods to healthy plants. Usually, infection starts from lowest leaves and progresses upward causing early defoliation, loss of vigour and in extreme cases death of the plant (Bhashkaram *et al.*, 1974). *D. rosae* has been differentiated into at least 15 physiological races (Whitaker *et al.*, 2007; Yokoya *et al.*, 2000; Debener *et al.*, 1998; Svedja and Bolton, 1980), though the use of uniform testing rose material may reduce the number of races. In the research group of Debener at the University of Hannover, Germany, AFLP and SSR analysis has been conducted to characterize the genetic diversity within a collection of *D. rosae* isolates. Preliminary results demonstrated a lower genetic complexity of populations indicating a low mobility compared to airborne pathogens (Blechert, 2005; Luhmann, 2009).

2.2.1. Pathology

The detailed infection cycle of *D. rosae* was originally described by Aronescu (1934) and Frick (1943), during the early 20th century. *D. rosae* is a hemibiotroph having a biotrophic phase characterized by haustoria and a necrotrophic phase characterized by necrotrophic intracellular hyphae (Gachomo *et. al.*, 2006). The hemibiotrophic fungus produces asexually two celled conidia that under favourable conditions germinates and penetrates the cuticle of the leaf producing hyphae and appressoria followed by haustoria. The host damage can be visualized macroscopically within 10-12 days of infection (Blechert and Debener 2005). The disease symptoms include brown or dark black spots usually surrounded by chlorotic areas on leaves leading to an early defoliation of the host (Horst, 1983). Moreover, Blechert and Debener (2005) characterized the morphology of various interactions of *D. rosae* and roses in 8 different types; 5 types of them were found as susceptible with different developmental levels of prolonged hyphae and formed acervuli, whereas 3 types of them were found as resistant interactions lacking any visible fungal structures beneath the cuticle or having penetration hyphae and haustoria in epidermal cells and necrosis of single or larger cell clusters (HR).

2.2.2. Disease control

Conventionally, black spot is controlled by integration of different approaches e.g. planting under sun-shine to keep the foliage dry, in good sanitation, without overhead watering and along with intensive application of fungicides throughout the growing season. Effective fungicides viz. propiconazole, mancozeb, chlorothalonil, benomyl, or

a copper-sulphur dust are sprayed shortly after bud break and usually continue at regular intervals until the first hard frost (Bowen and Roark, 2001). These regular sprays are very significant for proper disease control as black spot may develop at any time during wet and humid conditions (Walker *et al.*, 1996; Reddy *et al.*, 1992). On the contrary, the continuous use of fungicides poses serious legal restrictions and environmental concerns. Therefore, growing black spot resistant rose varieties could be the most effective and safe option.

2.3. Rose breeding for disease resistance (Black spot)

Introduction of natural resistance of wild species to cultivated roses through conventional methods of breeding is a lengthy process and requires about 10 or more years. This approach will add undesired wild background in existing regular varieties which needs many generations of crosses for elimination (Drewes-Alvarez, 2003). However, recent developments in biotechnology and genetic engineering, with particular reference to the transfer of foreign genes into plants, gene isolation, identification and functional analysis have opened new insights and novel prospects for the alteration of single traits in already successful varieties (Chandler and Lu, 2005). There are reports describing the transfer of genes for pathogenesis-related proteins (Marchant *et al.*, 1998), ribosome inactivating proteins (Dohm *et al.*, 2001) and phytoalexins (Lorito *et al.*, 2002) to increase the plant resistance against pathogens. Plants produce chitinases and glucanases in response to pathogen attack and their over-expression could improve resistance to fungi. A rice gene for chitinase was transferred into rose callus reducing the severity of black spot disease up to 13-43% (Marchant *et al.*, 1998). In addition to that, genes for ribosome inactivating proteins and barley genes for chitinases and glucanases were transferred into rose embryos by *Agrobacterium*. Their over expression reduced the black spot infection up to 60% (Dohm *et al.*, 2002). But this level of disease resistance achieved by transgenic plants is not enough to develop a resistant variety. On the contrary, the transfer of a disease resistance gene active against black spot using molecular tools is thought to be a very promising option of producing black spot resistant rose varieties in short period of time. The first step towards this landmark is to find the sources of black spot resistance in wild and cultivated roses. Many rose genotypes have been reported as resistant against many major diseases. Field and laboratory evaluations have proved many rose species highly resistant to black spot. These include *R. bella, R. californica* v. *plena, R. majalis, R.*

nanothamnus, R. multiflora, R. rugosa, R. wichuraiana, R. roxburghii, R. virginiana, R. carolina, and *R. laevigata* (Schulz, 2009; Drewes-Alwarez, 2003). Disease resistance could be conferred by single or many genes and these genes may reveal race specific or non-race specific resistance. Additionally, the alleles may be dominant or recessive. It is therefore, very important to know the genetic nature of disease resistance carried by these species to exploit the maximum potential of rose genotypes resistant against black spot.

2.3.1. Plant pathogen interplay

Plants have evolved multiple defense strategies to counteract biotic stresses. These include passive or pre-existing defenses as waxes, cuticle, apoplastic space, stable cell wall and inducible defenses (Goehre and Robatzek, 2008). Pathogens overcome some of the passive defenses by secreting hydrolytic enzymes or utilizing natural openings as stomata, hydathodes or wound sites to invade the apoplast space (Jones and Dangl, 2006). Some pathogens exclusively stay in the apoplast (Ellis *et al.,* 2009); other pathogens such as bacteria use a type III secretion system (T3SS) to inject effector proteins through the cell wall and plasma membrane, whereas fungi and oomycetes penetrate their hyphae through the cell wall and form haustoria (feeding structures) surrounded by the host plasma membrane (Ellis *et al*., 2009). The inducible or active line defenses can detect microbe associated molecular patterns (MAMPs or PAMPs) as a bacterial flagellin or fungal chitin by pattern recognition receptors (PRRs), which usually reside in plasma membrane and trigger MAMPs-triggered-immunity (MTI or PTI) (Bent and Mackey, 2007). MTI activates signaling cascades involving Ca++ fluxes and mitogen activated protein kinases (MAPKs) leading to defense reactions involving production of reactive oxygen species (ROS), deposition of callose in the cell wall, and expression of pathogenesis related proteins and defensins (Zipfel, 2009; Bolton, 2009; Pitzschke *et al*., 2009). However, some pathogens are able to suppress MTI by injecting effector proteins which can also reinforce plants defenses by encoding resistance proteins (RP). RPs recognize specific effectors directly (gene-for-gene hypothesis) or indirectly (guard hypothesis) resulting in effector-triggered-immunity (ETI); these R-genes are the classical single locus, race specific R-genes. The typical symptoms of ETI are hypersensitive response (HR) or programmed cell death (PCD) and systemic acquired resistance (SAR) (Shah, 2009; Zipfel, 2009; Goehre and Robatzek, 2008; Heath, 2000).

Table 1.3: Different families of plant resistance proteins (Vidhyasekaran, 2008)

RP-Families	Localization	Reference	
TIR-NBS-LRR proteins	Cytoplasm as peripheral membrane protein	*L6*- flax *N*- tobacco *RPP1- Arabidopsis*	Lawrence *et al.,* 1995 Whitham *et al.,* 1994 Botella *et al.,* 1998
Non-TIR-NBS-LRR proteins	Cytoplasm as peripheral membrane protein	*Lr10*- wheat *Mla1*-barley *RB*- potato	Feuillet *et al.,* 2003 Halterman *et al.,* 2001 Song *et al.,* 2003
LRR proteins without NBS domain	Extra-cytoplasmic LRRs anchored to a trans-membrane domain Or LRR-kinase with eLRR fused to a cytoplasmic serine-threonine kinase domain (KIN)	*Cf2/5*- tomato *Vfa1-4*- apple *Xa21*-rice	Dixon *et al.,* 1996 Xu and Korban, 2002 Song *et al.,* 1995
Proteins without LRR domain	NBS-LRD proteins Intracellular protein kinases *Serine/threonine protein kinase* Trans-membrane proteins *Small proteins with N-terminal trans-membrane and CC-domains* Lectin type proteins Heat shock protein like proteins NADPH-dependent reductase type proteins Photorespiratory peroxisomal enzyme proteins	*Pi-ta* – rice *Pto* – tomato *RPW8.1– Arabidopsis* *RTM1- Arabidopsis* *RTM2- Arabidopsis* *HM1*- maize *At1, At2*- melon	Bryan *et al.,* 2000 Martin *et al.,* 1993b Loh and Martin, 1995 Xiao *et al.,* 2001;2003 Chisholm *et al.,* 2000 Whitham *et al.,* 2000 Johal and Briggs, 1992 Taler *et al.,* 2004

2.3.1.1. Plant resistance genes

Resistance genes usually provide two types of resistance i.e. qualitative or quantitative. The qualitative resistance is governed by major dominant or recessive genes whereas quantitative resistance depends on many minor genes and is assumed as non-race specific resistance without any hypersensitive response (Ovesna *et al.,* 2000). The major genes confer race specific resistance following the gene-for gene hypothesis of Flor (1956; 1971). As plants solely depend on innate immunity they may contain hundreds of disease resistance genes (Young, 2000; Eckardt and Innes, 2003) that are amplified in high number and are positioned in the genome in a way that favors their rapid evolution (Fluhr, 2001). These resistance genes are classified in different families or groups depending on the structural domains of proteins they code (Table 1.3). Many recessive genes providing very high level of resistance are also cloned. The *mlo* gene of barley, not a classical R-gene, is a calmodulin binding transmembrane protein that confers non race specific resistance to powdery mildew (Bueschges *et al.,* 1997; Kim *et al*., 2002). A gene *pmr6* of *Arabidopsis* encodes pectate lyase-like protein and provide resistance against powdery mildew (Vogel *et al*., 2002). In addition to that *RRS1-R* recessive gene of *Arabidopsis* encodes a protein having a molecular structure of TIR-NBS-LRR domains and a WRKY motif (Deslandes *et a*l., 2002; 2003). According to Shirano and coworkers *ssi4* is a recessive resistance gene of *Arabidopsis* of TIR-NBS-LRR type (Shirano *et al*., 2002). This gene promotes constitutive expression of pathogenesis related proteins and suppress the growth of bacteria *Pseudomonas syringae* pv. *maculicola* and oomycete pathogen *Hyaloperonospora parasitica.*

2.3.1.2. NBS LRR gene family

So far, more than 40 plant resistance genes have been cloned from different plant species, the majority of which belong to the NBS-LRR resistance gene family (Lukasik and Takken, 2009; Jiang *et al*., 2007). These genes are found as single genes and/or as tight gene clusters. The only known function of this gene family in plants is elicitor recognition and activation of downstream signal pathways leading to disease resistance (Lorang *et al.*, 2007). This family is further subdivided into two groups based on the structural differences at the amino terminus. The first group contains a TIR motif with homology to toll/interleukin-1-receptor (TIR), whereas the second group has a coiled coil (CC) domain, also sometimes referred as leucine zipper (LZ) (Pan *et al*., 2000). In addition to that the TIR group has an aspartic acid (D) as the final amino acid of the

kinase 2 in the NBS domain, whereas non-TIR group has a tryptophan (W) on this place (Pei *et al*., 2007).

The NBS is a part of a nucleotide binding (NB)-ARC domain that belongs to the STAND (signal transduction ATPases with numerous domains) family of NTPases. These proteins are proposed to regulate signal transduction as NB domain hydrolyzes NTP and changes its conformational states (reviewed in Takken *et al*., 2006). The structure of the NB-ARC domain of RPs was derived from the crystal structures of APAF-1 or CED-4 (Takken *et al.*, 2006). The alignment of APAF-1 with RPs revealed three sub-domains conserved in NBS-LRR proteins: a P-loop NTPase fold forming a parallel β-sheet flanked by α-helices, an ARC1 consisting of a four-helix bundle, and an ARC2 adopting a winged-helix fold that is connected to the LRR domain by a short linker. LRR domains contain various numbers of tandemly repeated leucine-rich motifs with a conserved core consensus of L-X-X-L-X-L-X-X-N that form a series of β-strands (Jiang *et al*., 2007; Wroblewski *et al*., 2007; Fluhr, 2001). The arc-shaped structure of the LRR domain suggests its role in different intra and intermolecular interactions of direct recognition of pathogen effectors, regulating protein activation and signal transduction (Padmanabhan *et al.*, 2009). However, the mechanisms that make these dynamic functions possible await exploration. The N-terminus of NBS-LRR proteins is structurally diverse having homology to TIR or CC domain, as described earlier. The proposed functions of the N-terminal domain are downstream/ upstream signaling and pathogen recognition (Lukasik and Takken, 2009; Takken *et al.*, 2006; Fluhr, 2001).

Typically, RPs activate a HR/PCD to halt the growth of a pathogen (Goehre and Robatzek, 2008). However, sudden activation of these proteins can damage plants themselves which suggests a tight regulation of their activation. This inactivation of RPs is achieved by intramolecular interactions between the various domains (Autoinhibition). According to the proposed model for the activation of NBS-LRR proteins (Lukasik and Takken, 2009; 2006; Bent and Mackey, 2007), these proteins are in resting (ADP) or off state in the absence of a pathogen. Detection of pathogen elicitors releases this tight negative control by conformational changes in the LRR and ARC2 sub-domain (Induced state) followed by the exchange of a nucleotide that triggers the active state of RPs ready to interact with downstream signaling components and activate defense responses. The perception of pathogen elicitors is accomplished by

either direct (gene-for-gene model) or indirect manner (guard or decoy model) (Hoorn and Kamoun, 2008). However, it is reported that the TIR or CC domain mediates indirect recognition in the majority of such cases, whereas the LRR domain mediates direct recognition of the pathogen elicitors (reviewed in Padmanabhan *et al*., 2009).

2.3.2. Positional cloning of resistance genes

One of the most traditional and unbiased approaches for the identification of genes governing important heritable traits is the positional or map based cloning. This laborious method of discovering genes searches whole genome without any prior knowledge of the physiology, biology and/or the role of the genes. There are many examples of R-genes that were isolated by positional cloning and demonstrated to act against a range of pathogens (Staskawicz *et al.,* 1995; Ballvora *et al.,* 1995; Bent, 1996; Hammond-Kosack and Jones, 1996; and 1997; Gebhardt, 1997). However, this approach is limited by the genome size, number of the genes within the locus of interest, presence of transposons/ repetitive sequences in the species being investigated and time required for the complementation test. An alternative strategy could be the candidate gene (CG) approach, based on the assumption that the loci controlling the trait of interest are carrying the genes of biologically known function (Pflieger *et al.,* 2001). Human, animal and plant geneticists have successfully utilized this approach to reduce the number of candidate genes since the 1990s (Rothschild and Soller, 1997; Byrne and Mc-Mullen, 1996). These candidate genes can be classified as functional CGs when based on molecular or physiological studies or as positional CGs when based on linkage data of the locus of interest. Several genes have been screened and mapped using this approach including the *CO* (constans) gene of *Arabidopsis*, involved in late flowering (Putterill *et al.,* 1995), CGs for fruit quality in peach (Etienne *et al.,* 1999) and in tomato (Causse *et al.,* 1999), plant height QTLs in maize (Beavis *et al.,* 1991), QTLs affecting flowering time in *Arabidopsis* (Koornneef *et al.,* 1998), cloning and isolation of major disease resistance genes in several species as potato(Paal *et al.,* 2004), tomato (Pan *et al.,* 2000), pepper (Pflieger *et al.,* 1999), lettuce (Woo *et al.,* 1998; Shen *et al.,* 1998), common bean (Geffroy *et al.,* 1999a; Rivkin *et al.,* 1999) soybean (Kanazin *et al.,* 1996; Yu *et al.,* 1996), *Rosaceae* family (Samuelian *et al.,* 2008; Martinez Zamora *et al.,* 2004; Lee *et al.,* 2003; Baldi *et al.,* 2004; Lalli *et al.,* 2005; Soriano *et al.,* 2005; Xu *et al.,* 2005). These are structural similarities between resistance genes isolated from different plants that made it possible to clone R-genes using the candidate gene

approach. The cloning of potato Gro1 gene, conferring resistance to nematodes, was reported without prior construction of a physical map (Paal *et al*., 2004). The *Gro1* resistance locus active against all pathotypes of *G. rostochiensis* initially localized on potato chromosome VII (Barone *et al.,* 1990) followed by high resolution mapping that restricted *Gro1* locus to 1.4 cM (Ballvora *et al.,* 1995). Later on conserved sequences between resistance genes *N* (tobacco- Whitham *et al.,* 1994) and *RPS2* (*Arabidopsis*- Bent *et al.,* 1994; Mindrinos *et al.,* 1994) were used to isolate RGAs from potato genome; two of the isolated RGA fragments *St322*, and *St334* found to co-localized with *Gro1* and identified a cluster of genes at *Gro1* locus (Leister *et al*., 1996). Paal and colleagues utilized this information and assumed that the resistance gene like marker *St322*, co-localized with *Gro1* was identical to the nematode R gene. Using this marker they isolated 15 candidate genes from genomic libraries. Inheritance analysis, linkage mapping and sequencing reduced the number of candidates to three. Stable genetic complementation of potato validated that the gene *Gro1-4* provided resistance against *G. rostochiensis* pathotype *Ro1*. In soyabean degenerate primers identified nine classes of RGAs those were located to eight different linkage groups by genetic mapping near known resistance genes (Kanazin *et al.,* 1996). However, the most successful and updated strategy for the identification of R-genes is the combination of positional cloning and candidate gene approaches. The final validation of a CG is usually provided through physiological analysis, genetic transformation and/or sexual complementation (Byrne and McMullen, 1996; de Vienne, 1999). The genetic complementation of a deficient phenotype and/ or silencing the under test gene in a non-deficient phenotype are the most popular approaches to prove the functionality of a CG (Pflieger *et al.,* 2001). In addition genetic complementation could be achieved by generating transformants that are expressing the CGs stably or transiently depending on the number CGs and time frame needed for the complementation assays.

2.3.3. *Rdr1* background studies

The genetic characterization of *Rdr1* was started in our research group by investigating the interaction of single conidial isolates of black spot on wild and cultivated roses. This interaction resulted in the identification of five different physiological races of black spot, *Diplocarpon rosae*, for roses (Debener *et al.,* 1998). In this study, a so called quadratic check implied as first evidence for the presence of a gene-for-gene relationship between black spot and roses. Meanwhile extensive phytopathological

analysis in tetraploid rose populations were performed and the segregation ratios of the resistance reaction against the black spot isolate DortE4 indicated the presence of a single dominant resistance locus in the duplex configuration (RRrr), which they called *Rdr1*.

The first linkage map for diploid roses was constructed using RAPD and AFLP markers in a population of 60 F1 plants (Debener and Mattiesch, 1999). The hybrid population resulted from a cross between the diploid rose genotypes 93/1–117 and 93/1–119. In addition to molecular markers the map also showed the location of two genes controlling important morphological traits, petal number and flower colour. During the following year, seven AFLP markers linked to *Rdr1* within the distances of 1.1 and 7.6 cM were developed using the tetraploid progeny 95/3, segregating for the presence of the black spot resistance gene *Rdr1* (von Malek *et al.*, 2000). The most closely linked AFLP marker M10 was converted into a SCAR marker and screened in a larger population. The SCAR marker was found to be linked to *Rdr1* at a distance of 0.76 cM. The closely linked markers developed by von Malek should have enabled the localisation of *Rdr1* on the rose linkage map developed by Debener and Mattiesch in 1999. The direct mapping of *Rdr1* in this population was not possible because no clear segregation of black spot resistance was observed. Moreover, the direct integration of the markers was also not possible since none of the AFLP markers could be detected in the parental lines (93/1–117 and 93/1–119), and the SCAR marker SCM10 did not show any polymorphism between both genotypes even when applied as a CAPS marker. The indirect localisation of *Rdr1* to the distal ends of linkage groups A1 and B1 of 93/1-117 and 93/1-119, respectively was facilitated by developing the RFLP markers BMA 1-4 from the AFLP marker M10 (von Malek *et al.*, 2000).

The first crucial step to start map based cloning of *Rdr1* was the construction of a BAC library from a *R. rugosa* genotype (Kaufmann *et al.*, 2003). This BAC library comprised about 27,300 clones with an average insert size of 102 kb, containing 5.2 genome equivalents. The probability of recovering any given sequence of rose genomic DNA from this library was greater than 99%. Meanwhile, to facilitate positional cloning the mapping resolution in the *Rdr1* region was improved by bulked segregant analysis using 538 plants of three diploid sister backcross populations segregating for *Rdr1* (Kaufmann *et al.*, 2003). The SCAR marker SCM10 and the other *Rdr1*-linked AFLP markers that

were identified in the diploid populations could not be analyzed in the tetraploid population due to the lack of polymorphism between the parents (von Malek *et al.*, 2000). Further analysis located three new AFLP markers on one side of *Rdr1*, the closest of which was at a distance of 0.18 cM to *Rdr1* and one AFLP marker co-segregated with black spot resistance in the 538 plants. In addition to that one CAPS marker was located on the telomeric side of *Rdr1* at a distance of 0.93 cM. In this way the gene was located between two closely linked markers (Kaufmann et al. 2003).

However, the *R. rugosa* genotype used to construct the BAC library does not possess the black spot resistance allele *Rdr1*, the reason for selecting *R. rugosa* was the small genome size (2C< 1.10 pg) and future use of this contig for the establishment of a syntenic contig in some resistant genotype harbouring *Rdr1*. Therefore, a second library was established from the *R. multiflora* genotype 88/124-46. This genotype obtained the resistance by introgression from the wild rose species *R. multiflora* and is homozygous for the resistant allele (Biber *et al.*, 2009). This BAC library was constructed in the transformation competent vector pcLD04145 with a smaller average insert size (46 kb) compared to the *R. rugosa* library. The *multiflora* library consists of 60,000 clones providing genome coverage of 4.8. Markers from the *R. rugosa* contig were taken to identify the respective *R. multiflora* clones via hybridization. The contig was constructed by both hybridization and PCR analysis of end sequences of the clones. Although the new 88/124-46 contig differed in physical distances between some of the molecular markers, it turned out to be co-linear and the ends of both contigs could be linked to each other. The new contig was represented by a minimum of six clones with a maximum size of about 400 kb. The contig borders were determined by one recombinant among 538 plants showing recombination to the BAC-end derived markers 20T and 55T, respectively. During the process of map based cloning of *Rdr1* authors realised that the progress was very slow because of a high degree of heterozygosity and presence of several repetitive elements on the BAC contig (Kaufmann *et al.*, 2003). To speed up the process for the identification of *Rdr1*, candidate gene approach was applied to isolate RGAs from roses. For this purpose, the sequence information of already cloned resistance genes was utilized and different degenerate primers (Kanazin *et al.*, 1996) targeting the conserved motifs of NBS region of NBS-LRR genes were used to isolate NBS-like sequences in a PCR-based approach (Hattendorf and Debener, 2007). Several degenerate primer combinations that amplify diverse NBS-LRRs and serine

threonine kinase candidate sequences from rose DNA were employed to check the contig clones for the presence of other candidate genes. No amplification apart from the TIR-NBS-LRR gene family could be detected. Based on the consensus primers all the RGA elements from diverse BAC clones were reliably amplified and verified with hybridization and sequencing experiments. Further analysis gave a hint for the presence of a family of resistance gene analogues with high similarity to the TIR-NBS-LRR gene *N* from tobacco with 8-10 copies on this contig (Hattendorf and Debener, 2007).

Based on the observation that the contig contains at least 8-10 resistance gene analogues of the TIR-NBS-LRR class, initially one of the clones in the centre of the contig (155F3) was completely sequenced to obtain full sequence information of the RGAs. Two complete and one partial TIR-NBS-LRR genes with highly significant similarity to the *N*-gene from tobacco and the potato *Gro1* genes were identified along with some copia like retrotransposons and three microsatellites. Moreover, conserved primers were used to analyse the expression of RGAs in rose leaves. A total of five different copies of expressed RGAs were identified and one of which was located on the fully sequenced clone 155F3. Later on, sequencing analysis of the complete contig identified a total number of 9 RGAs on three BAC clones within a 220 kb interval on the *R. multiflora* contig (Biber *et al.*, 2009; Figure 1; Table 4). *Rdr1* paralogues on this locus share a sequence similarity that ranges between 85-99% and can be assumed to be a single gene family or simple cluster (Friedman and Baker, 2007). All nine candidates also share a common gene structure, having four exons and three introns. The first two exons code for the TIR and NBS domains, respectively, while the third and fourth exon, interrupted by an intron, represent the LRR domain. All RGAs encode open reading frames (ORF) of about 1100 amino acids except RGA4 and RGA9 (Kaufmann *et al.*, 2010). RGA4 is a pseudogene as it is interrupted by a large transposon insertion (7kb) in the non coding region of its first intron. Although RGA9 has a stop codon within the third exon that reduces the ORF to 794 amino acids there is some part of LRR region present, making it possible to encode a functional protein. In the light of above facts there are about 8 possible candidates of *Rdr1* (RGAs 1, 2, 3, 5, 6, 7, 8, and 9) and it is highly probable that one of the RGA copies on the contig represents active and functional *Rdr1* gene.

BAC clones spanning *Rdr1* resistance locus of *R. multiflora*

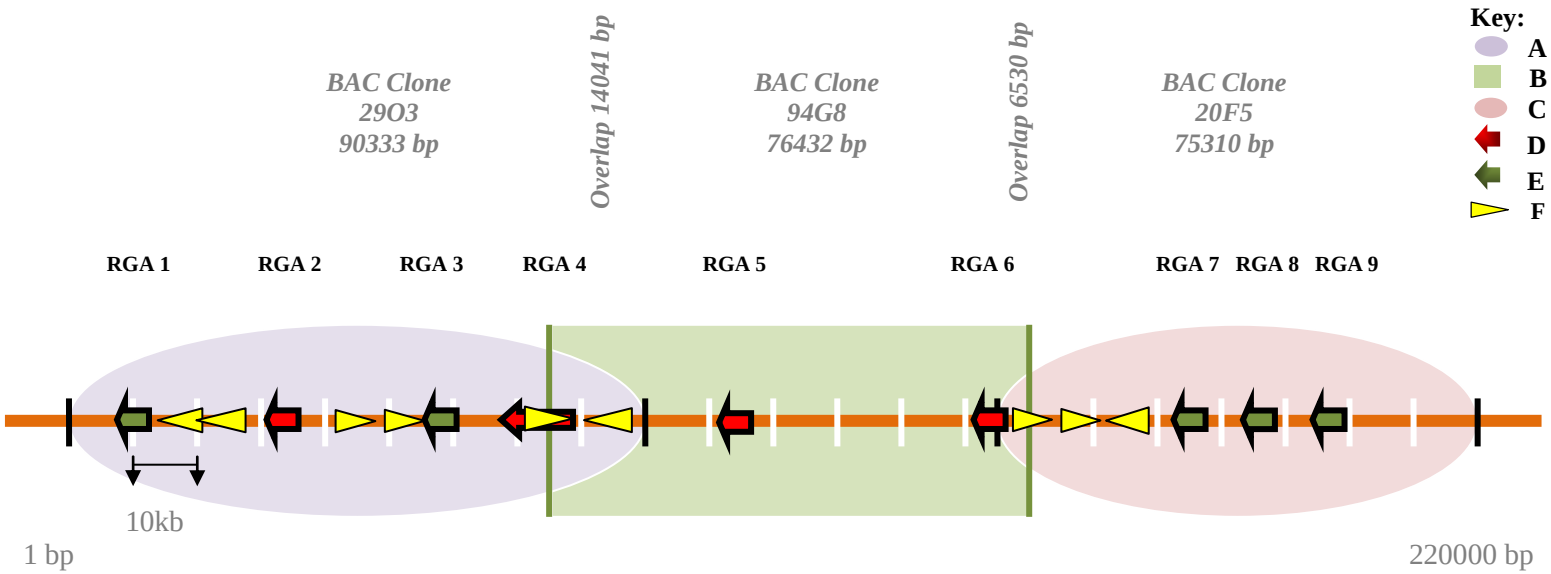


Figure 1.1: Overlapping BIBAC clones spanning *Rdr1* resistance locus of *R. multiflora* with 9-candidates genes.
The above diagram is based on the previous molecular and sequence analysis of three BAC clones (**A**- 29O3, **B**- 94G8, **C**- 20F5) which are part of the genomic library constructed for *R. multiflora* genotype 88/124-46, homozygous for *Rdr1* (Terefe, unpublished data). **D**- Green arrows represent RGAs that are found to be expressed in leaves of rose resistant genotypes whereas; **E**- red arrows are for non-expressed RGAs. **F**- The yellow triangles are representing copia elements within this interval of DNA. The arrows and triangles are pointing the orientation of RGAs and copia elements in the contig, respectively. Table 1.4 summarizes the exact positions of these elements on single BAC and on contig. It also provides some insights in previous functional data available for *Rdr1* candidates.

Table 1.4: Genomic and functional data available for *Rdr1* candidates*.

Candidates of *Rdr1* (CG)	BACs Carrying RGAs		Position on BAC	Final size of RGAs***	Position on contig	Copia Elements (CE)** (total-09)	Previous Info
RGA1	29O3 90333bp (1-90333bp)	Overlap b/w 29O3 & 94G8 76292-90333=14041bp	8671-12500	*3830bp*	8671-12500	2 CE b/w RGA1 & 2 : 14663 ◂ 27523-type ab 2 CE b/w RGA2 & 3 41617 ▸ 48142-a 50202 ▸ 56680-b	Expression confirmed cDNA-
RGA2			32656-36620	*3965bp*	32656-36620		Expression not confirmed RGA2&7 (sequence similar)
RGA3			56946-60848	*3903bp*	56946-60848		Expression confirmed 5' & 3' Race products
RGA4	94G8 76432bp (76292-152724bp)		68148-79018	*10866bp*	68148-79018	1 CE (c type) is within RGA4: 71531 ▸ 78486-c	Likely to be not expressed Insertion of 7kb of CE
RGA5			25140-29024	*3883bp*	101431-105313	1 CE (d type) b/w RGA 4 & 5 : 80614 ◂ 87779-d	No information
RGA6		Overlap b/w 94G8 & 20F5 146195-152724=6530bp	65332-69288	*3957bp*	141621-145577	3 CE b/w RGA 6 & 7 146068 ▸ 152947-e 155448 ▸ 161924-ab 162463 ◂ 169678-d2	No information
RGA7	20F5 75310bp (146195-221504bp)		27441-31370	*3932bp*	173635-177564		Partial cDNA sequence
RGA8			38655-42513	*3859bp*	184849-188647	No CE b/w RGA 8 & 9	Partial cDNA sequence
RGA9			49874-53787	*3914bp*	196068-199981		Partial cDNA sequences

*****This data is part of the previous studies carried out in the research group of Prof. Dr. Debener at the department of molecular plant breeding, University of Hannover, Germany. **The contig contains 9 copia like retrotransposons classified in different types according to their sequence similarities as type ab: 5 members, type c: 1 member, type d: 2 members and type e: 1 member). ***The final size of RGAs mentioned here is the ORF (as predicted by software FGENESH 2.6) with introns starting at ATG and ending at TGA.

3. Aims and objectives of the project

The ultimate objective of the project was the genomic and functional characterisation of *Rdr1* candidates (8-CGs) for the identification of the functional gene for *Rdr1* that confers resistance to black spot (*Diplocarpon rosae*). To attain the goal, following strategic plan was pursued to reduce the number of candidates step by step.

- Isolation of 8 single CGs (*Rdr1* paralogues) under the control of their endogenous regulatory elements
- Expression and functionality check of the CGs in tobacco heterologous system
- Expression analysis of CGs in different tissues of homologous system i.e. leaves, petals and roots of resistant rose varieties (88/124-46 and 91/100-5).
- Transient genetic complementation of susceptible rose variety (*Pariser Charme*) using single CGs and analysis of their activity against two isolates of *Diplocarpon rosae* (race 5 and 6).
- Stable genetic complementation of susceptible rose variety (*Pariser Charme*) and *Arabidopsis* using the most likely CGs for *Rdr1*.
- RNAi knock outs of the *Rdr1* gene family to prove the functionality of the locus against black spot.
- Isolation of 5’ and 3’-RACE products for the functional gene for *Rdr1* to get full length cDNA.

4. Materials and Methods

4.1. Materials

4.1.1. Plant material

Five rose genotypes, *Nicotiana benthamiana* and *Arabidopsis thaliana* [Columbia-0 (wild) and PEN-2 (mutant; Lipka *et al.,* 2005)] were used in the present study. The PEN2-1 mutants were kindly provided by P. Schulze-Lefert (Max Planck Institute for Plant Breeding Research, Cologne, Germany). Among the five rose genotypes *Pariser Charme* (4x), *Heckenzauber* (4x), *Marvel* (4x), are susceptible to black spot infection whereas *88/124-46* (2x) and *91/100-5* (4x) are resistant. The plants used here are part of the genotype collection of the Institute of Plant Genetics, Leibniz University of Hannover, Germany and were maintained in greenhouses under semi-controlled conditions.

4.1.2. Bacterial strains

The *Agrobacterium* strains used in this study were GV3101::pMP90, C58C1, EHA105 (Hellens *et al.,* 2000) and WT 80.1 (University of Hannover, Germany). *Escherichia coli* DH10B (T1R: F- mcrA Δ (mrr-hsdRMS-mcrBC) Δ 80lacZ_M15 Δ lacX74 deoR recA1 endA1 ara Δ 139 Δ (ara, leu)7697 galU galK Δ - rpsL(StrR) nupG tonA; Invitogen GmbH, Germany) was used for standard cloning procedures.

4.1.3. Fungal isolates

The single conidial isolates *Dort* E4 and race 6 of *D. rosae* were used in transient heterologous disease assay. *Dort* E4 represents the physiological race 5 that was used to identify resistance gene *Rdr1* (Debener *et al.,* 1998). These two single conidial isolates were conserved and propagated by repeated inoculation on excised leaves of 'Pariser Charme' (PC) as described by Debener *et al.,* (1998). The PC plantlets used for fungal inoculations were propagated *in vitro* by shoot tip culture and maintained in a disease free chamber.

4.1.4. BAC clones

The BAC clones 155F3, 29O3, 94G8 and 20F5 in vector V41 (pCLD04541: a binary cosmid vector of ~ 29 kb) were utilized during this study. These overlapping BIBAC clones are spanning the *Rdr1* resistance locus and are part of the genomic library constructed from the *R. multiflora* genotype 88/124-46 which is homozygous for *Rdr1*

(Kaufmann *et al.*, 2010). The BAC 155F3 with an insert size of about 60 kb is carrying RGAs 2, 3 and 4. The BAC 29O3 (~ 90kb) is carrying RGAs 1, 2, 3 and 4. The BAC 94G8 (~ 76kb) has RGAs 5 and 6. Remaining RGAs 7, 8 and 9 are on BAC 20F5 (~ 75kb) (Kaufmann *et al.*, 2010).

4.1.5. Cloning vectors

Binary expression vectors pBINPLUS [size: 12300bp; npt-Kanamycin resistance; MCS allow lacZ-Gen (β-Galactosidase) selection (Van Engelen *et al.*, 1995)], pBIN19 [size: 11777bp; npt-Kanamycin resistance; MCS allow lacZ-Gen (β-Galactosidase) selection (Bevan, 1984)] and p9U10-RNAi [size: 11853bp; Sm/Sp-Streptomycin resistance (DNA cloning services, Hamburg, Germany)] were used to transfect the bacterial strains mentioned in 4.1.2. pUC19 [size: 2686bp; bla-Ampicillin resistance (Invitrogen GmbH, Germany)] was used as transformation control vector. The pGEM-T easy vector [size: 3000bp; bla-Ampicillin resistance; lacZ-Gen (β-Galactosidase) selection (Promega Co.)] was used for the cloning of PCR products.

4.1.6. Enzymes

The enzymes used in this study and their sources are listed in Table 7.1 in the appendix.

4.1.7. Primer sequences

The Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was utilized for the development of different PCR primers. The oligonucleotides were synthesized by Invitrogen GmbH, Germany. Primer sequences, their annealing temperatures and product sizes are summarized in Table 7.7-7.10 (appendix).

4.1.8. Miscellaneous materials

During the course of this study different kits were utilized for the isolation of BAC DNA, plasmid DNA, RNA, 5' RACE etc. their description, purpose and sources are given in Table 7.2. In addition, fine chemicals (Table 7.3), Equipments (Table 7.4), media (Table 7.5), solutions and buffers (Table 7.6) used during this study are summarized in the corresponding tables of the appendix with their sources.

4.2. Methods

4.2.1. Isolation and manipulation of nucleic acids

4.2.1.1. Isolation of BAC DNA

Alkaline lysis (SDS) method was used to isolate DNA from 500 ml overnight grown culture of the BAC clones 155F3, 29O3, 94G8 and 20F5 (Sambrook and Russel, 2001). Table 7.6 summarizes the preparation of different solutions required to carry out alkaline lysis method. The BAC vector V41 (pCLD04541) was selected by adding tetracycline (15mg/l) to the culture medium. The cocentration of the isolated BAC DNA was determined by diluting 2µl of preparation in 7 µl of nuclease free water and 1µl of bromophenol blue buffer (Table 7.6) and running samples along with different concentrations (10, 30, 50, 80 and 100 ng) of standard lambda DNA on 1 % agarose gels (100V for 1 hour). The gel picture was taken with gel documentation system and analysed using Gel Pro Analyzer.

4.2.1.2. Isolation of plasmid DNA

All plasmid isolations (mini preps: 5 ml of overnight grown culture) were carried out using NucleoSpin®Plasmid kit according the instructions of the manufacturer. After isolation, the plasmid preparations were quantified as described in section 4.2.1.1.

4.2.1.3. RNA extraction and cDNA synthesis

RNA was extracted using the Invisorb® Spin Plant RNA Mini Kit. The collected leaf material (30-50 mg) was frozen immediately in liquid nitrogen and ground in a bead mill for 3 min at a frequency of 24 cycles/ second. Further processing of the material was carried out according to the manufacturer's instructions. The contaminating DNA was removed from the extracted RNA using the DNase free kit (Ambion) as recommended by the manufacturer. The quality of isolated RNA was initially checked on 1% agarose gels (100V for 30 min) using RNase free reagents and later in a photometer (Safas) taking the value of 260/280nm ratio of the samples. The quantification of RNA samples was performed using 2 µl of RNA preparation directly on Hellma TrayCell cuvette of Safas photometer. The purified and quantified RNA (300-500ng) was used as a substrate for the preparation of total cDNA using random primers of the high capacity cDNA reveres transcription kit (Applied Biosystems). The quality of RT-PCR products was finally checked on agarose gels using Actin primers in the PCR (section 4.2.3.4).

4.2.1.4. Enzymatic digestion of BACs

The single candidate genes (CGs) were isolated by either partial or complete enzymatic digestion of BACs 155F3, 29O3, 94G8, and 20F5. Five µg of isolated BAC DNA was partially digested with 4 U of *Sau 3A1* for 15 min at 37 ºC. The digested fragments of these BACs were separated on a 0.8% agarose gel (80V for 30 min) and bands with ~ 7-12 kb size were excised from the gel and purified using the QIAquick gel extraction kit (Qiagen). The purified fragments were quantified by running an aliquot of the samples along with a dilution series of λ-DNA as described in section 4.2.1.1. Sequence based complete digestion of BACs 29O3 and 20F5 was carried out using a variety of endonucleases. Five to ten µg of BAC DNA was completely digested by 10U of enzyme overnight at 37 ºC. BAC 29O3 was at first digested by *EcoR1* to isolate RGA 1 and secondly by *Kpn1* to isolate RGA 2. BAC 20F5 was digested by *Xba1* for the isolation of RGA 7, 8, and 9, simultaneously. The obtained fragments were separated on a 0.8% agarose gel (30-80V for 3-16 hours) and bands of expected sizes were extracted and quantified as describe earlier for the partial digest.

4.2.1.5. Enzymatic digestion of pBINPLUS

The binary expression vector pBINPLUS was digested by different endonucleases (*BamH1 or EcoR1 or Kpn1 or Xba1*) one at a time to accommodate the corresponding fragments generated from BAC enzymatic digestions. The enzyme digestion mix was prepared by mixing 5 to 10 µg of pBINPLUS DNA, 10U of endonuclease and 1/10 of the corresponding buffer in a total volume of 500µl and incubating the mix at 37 ºC for 3 hours. The quality of the digest was verified by loading 100ng of the treated plasmid along with untreated plasmid on 0.8% agarose gel for 1 hour at 100V. Properly digested samples were purified and concentrated by performing a phenol:chloroform (1:1) extraction according to Sambrook and Russel (2002) with following modifications. The upper layer obtained from organic extraction was mixed with 1/10 volume of 3M sodium acetate and 1 volume of 100% isopropanol and DNA was pelleted (centrifugation 10,000 rpm 15 min), washed (70% ethanol) and dissolved in 100µl of TE after 1 hour incubation on ice. The 1 µl of the preparation was diluted as 1:30 and quantified as described in section 4.2.1.1. The digested pBINPLUS (4 µg) was treated with Shrimp Alkaline phosphatase (Fermentas) according to manufacturer's guidelines to get 1 p moles of de-phosphorylated 5' ends of a 12kb plasmid. At the end of reaction SAP was destroyed by heat inactivation.

4.2.1.6. Enzymatic digestion of clones carrying single CGs

Five µg of plasmid DNA of each isolated sub-clone carrying single CGs was digested by 10 U of different endonucleases ,*EcoR1, BamH1* and *Hind III* one at a time, at 37 ºC overnight to determine and/ or varify the insert size. The resultant products were separated on a 0.8 % agarose gel (80V for 3-6 hours) parallel with 1 kb ladder (Invitrogen). The fragments generated by each endonuclease were summed up in terms of size in kb to get an approximate estimate of the insert size. The used endonucleases have only one restriction site in the pBINPLUS vector that has a total size of about 12 kb.

4.2.1.7. Ligation mixes

Ligation mixtures were usually prepared in a total volume of 10-15 µl by adding 15-30ng of deposphorylated pBINPLUS vector, 30-90ng of insert (section 4.2.1.4), ATP to a final concentration of 1mM, 1/10 volume of buffer and 2.5 Weiss units of T4 DNA ligase. In addition to that a negative (without insert) and positive controls (pUC19) were prepared. Ligation mixes were incubated at 14 ºC overnight.

4.2.2. Transfecting bacteria

4.2.2.1. *E. coli* (DH10B)

All bacterial transformations were carried through electroporation using BioRad Micropulser with EC2 program i.e. 2.5 kV for 0.2cm cuvettes, 25 uFD, 400 Ohm, Pulse time 8 – 12 ms. One-two µl of the ligation reaction mixture were mixed with 40 µl of electro-competent *E. coli* (DH10B) cells and the cells were pulsed followed by immediate addition of 1 ml SOC medium. The cells in suspension were allowed to recover by continuous shaking at 37 ºC for 1 hour. Afterwards, 10 and 100 µl of the bacterial suspension were plated onto LB agar plates with kanamycin (50 mg/l) or ampicillin (50mg/ l) depending on the vector properties and incubated at 37 ºC overnight. On the following day initial positive clones were picked in 96 well plates, using sterile tooth picks, through blue white screening.

4.2.2.2. *Agrobacterium* species

Agrobacterium transformation competent cells were prepared by inoculating 100ml LB with a single colony of *Agrobacterium* and incubating overnight at 30 ºC under vigorous shaking. By the next day, the bacterial cells were washed 3 times with 50 ml

autoclaved and ice cold 10% glycerol by spinning at 5000 rpm for 5 min at 4 ºC. Finally, the cells were resuspended in 1 ml of 10% glycerol and stored in 40 µl aliquots at -80 ºC. About 50-200 ng of plasmid DNA was mixed with 40 µl of electro-competent *Agrobacterium* cells and transferred to a pre-chilled 0.2 mm electroporation cuvette. The cells were pulsed using Biorad GenePulser (EC2 = 2.50 kV, 25 uFD, 400 Ohms, pulse length: 8 to 12 ms) followed by immediate addition of 1 ml liquid LB. The electro-shocked cells in suspension were recovered by continuous shaking at RT for 2 - 4 hours. Ten and 100 µl of the bacterial suspension were plated onto LB agar plates with the appropriate antibiotic and incubated at 28 ºC for 2 days. Colonies were picked and checked by PCR (primer pairs as consensus cDNA, consensus P1, npt, and / or GUS) as described in section 4.2.3.1. *Agro*-clones carrying single CGs were grown overnight at 28 ºC to prepare stocks for future use; therefore 1.5 ml of overnight grown bacteria were mixed with of 0.5 ml 60% glycerin and those suspensions were stored at -80 ºC. These agro-clones were also streaked out on solid LB medium (Rifampicin: 10-50mg/l and kanamycin: 50mg/l). In case of GV3101::pMP90 Gentamycin: 25mg/l was also included in media.

4.2.3. Polymerase chain reactions

The PCR reaction mixture for most of the PCRs was prepared by mixing Williams buffer with Mg^{2+} (2mM), dNTPs (200 µM), forward primer (0.4 µM), reverse primer (0.4 µM), Bioline DNA polymerase (1U), template DNA (0.5-10ng) and ddH_2O to a final volume of 25µl. After completion of the PCR the products were separated on 1-1.5 % agarose gels (100V for 1-2 hours).

4.2.3.1. Colony PCRs

During this study, colony PCRs were usually carried out to confirm the presence of inserts in the vector by using inserts specific primers. For these PCRs 25µl of PCR reaction mix was inoculated by bacterial colony or bacterial suspension (template) using a sterile toothpick. The primer pairs used for colony PCR were consensus P1, consensus cDNA, Kuehr3, Aneela 1F-1R, GUS and Npt. The sequence, annealing temperature, product size and purpose of these primers is summarized in Table 7.8. The conditions used for colony PCR were: denaturation at 95 °C for 4 min; 30-35 repeats of denaturation (94 °C for 1 min), annealing (temperature adjusted according to primer Tm

for 30s-90s) and elongation at 72 °C (time depends on product size and activity of DNA polymerase) followed by final elongation at 72 °C for 10 min only once.

4.2.3.2. Insert PCRs

Insert PCR was performed using an M13 primer pair to check the insert size and to exclude false positives after transformations. PCR mix for insert PCR was the same as described in section 4.2.3 and template was the isolated plasmid of transformants. However, the conditions were initial denaturation at 95 °C for 4 min followed by 30 repeats of denaturation (94 °C for 1 min), annealing (56 °C for 1 min) and elongation at 72 °C (time depends on the expected product size and activity of DNA polymerase) and a final elongation at 72 °C for 10 min.

4.2.3.3. SSR PCR

The SSR primer pair Rd1LRR is specific for RGAs and able to differentiate all RGAs on the basis of fragment size (Terefe and Debener, 2010). The PCR mix and conditions used for Rd1LRR primer pair are summarized below. One of the primers was labelled with IRD700. The PCR products obtained through SSR primer were separated on 6% acrylamide gels using a Li-COR automated sequencer (section 4.2.5.2).

SSR PCR reaction mix

Reagents	Target Concentration
Bioline buffer without Mg^{2+}	1x
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Rd1LRR F	2.0 pmol
Rd1LRR R	2.0 pmol
cDNA template	10 ng
Bioline DNA polymerase	1 U
dd H_2O to total vol.	20µl

SSR PCR program

PCR-Steps	Time	Temp.	Cycles
Initial denaturation	5 min	95 °C	1 x
Denaturation	1 min	94 °C	30 x
Annealing	1 min	62 °C	
Elongation	1 min	72 °C	
Final Elongation	10 min	72 °C	1 x

4.2.3.4. Actin PCR

The actin primer pair was used to evaluate the quality of the cDNA synthesized from extracted RNA. The PCR mix was prepared as described in section 4.2.3 and the PCR conditions were as follows:

Conditions to perform Actin PCR

PCR-Steps	Time	Temperature	Cycles
Initial denaturation	4 min.	95 °C	1 x
Denaturation	1 min.	94 °C	
Annealing	1 min.	58-60 °C	30 x
Elongation	1 min.	72 °C	
Final Elongation	10 min.	72 °C	1 x

4.2.4. Isolation of 5' and 3'-RACE products for RGAs

RACE (Rapid Amplification of cDNA Ends) technique was used to obtain the 5' and 3' ends of RNA transcripts of RGAs transiently expressed in tobacco. For this analysis tobacco plants were infiltrated using *Agrobacterium* suspensions carrying the single RGAs 1, 7, 8, 9 as described in section 4.2.7.1. Samples were collected 3 days after infiltration. The leaf discs of 30-50 mg were immediately frozen in liquid nitrogen. RNA was extracted from the frozen leaf samples using Invisorb® Spin Plant RNA Mini kit as summarized in section 4.2.1.3. The 10 µg of RNA were used to isolate 5' RACE products only for RGA8 using FirstChoice® RLM-RACE Kit (Ambion). The specific primers used for this purpose are mentioned in Table 7.9. For the isolation of 3' RACE products the RNA preparations of RGA 1, 7, 8 and 9 were used to synthesize cDNA using the AP_Xma-primer 0.5 µM (Table 7.9) with the high capacity cDNA reveres transcription kit (Applied Biosystems). The reaction mix for cDNA synthesis was prepared by mixing 0.25-0.5µg of total RNA, AP_Xma primer (0.5µM), dNTPs (4mM) and ddH_2O up to 16µl of final volume. This reaction mixture was incubated for 5 min at 65 ºC followed by 5 min at 55 ºC and chilled on ice. Afterwards, 1x RT buffer, 50U of MultiScribe™ reverse transcriptase and 40 U RNase inhibitor were added. The reaction mixture was incubated for 2 hours at 37 ºC and 5 s at 85 ºC to inactivate the RNase inhibitor. Actin primers were used to check the quality of RT-PCR products (sections 4.2.3.4). Good quality cDNAs of RGA1, 7, 8, and 9 were used to isolate 3' RACE products of these specific RGAs. For this purpose, PCR reactions were set up using gene specific primers (GSP) for each RGA separately in combination with AUAP_Xma primer (Table 7.9) and Takara prime star HS DNA polymerase. The details of PCR reaction mix and conditions used to isolate 3' RACE products are described on page 29.

The PCR products were visualized and separated on 1.5 % agarose gels (100V for 1 hour). When no PCR products were obtained using 1st GSP, a second PCR reaction was started using 0.2-1 µl the PCR products of the first PCR (1:10 or 1:20 diluted in

nuclease free water) as template and a 2nd GSP primer in combination with AUAP_Xma primer. This process of PCRs was repeated using different GS primers until amplicates of expected size were obtained. These PCR products were extracted from the gel using the Mini Elute Gel Extraction Kit (Qiagen). The purified blunt-ended PCR fragments were A-tailed by mixing 30-100ng of the purified PCR fragments with 0.2mM dATPs, 1x reaction buffer, 5U Bioline Taq DNA polymerase and dd H_2O up to 10 µl. The reaction mix was incubated at 70 ºC for 15-30 min. An aliquot of 1-2 µl of the A-tailed fragments was ligated into the pGEM® T- Easy Vector according to the protocol supplied by the manufacturer (Promega). Electro-competent cells of *E. coli* (DH10B) were transfected by 1-2 µl of ligated fragments employing electroporation (section 4.2.2.1). The transfected bacteria were plated and incubated overnight at 37 ºC. On the following day, positive clones were picked through blue white screening and an insert PCR (M13 primer pair) was performed to confirm the size of the insert and to exclude false positives having an insert size less than the expected one (section 4.2.3.2). After size confirmation, plasmid mini preps were prepared (section 4.2.1.2) and end sequences of the inserts were determined as described in section 4.2.6.

3' RACE PCR reaction mix

Reagents	Target concentration
5× Takara buffer with Mg^{2+}	1 mM
dNTPs (2.5 mM)	0.2 mM
GSP primer (5µM)	0.2 mM
AUAP_Xma primer	0.2 mM
cDNA template	< 200 ng
Takara DNA polymerase	0.025 U
dd H_2O to final volume	25µl

3' RACE PCR program

PCR-Steps	Time	Temperature	Cycles
Initial denaturation	5 min	95 °C	1 x
Denaturation	10 s	98 °C	35 x
Annealing	05 s	>60 °C	
Elongation	3 min	68 °C	
Final Elongation	10 min	68 °C	1 x

4.2.5. Gel electrophoresis

4.2.5.1. Agarose gel

0.8-4% agarose gels were prepared according to sample requirements (Sambrook and Russel, 2001). The agarose was mixed in 1 x TAE buffer and boiled to get a homogenized solution which was poured in assembly after adding 0.5 µg/ml ethidium bromide. The gel pictures were taken by gel documentation system (Intas, Göttingen).

4.2.5.2. Polyacrylamide gels

Denaturing polyacrylamide gels (6%) were prepared by mixing readymade solutions of Ultra Pure SequaGel® XR and Ultra Pure SequaGel Complete® Buffer Reagent (National Diagnostics) plus 10% APS according to the support protocol. The prepared solution was poured between two glass with 0.25 mm spacers. After polymerization (3 hours to overnight) the gel was loaded in Li-COR-Sequencer and a pre-run was started to set required conditions for 45 cm long sequencing gels (2000 V, 40 mA, 40 W and 45 ºC for 30 min) or for SSR 18cm long PA gels (1500 V, 35 mA, 31.5 W und 40 °C for 10 min), after pre-run 0.3-1 µl of the samples were loaded. The samples were prepared by adding equal volume of stop solution in it and denaturing it at 70 ºC for 2 min. The SSR gels took about 3 hours for proper and complete separation of fragments whereas sequencing gel took about 13 hours. The Li-COR system works with a dye-primer chemistry and analyzer can detects IRD700 or IRD800 or both dyes at the same time.

4.2.5.3. SSCP gels

Single strand conformation polymorphism gels (Orita *et al*., 1989) were prepared by using 0.5x MDE (mutation detection enhancing) gel solution. The prepared solution for SSCP gels was poured between two clean glass plates with 0.4mm spacer. The front plate was treated with 'GelSave' solution to ensure easy release from the gel after the run and the rear plate was treated with bind-silane to fix the gel. Forty µl of the samples were loaded after denaturation by adding an equal volume of SSCP dye (Table 7.6), then heating the samples for 3 min to 95 °C and chilling them immediately on ice. The gel was run for 16 hours in a vertical electrophoresis system maxi-gel (Biometra) at 4 °C, 120V and 20mA. The DNA was visualized after silver staining of the gel. For silver staining the rear plate with the fixed gel was incubated in fixing solution (7.5 % acetic acid) for 30-45 min and washed for 5 min in ddH_2O. Then the gel was incubated in staining solution for 45 min with continuous shaking and washed with ddH_2O for few seconds to remove surplus silver nitrate and rinsed with pre cooled developing solution for 10 min. When the bands became clearly visible, the developing reaction was stopped by rinsing the gel in 7.5 % acetic acid for 2 - 3 min. The gel was washed with demineralised water for about 5 min and dried for 2 hours in oven at 80 °C. All solutions used for this purpose are describd in Table 7.6 in appendix.

4.2.6. Sequencing

The end sequences of an insert were determined by performing sequencing reaction using the Thermo Sequenase Cycle Sequencing Kit from USB. According to the support protocol, 1µg of plasmid DNA and IRD labeled primers are prerequisite. Primer pair M13 uni [(-21) IRD-700 labeled] and M13 rev (-29) [IRD-800 labeled] were used to sequence the inserts from both ends. To detect the products of sequencing reaction 6% denaturing polyacrylamide gels were used in Li-COR DNA-Analyser as described in section 4.2.5.2. The fragment pattern was analyzed using the E-seq™ Software (Li-COR) and saved as text files. The obtained sequences were further analyzed using different bioinformation softwares as described in section 4.2.6.1.

4.2.6.1. Bioinformatic of DNA and protein sequences

The Bioedit Version 7.0.9 (Hall, 1999) was used for sequence editing, alignment and local BLAST searches to analyze the obtained DNA sequences. As the full sequences of BACs spanning the *Rdr1* resistance locus were available, this source was utilized as local BLAST to confirm the presence, find the position and evaluate the insert size by using the end sequences obtained through sequencing reactions of positive clones. Later on Blastn and Blastx searches (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were performed using NCBI database to find the similar genes as of isolated genes from *Rdr1* resistance locus. The gene prediction and protein analysis were performed on different free internet sources as http://www.expasy.ch/, http://swissmodel.expasy.org/SWISS-MODEL.html, http://www.ebi.ac.uk/interpro/, http://linux1.softberry.com/berry.phtml. Neignbor-joining (NJ) analysis of protein sequences downloaded from NCBI database was carried out by MEGA4 (Tamura *et al.*, 2007; Kimura, 1980) whereas the ratio of synonymous (K_s) and non-synonymous (K_a) substitutions rates per synonymous/ non-synonymous site were calculated using software DnaSP v 5 (Rozas *et al.*, 2003).

4.2.7. Expression studies

4.2.7.1. Transient heterlogous expression studies in tobacco

Heterologous expression of the isolated single CGs was carried out in *N. benthamiana*, a model plant for transient expression studies. The fourth and older true leaves of *N. benthamiana* (Wroblewski *et al.*, 2005), were infiltrated by *Agrobacterium* suspension of GV3101, harboring the helper plasmid pMP90 and single CGs in pBINPLUS, for the majority of these experiments. A single colony of *Agrobacterium* carrying a single CG

was used to inoculate an overnight culture of 50 ml YEP medium (Table 7.5) containing specific antibiotics (Rifampicin: 10-50mg/l, Gentamycin: 25mg/l, and Kanamycin: 50mg/l) at 28 ºC. The following day, 50 ml of bacterial culture was processed according to Wroblewski and colleagues (2005) and infiltrations were done in tobacco using a 1 ml syringe without a needle (Schöb *et al.,* 1997; Wroblewski *et al.,* 2005). The samples for RNA extraction and cDNA synthesis were collected from infiltrated leaves 3 days after infiltration (section 4.2.1.3). Good quality cDNA was evaluated through a series of primer pairs for the expression of single RGAs. These primer pairs were consensus P1, consensus cDNA, Kuehr3, Rd1LRR and Aneela 1F-1R. The sequence, annealing temperature, product size and purpose of these primers are summarized in Table 7.8. PCR mix and conditions are described in section 4.2.3. In addition to that after 24 hours the single RGA infiltrated plants were challenged by *Dort* E4 (5 x 10^5 spores/ ml) to observe the activity of infiltrated CG against black spot.

4.2.7.2. Homologous expression analysis of single RGAs

Homologous expression analysis of single RGAs was carried out in different tissues (leaves, petals, and roots) of the resistant rose genotypes 91/100-5 and 88/124-46. For this purpose, RNA was extracted from the different tissues followed by cDNA synthesis and its quality check as described in section 4.2.1.3. This cDNA was utilized to evaluate and confirm the expression of different RGAs in different tissues of resistant rose genotypes using a range of specific primer pairs for different RGAs. The collection of primers were used, is listed in Table 7.10. The PCR products of these primers were evaluated on 3-4% agarose gel, 6% denaturing PA gels and SSCP gels as described in section 4.2.5.

4.2.7.3. Transient expression studies in rose petals and leaves

Primarily all important parameters for a successful transient expression assay were optimized using GUS (Intron) reporter gene. Petals of five rose genotypes, [Pariser Charme (4x), Heckenzauber (4x), 91/100-5 (4x), 88/124-46 (2x) and Marvel (4x)] and leaves of three genotypes, [Pariser Charme (4x), 91/100-5 (4x) and 88/124-46 (2x)] were used at the outset to optimize the transient expression assay. For resistant genotypes 91/100-5 and 88/124-46 leaves were collected from plants growing in greenhouse, under plastic tunnels (high humidity and temperature) and *in vitro.* The *Agrobacterium* strains GV3101::pMP90, C58C1, EHA105 (Hellens *et al.,* 2000) and

WT 80.1 (University of Hannover) harbouring the construct 35S:GUS-intron in pBINPLUS (Van Engelen *et al.,* 1995) were grown overnight in YEP liquid medium supplemented with Kanamycin (50mg/L) and Rifampicin (10mg/L) according to Wroblewski *et al.* (2005). In addition to these two antibiotics, Gentamycin (25mg/L) was added in GV3101 cultures for the selection of pMP90. The following day, bacteria were collected through centrifugation at RT, and 4500 rpm for 15 min. The pellets were washed once using sterile distilled water and resuspended in 1-2ml of sterile distilled water; the bacterial suspensions were adjusted to OD_{600} of 0.4-0.5 in case of petals (Wroblewski *et al.,* 2005) and 1.5-2.0 in case of leaves. The bacterial suspensions were supplemented with 0, 100, and 200 μM of acetosyringone and the non-ionic surfactant Breakthru (Joh *et al.,* 2005) at final concentrations of 0, 10, 100, and 1000 ppm (v/v). The bacterial suspension were infiltrated from a hole punctured at the base of the petal and as 2-10 spots in a detached leaf using a 1ml syringe without a needle (Schöb *et al*., 1997; Wroblewski *et al.,* 2005). The infiltrated leaves/petals were kept on a wet tissue paper in a rectangular transparent box fitted with a cover at 22 ºC in the dark until monitored for GUS expression; 3 days after infiltration. The histochemical GUS assay was performed according to Jefferson *et al*. (1987). On average 30 petals and 20 spots on leaves per condition for seven independent replicated experiments were evaluated. Vacuum (5 min at 200 mbar with 3 breaks) was used to facilitate infiltration of staining solution in petals and tobacco leaves. The samples merged in staining solution were incubated overnight at 37 ºC and washed in 70% ethanol many times until chlorophyll was removed completely. GUS expression levels were visually rated on a scale from 0 to 3 indicating no expression (score 0) to very high expression (score 3). Infiltrated *N. benthamiana* leaves were used as a positive control in all experiments. Non-infiltrated leaves and petals of all genotypes were used as negative control.

4.2.8. Transient disease assay

4.2.8.1. Transient homologous disease assay in rose leaves and petals

In order to evaluate the effect of the different single RGAs in restricting the infection caused by race 5 and 6 of *D. rosae* compared to non-RGA infiltrated and GUS infiltrated samples, a bioassay was established. The petals and leaves of Pariser Charme, susceptible rose genotype to black spot, were infiltrated as described earlier in section 4.2.7.3 using bacterial suspensions harbouring single RGAs. These leaves and petals were challenged by black spot spores (*Diplocarpon rosae* conidia) at the same time as

RGAs were infiltrated or after 24 hours of agro-infiltrations. The *D. rosae* isolates used were single spore isolates Dort E4 and race 6. The spore concentrations were adjusted to $5x10^2$– 10^5 conidia/ml as described by Dohm and colleagues (2001). Infiltrated samples were incubated at 22 °C in dark and the samples were collected for fluorescence microscopy 4 days after bacterial infiltrations in both cases. Aniline blue staining was done by cutting out infiltrated and inoculated leaf/petal areas (approx. 1 cm^2) using a scalpel and cleared in 1 M KOH solution for 15 min at 121 °C and 1.2 bar in an autoclave. After cooling, the samples were washed in distilled water and stained in the staining solution (0.067 M K_2HPO4 with 0.05% aniline blue) as described by Hood & Shew (1996). The samples were examined under a fluorescent microscope [Zeiss epifluorescence microscope (excitation 450–490 nm, dichronic mirror 510 nm, barrier 520 nm)] and fungal growth was scored as number of colonies/ spot. Colony can be described as the fungal growth with different developmental levels of prolonged hyphae and formed acervuli and spot is the area with a diameter of 0.3-0.5mm where bacterial suspension and spores are infiltrated or inoculated. The generated data was analyzed statistically as described in section 4.2.10.

4.2.8.2. Transient RNAi knockouts of *Rdr1* family

The exon 2 of RGA8 (1104 bp) was selected to generate an RNAi construct as it has a homology of about 86-96 % to the exon 2 of the remaining RGAs. The RNAi vector was constructed in plasmid p9U10 (streptomycin resistant) by the company DNA cloning service, Hamburg, Germany. The construct was used to transfect GV3101 through electroporation as described in section 4.2.2.2. Rose genotypes 91/100-5 and PC were used to carry out these experiments. The selected genotypes were infiltrated as described in section 4.2.7.4. and challenged by fungal isolates *Dort* E4 and race-6 after 24 hours of bacterial infiltration (Section 4.2.8.1). The samples were collected for fluorescence microscopy 4 days after bacterial infiltrations and processed as described earlier (Section 4.2.8.1). The data were collected as number of colonies / spot and analyzed as described in section 4.2.10.

4.2.9. Generation of *Arabidopsis* lines homozygous for single CGs

Arabidopsis varieties *Columbia (wild)* and *PEN-2* (mutant) were used to generate homozygous lines for single RGAs. *Agrobacterium* mediated transformation of *Arabidopsis* was carried out using the floral dip method (Zhang *et al.,* 2006) in which

the female gametes of plants are transformed just by dipping their developing inflorescences in *Agrobacterium* suspension. *Agrobacterium* suspensions (carrying the genes to be transferred) for floral dip were prepared by pelleting an overnight culture (500ml) and re-suspending the pellet into a 5% sucrose solution containing 0.02% (vol/vol) Breakthru (a non-ionic detergent). The plants with healthy inflorescences consist of immature flower clusters with some maturing siliques were then dipped in the suspension for 10 s and transferred to greenhouse to allow seed set which were then plated *in vitro* on a selective medium to screen for transformants. Generation of *Arabidopsis* transgenic homozygous lines require minimum 6 months using this method.

4.2.10. Data analysis

Different effects as effect of different parameters (section 4.2.7.3) on GUS expression, effect of different RGAs on the fungal growth in a susceptible genotype (section 4.2.8.1) and effect of silencing *Rdr1* family on the fungal growth were evaluated using the Kruskal-Wallis and Wilcoxon exact tests as implemented in the R-software (R Development Core Team 2009).

5. Results

5.1. Isolation of genomic DNA fragments carrying single Candidate Genes (CGs)

To isolate single CGs, BAC clones 155F3, 29O3, 94G8 and 20F5 of *R. multiflora* BAC-gene bank were selected; they span the *Rdr1* resistance locus with 9 RGAs, potential candidates of *Rdr1*. The partial digestion of BACs 155F3, 29O3, 94G8 and 20F5 generated 288, 864, 960 and 1632 sub-clones, respectively. The generated BAC sub-libraries were screened with RGA general primers (Table 7.8 in appendix) followed by enzymatic digestion of positive clones to estimate the size of inserts. The clones carrying 7 kb or bigger inserts were subjected to insert end sequencing analysis. The end sequencing of inserts, limited the number of real positive clones to 4 (RGAs 3, 5, 6 and 7). These clones were carrying enough upstream and downstream DNA regions to ORF of genes expecting to harbor necessary endogenous regulatory elements for expression (Figure 5.1). The complete digestions of BACs resulted in the isolation of RGAs 1, 2, 7, 8 and 9 (Table 5.1; Figure 5.1). RGA 4 was not isolated as available data revealed its interruption by a retro-transposon of about 7 kb due to which it is likely that it is non-functional (Kaufmann *et al.*, 2010). All isolated single RGAs were cloned into binary transformation vector pBINPLUS and subjected to further analyses.

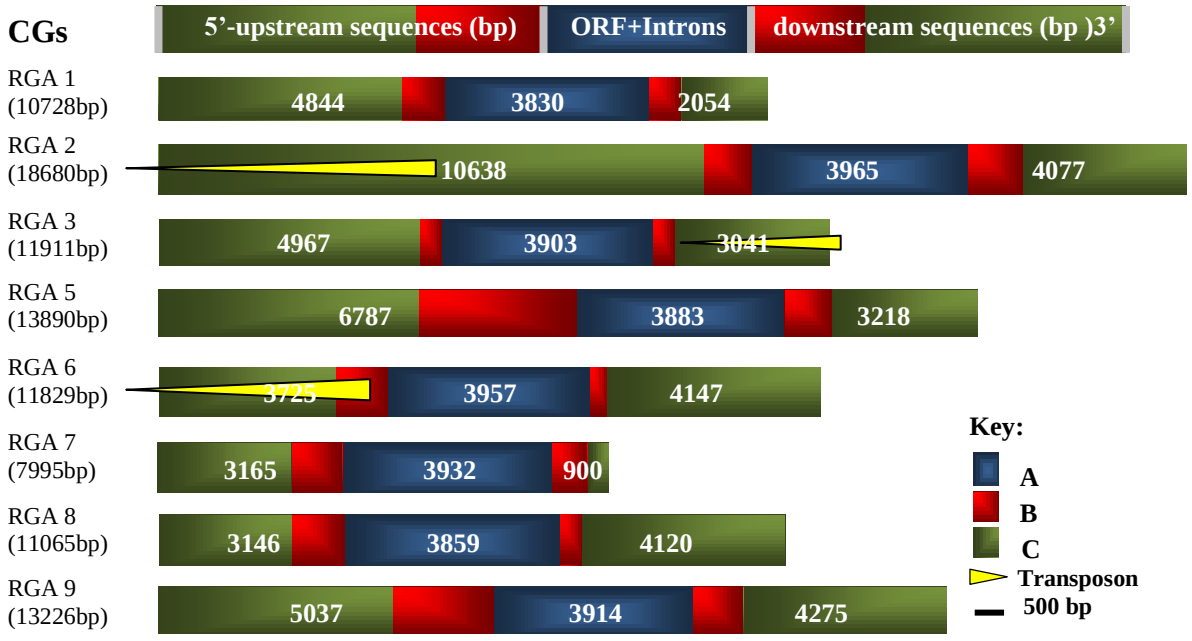


Figure 5.1: Structure of genomic fragments carrying *Rdr1* candidates. (**A**) The isolated genomic sub-clones of single RGAs represent software predicted open reading frame with introns; (**B**) software predicted upstream and downstream sequences of isolated genes; (**A+ B+C**) the full length of the isolated single RGAs with endogenous regulatory elements. The yellow triangles are showing the presence of incomplete retrotransposons on the isolated genes.

Table 5.1: *Rdr1* candidates generated by partial or complete enzymatic digestion of whole BACs.

Candidates of *Rdr1*	BACs Carrying RGAs	ORF of RGAs*	Isolated single RGAs in size/ clone	Method of isolation CD or PD**	Position on single BACs	Position on contig
RGA1	29O3-90333bp (1-90333bp)	3830bp	10728 bp/ 1C2	CD by *Eco*RI	6617-17344	6617-17344
RGA2		3965bp	18680 bp/ 1C4	CD by *Kpn*I	28579-47258	28579-47258
RGA3		3903bp	11911 bp/ 3C11	PD by *Sau*3A1	53905-65815	53905-65815
RGA4		10866bp	Not isolated			
RGA5	94G8-76432bp (76292-152724bp)	3883bp	13890 bp/ 2D7	PD by *Sau*3A1	21922-35811	98213-112100
RGA6		3957bp	11829 bp/ 3H3	PD by *Sau*3A1	61185-73013	137474-149302
RGA7	20F5-75310bp (146195-221504bp)	3932bp	7995 bp/ 1C7	CD by *Xba*I	26541-34535	172735-180729
RGA8		3859bp	11065 bp/ 2B7	CD by *Xba*I	34535-45599	180729-191793
RGA9		3914bp	13226 bp/ 3D10	CD by *Xba*I	45599-58824	191793-205018

* ORF of RGAs corresponds to the software predicted open reading frame with introns starting at ATG (start codon) and ending at TGA (stop codon).
** CD represents complete digestion and PD is for partial digestion by endonucleases.

5.2. Functional analysis of CGs

5.2.1. Expression in heterologous system

The expression and activity of isolated RGAs against Dort E 4 were assessed in seven independent transient heterologous (tobacco) complementation assays performed in triplicate. The leaves of *Nicotiana benthamiana* plants were infiltrated with GV3101 suspensions carrying relevant RGA constructs and after 24 hours these were challenged by Dort E4 in intact plants. The expression of RGAs was detected using different RGA general and specific primers (Table 5.2). All RGAs were found to be expressed in the heterologous system showing the presence of regulatory elements on the flanking 5' and 3' regions of isolated single RGAs necessary for their expression (Figure 5.2). However, the expressed RGAs did not respond to Dort E4 even at a very high spore concentration ($5x10^5$ spores/ ml) in form of hypersensitive response (HR) or necrosis and microscopy revealed the presence of spores without germination. On the basis of this observation tobacco was found to be non-host species for Dort E4, representing the phenomenon of non-host type resistance and the number of *Rdr1* candidates could not be reduced as all isolated RGAs were expressed in heterologous system.

Table 5.2: Heterologous expression analysis of single RGAs

Expressed RGAs	Expression detected by primer pair
RGA 1, 3, 7, 8 and 9	consensus cDNA (Bw1 Fw1)
RGA 5	consensus P1
RGAs 1, 3, 7, 8 and 9	Kuehr3
All RGAs (1-3 and 5-9)	Aneela 1F-1R
All RGAs (1-3 and 5-9)	Rd1LRR

5.2.2. Expression analysis of CGs in homologous system

To reduce the number of potential *Rdr1* candidates, the expression analysis of single CGs was carried out in different tissues (leaves, petals and roots) of resistant rose varieties (88/124-46 and 91/100-5). According to the available information, the leaves and petals of resistant rose genotypes used in this experiment displayed the same degree of resistance against Dort E 4 infections therefore by comparing the expression profile of RGAs in different tissues; the similarly expressed RGAs can be designated as potential *Rdr1* candidates reducing the number of *Rdr1* potential candidates. With this idea the cDNA of above mentioned genotypes was tested by a range of RGA specific primers (Table 7.10 in appendix). In the homologous system all RGAs were expected to be expressed and their very high DNA sequence similarities (85-99%; Kaufmann, 2010)

technically made it difficult to develop an specific primer pair for each single RGA separately that could give a clear cut proof of the expression of corresponding single RGA among the others, due to that many specific primer pairs were developed for each RGA and obtained PCR products were analyzed on agarose gels, polyacrylamide gels and SSCP gels. The analysis revealed the expression of RGAs 1, 2, 3, 7, 8, and 9 whereas RGAs 4, 5 and 6 were never found to be expressed in any kind of checked tissues (Table 5.3). In addition to that RGA2 was found to be expressed only in the leaves and not in the petals of both genotypes whereas petals already displayed a resistant reaction against Dort E-4. This approach reduced the number of *Rdr1* candidate genes to five i.e. RGAs 1, 3, 7, 8 and 9.

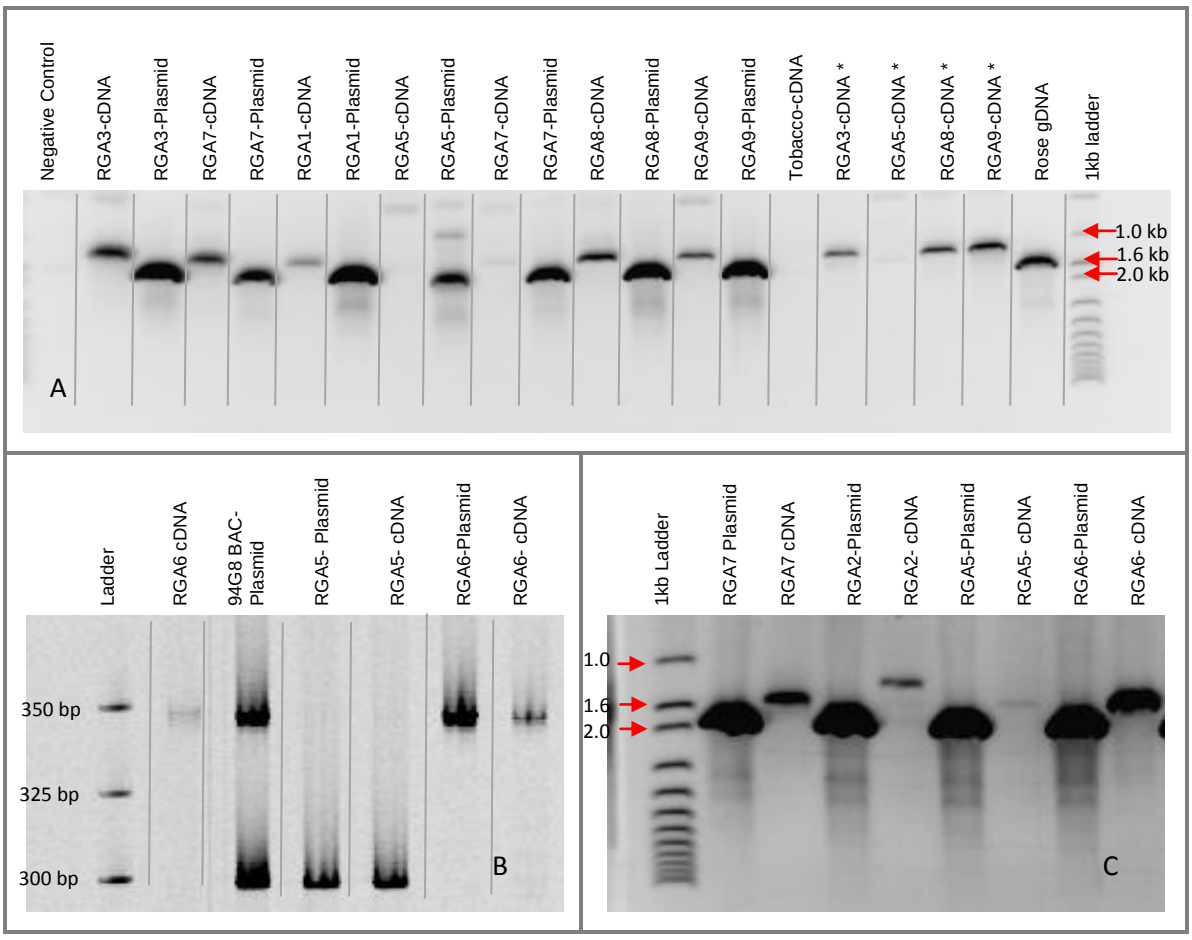


Figure 5.2: Detection of single CGs' expression in heterologous system. (A) Primer pair Kuehr3 is able to differentiate cDNA (1180bp) and gDNA (1550bp) in 1% agarose gel and it proved the expression of RGA 1, 3, 7, 8 and 9. * indicates the repeated loading of the cDNAs of corresponding RGAs ; (B) Primer pair Rd1LRR is able to amplify and differentiate all RGAs on the basis of fragment size in 6% polyacrylamide gel, here is an example of BAC 94G8 carrying RGA 5 and 6; (C) Aneela 1F-1R can amplify all RGAs and can differentiate cDNA (1600bp) and gDNA (1950bp) in 1% agarose gel. The freshly synthesized cDNAs before freezing were tested in these experiments.

Table 5.3: Expression profile of single CGs in homologous system

RGAs	Rose genotype 88/ 124-46			Rose genotype 91/ 100-5		Rose genotype PC	
	Leaves	Petals	Roots	Leaves	Petals	Leaves	Petals
1	+	+	+	+	+	+	+
2	+	-	+	+	-	-	-
3	+	+	+	+	+	+	+
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+
8	+	+	+	+	+	-	-
9	+	+	+	+	+	+	+

Key: + = expressed - = not expressed

5.3. Transient homologous disease assay

To validate the functionality of CGs, genetic complementation of a deficient plant is an important tool. Pariser Charme, a susceptible rose genotype, was selected to generate stable transformants harbouring CGs; due to technical problems and fungal contaminations initial effort resulted no transformants followed by new repeats. The putative transformants are in process of screening. While, generation of stable transformants is a lengthy process with low efficiency (Dohm et al. 2001; Marchant et al. 1998a), *Agrobacterium* mediated transient transformations were used as an alternative to evaluate the functionality of CGs.

5.3.1. Optimization of transient GUS expression assay in rose petals and leaves

For a successful transient expression assay a number of variables including host genotypes and their culturing methods, *Agrobacterium* genotypes, flower age, petal position within a flower, leaf type, additives to the bacterial growth media, bacterial density, temperature during incubation of infiltrated samples and incubation time required for significant GUS expression were optimized using GUS-intron reporter gene. First of all two different infiltration methods were tested using *Agrobacterium* strain GV3101 harbouring pBINPLUS::GUS-Intron in petals and leaves of Pariser Charme, for their feasibility and effectiveness. Infiltrations with l ml syringes without needles found to be the best option to infiltrate the whole petal completely or to perform spot infiltrations in leaves, whereas vacuum infiltration (5 min at 200 mbar with 2-5 breaks) made the delicate petals soaky and resulted in their early senescence; in case of leaves, vacuum infiltrations found comparable to syringe infiltrations followed by no or very little GUS expression when compared to samples infiltrated using a 1 ml syringe

without needle. These observations were consistent to all rose genotypes tested here. Therefore, in all subsequent experiments petals and leaves were infiltrated with syringes.

5.3.1.1. Effect of host genotypes

The petals of five and leaves of three host genotypes were evaluated to optimize GUS expression. GUS expression was monitored visually and rated as described in Figure 5.3. Host genotypes found to have a highly significant effect on the level of GUS expression (Kruskal Wallis Test $p = 2.2\ e^{-16}$).

In case of petals two rose varieties Pariser Charme (Figure 5.4) and Marvel displayed very high GUS expression whereas genotypes 91/100-5, 88/124-46 and Heckenzauber were resistant to agro-infection showing little or no GUS expression. Pariser Charme revealed the highest intensity of GUS expression and in some petals even better than in leaves of *N. benthamiana* that were used as positive control in all experiments (Figure 5.5). Therefore, Pariser Charme was selected to optimize different physical and biological factors that could influence the expression of a foreign gene in this system.

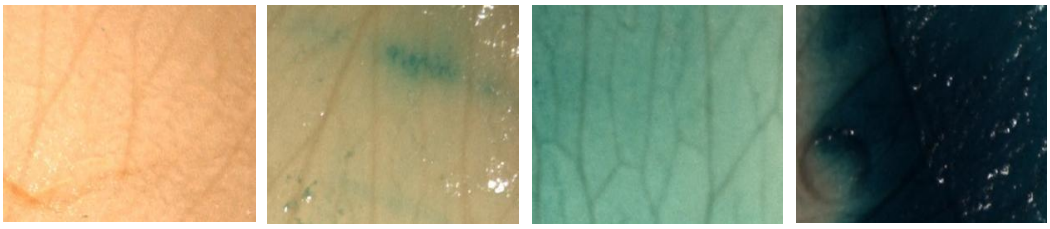


Figure 5.3: Pattern of scoring for the histochemical GUS assay in rose petals. Scores are indicated below the pictures of four different staining intensities.

In case of leaves GUS expression was optimized in three rose genotypes growing in different semi-controlled conditions (greenhouse, under plastic tunnels and in vitro as described in section 4.2.7.3). These culturing environments effected GUS expression significantly (Kruskal Wallis Test $p = 6.37\ e^{-16}$). Pariser Charme and 91/100-5 genotypes grew under tunnels with high humidity and temperature (above 30ºC) displayed the highest GUS expression (Figure 5.6a and b). Genotype 88/ 124-46 again found to be resistant to agro-infection and showed no GUS expression at all. Pariser Charme and 91/100-5 rose genotypes were selected to perform homologous disease assay and RNAi knock outs of *Rdr1* family.

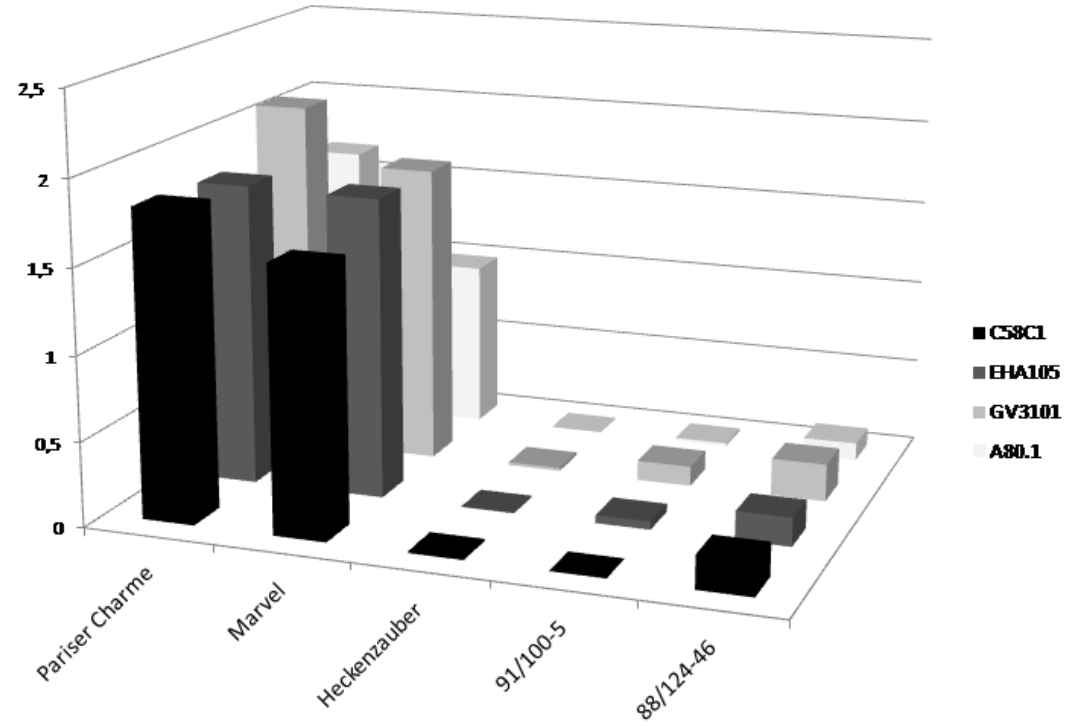


Figure 5.4: Effect of rose genotypes and *Agrobacterium* strains on the expression of GUS in rose petals. Indicated on the vertical axis are the mean values for the GUS scores, from 0 to 2.5, as shown in Figure 5.3.

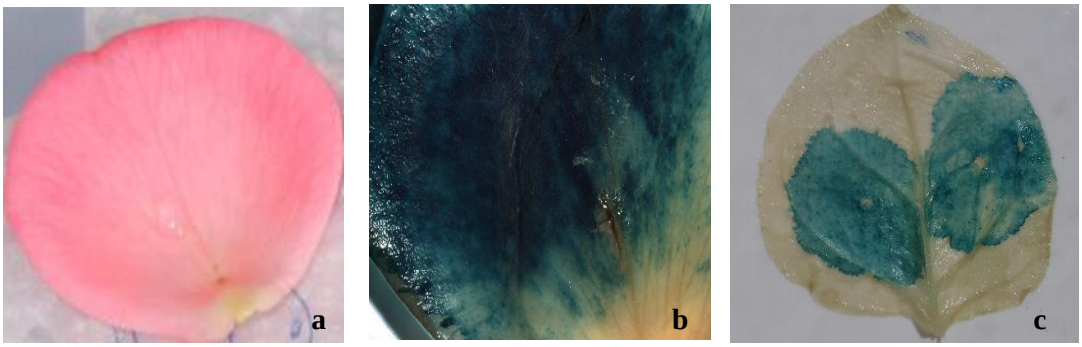


Figure 5.5: Samples infiltrated by *Agrobacterum* harbouring GUS-Intron construct at OD_{600} = 0.5. a- PC petal before GUS staining; **b**- PC petal after GUS histochemical assay; **c**- *N. benthamiana* leaf after GUS staining.

5.3.1.2. Effect of *Agrobacterium* genotypes

All tested *Agrobacterium* strains GV3101, EHAI05, C58C1 and 80.1, each harbouring 35S::GUS-Intron showed almost the same degree of GUS expression on each of the rose genotypes and no significant differences were detected. Although significant differences occur after inoculation on genotypes Marvel (Kruskal Wallis $p = 3.7\ e^{-7}$) and 88/124-46 (Kruskal Wallis p = 0.0023). On both Marvel and 88/124-46 strain WT-80.1 produces significantly weaker GUS-signals as all the other strains (p values between 0.00023 and $8.16e^{-7}$ for Marvel and between 0.0002 and 0.028 for 88/124-46)-Figure

5.4. GV3101 was selected for further studies as it gave the highest average expression level and as it had been used in several published studies for agro-infiltration (Zottini et al. 2008; Kim *et al.*, 2009; Bhaskar *et al.*, 2009).

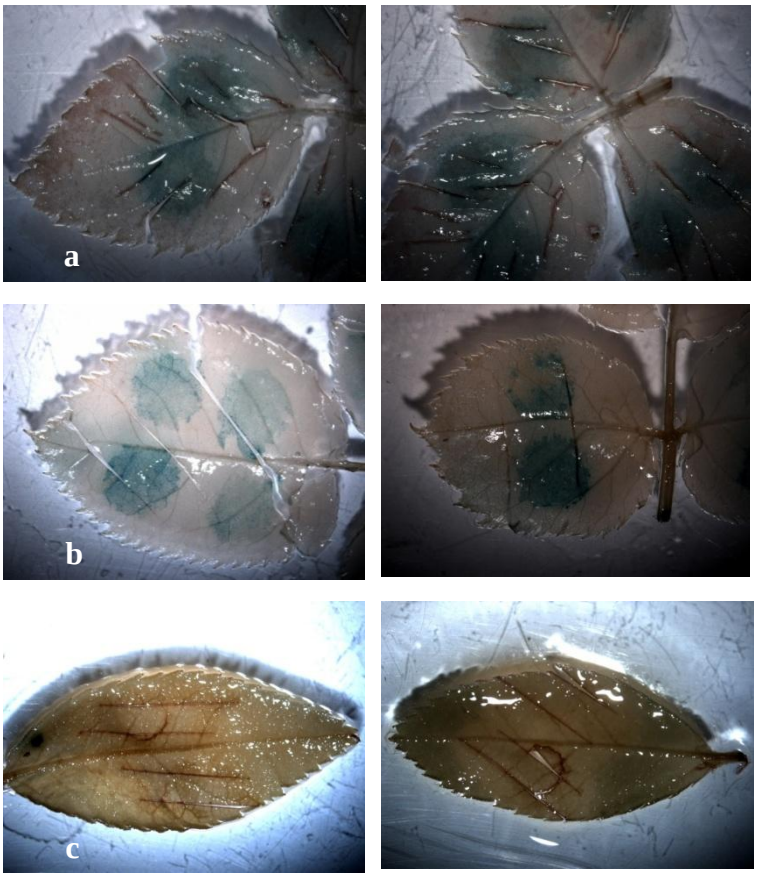


Figure 5.6: Leaves of different rose genotypes after GUS histochemical assay. These were infiltrated by *Agrobacterum* harboring GUS-Intron construct at OD_{600} = 1.5. **a-** PC (susceptible to Dort E4 and race6); **b**- 91/ 100-5 (resistant to Dort E4; susceptible to race 6); **c**- 88/ 124-46 (resistant to Dort E4; susceptible to race 6).

5.3.1.3. Effect of flower/ leaf age and petal position

GUS-expression was compared among petals from buds of Pariser Charme before opening (stage 1), flowers just opened (stage 2) and fully opened flowers (stage 3). The highest level of expression was found in stage 2 flowers (mean value = 2.13) as compared to stage 1 (mean value of 0.69 p = $2.0e^{-5}$) and stage 3 (mean value = 1.47 p = 0.016) flowers. Within the stage 2 flowers, petals from the middle of the flowers displayed the highest GUS-expression as compared to the outer and inner whirl petals (p values between 0.0003 and 0.0029). However, the variation between petals of the same flower stage and the same whirl was very high with standard deviations between 0.64 and 0.95. GUS-expression was evaluated in different types of leaves of Pariser Charme; Leaves with a green upper side and red lower side (type A), light green leaves with dark

green prominent veins and red edges (type B), complete light green young leaves (type C) and dark green old leaves (type D). Although, the infiltration was very easy in old leaves, type B leaves showed significantly higher GUS expression as compared to others (Kruskal Wallis Test p = 0.0008337).

5.3.1.4. Effect of additives in growth media

The effect of acetosyringone on GUS expression was found to be non-significant (Kruskal Wallis p = 0.326). In addition, a surfactant (Breakthru) was used to promote an even distribution of bacterial suspensions in petals and no significant differences in GUS-expression were noted. At higher concentrations (100 and 1000 ppm) it promoted early senescence in petals; the highest concentration even being lethal to petals leading to necrosis within 24 hours.

5.3.1.5. Effect of bacterial density

The bacterial suspensions were adjusted to OD_{600} of 0.1, 0.3, 0.5 0.8, 1.0, 1.5, 2.0, 3.0 and 4. In case of petals, the GUS expression was observed only for bacterial densities between $OD_{600 =}$ 0.5-and $OD_{600 =}$ 4.0 (Figure 5.5a). Among these densities, no significant differences were detected. The optimal OD was found to be 0.5 for petals of Pariser Charme and Marvel. In contrast to this, even the highest densities did not lead to GUS signals in the petals of remaining rose genotypes. The optimal concentration of bacteria for GUS expression in leaves of Pariser Charme and 91/ 100-5 was found to be 1.5-2.0 (Figure 5.6). The GUS expression in leaves was observed only for bacterial densities between $OD_{600 =}$ 1.5-and $OD_{600 =}$ 3.0. Tobacco leaves displayed highest GUS expression at OD_{600} = 0.5 (Figure 5.7).

5.3.1.6. Effect of incubation temperature and time

GUS expression in infiltrated rose petals was recorded at four different incubation temperatures, 19ºC, 22ºC, 25ºC, and 28ºC. The effect of the temperature during co-cultivation was found to be significant (Kruskal Wallis p = $2.2e^{-16}$). Temperatures of 19ºC and 25ºC revea1ed significantly lower GUS expression levels as compared to 22ºC (Figure 5.8). At 28ºC GUS expression levels were very low and almost no detectable. The time of co-cultivation had a significant effects on the level of GUS-expression (Figure 5.9c, Kruskal Wallis p = 7.3 e^{-14}). GUS expression was detectable from the second day after infiltration. However, significant levels of GUS expression occurred

only after day three. The highest intensity of GUS expression was detectable between days three and seven after which expression decreased significantly.

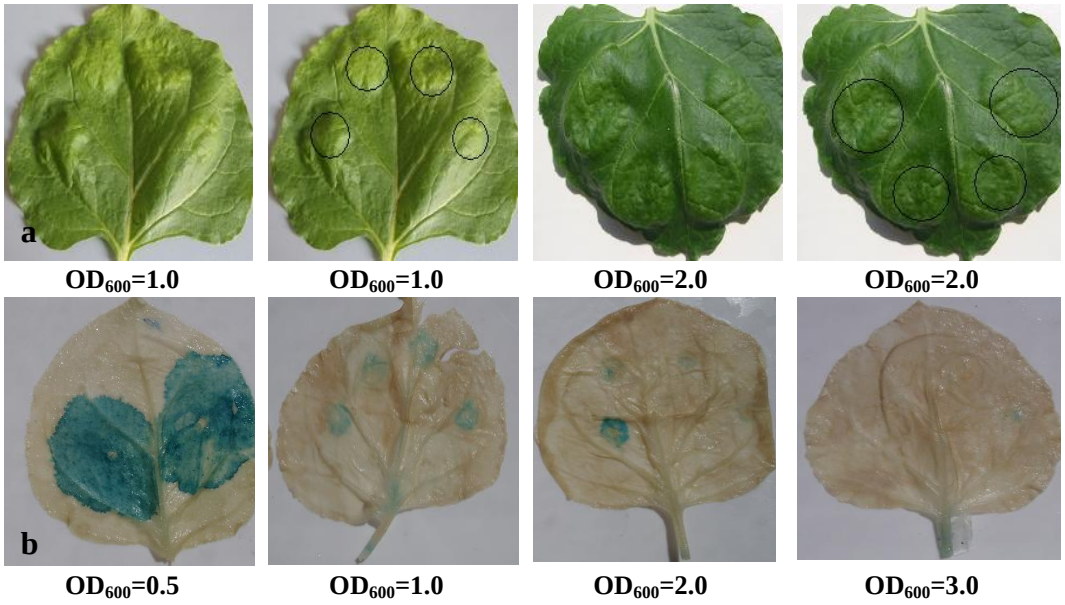


Figure 5.7: *N. benthamiana* leaves were infiltrated by *Agrobacterum* harbouring GUS-Intron construct at different OD_{600}. a- Infiltrating higher bacterial densities showed withering in leaves before GUS histochemcal assay. ; b- Infiltrating higher bacterial densities displayed low GUS expression as shown after GUS histochemcal assay; Infiltrated bacterial OD_{600} are indicated below the pictures.

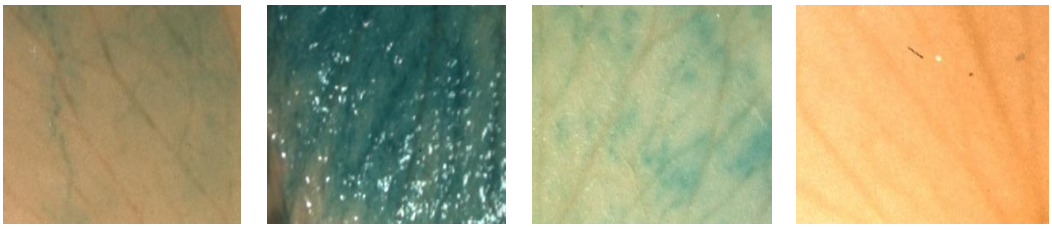


Figure 5.8: Effect of incubation temperature on GUS expression. Petals of Pariser Charme were infiltrated by *Agrobacterium* harbouring GUS-Intron construct at OD_{600} = 0.5 and incubated at different temperatures (as indicated below the pictures). Samples were collected on 4th day post-infiltration and GUS histochemcal assay was carried out.

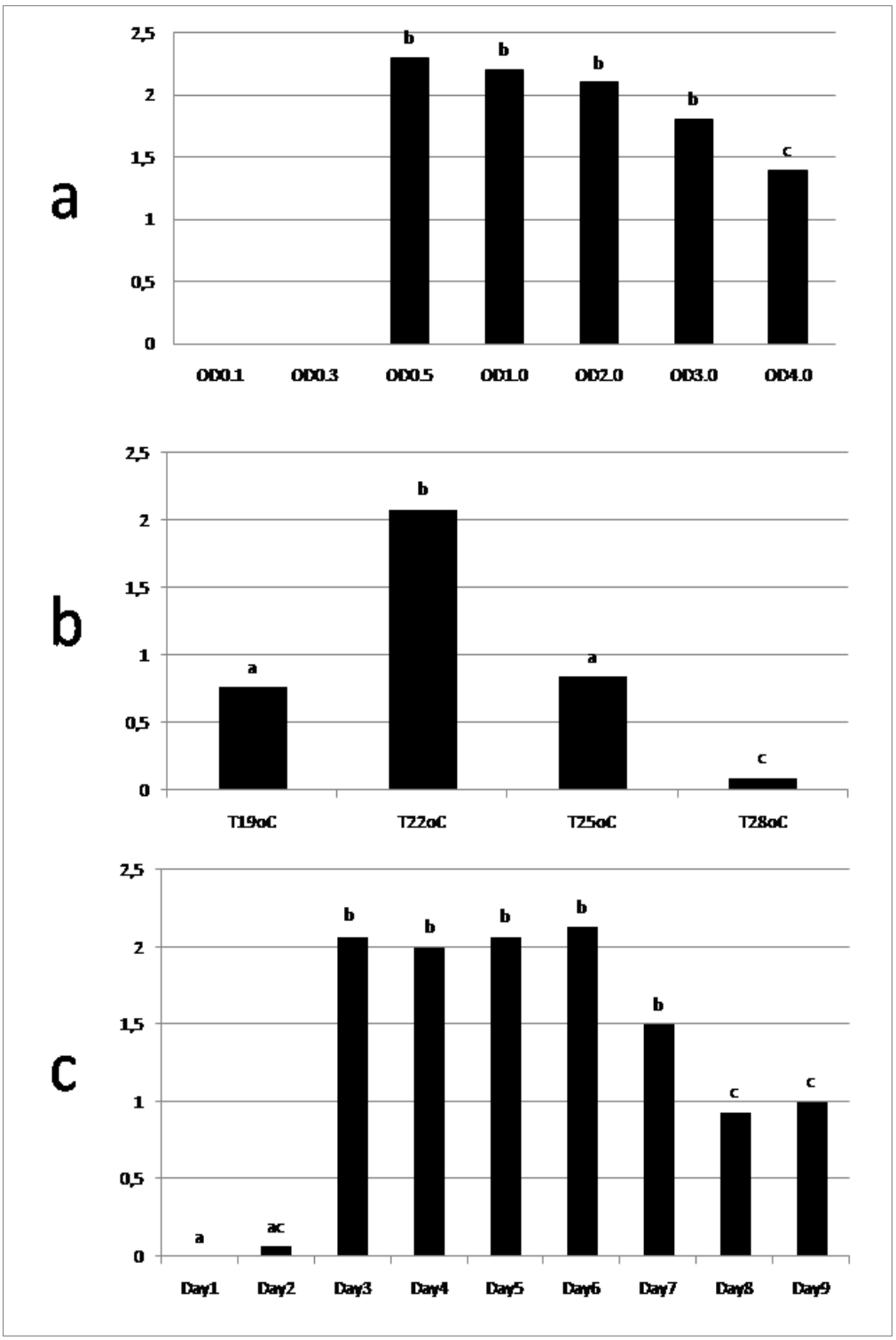


Figure 5.9: Optimization of different parameters for a successful *Agrobacterium* mediated transient GUS expression assay in the petals of Pariser Charme. a- The effect of bacterial density; b- The effect of cocultivation temperature; c- The effect of co-cultivation time. The y-axis indicates the mean values of the GUS scores; the x-axes indicate the different treatments within each factor. Different letters above each column indicate significant differences of the mean values at $P < 0.05$.

5.3.2. Verification of the expression of single CGs

Although petals were proved to be very efficient system to carry out transient expression studies this system was not found suitable to study the interaction of rose and black spot because of their short lifespan and limited seasonal supply. Therefore rose leaves were selected to carry out disease assays after optimizing GUS expression (Table 5.4) and proving the expression of single CGs post-infiltrations. According to available unpublished data it was demonstrated that the PC variety, susceptible to black spot, does not contain RGA8 (Terefe D, personal communication). Utilizing this information and already optimized parameters for transient GUS expression in leaves, the leaves of PC were infiltrated with agro-suspension of GV3101 harbouring RGA8 single CG and samples were collected for RNA extraction and cDNA synthesis 3 days after infiltrations (section 4.2.1.3). cDNA was evaluated using Rd1LRR primer pair in 6% polyacrylamide gel for the expression-detection of infiltrated RGA8. In infiltrated samples RGA8 was expressed as compared to control leaves that lack expression band for RGA8, as shown in Figure 5.10.

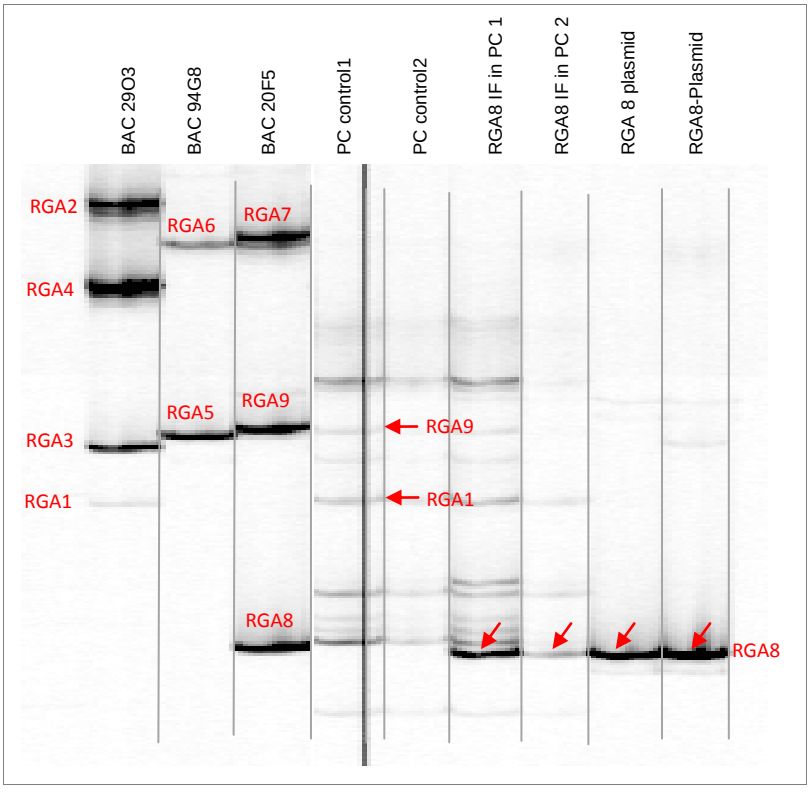


Figure 5.10: Detection of RGA8 expression in RGA8-infiltrated leaves of PC. Primer pair Rd1LRR was used to detect expression of different RGAs in PC control leaves and RGA8-infiltrated leaves in 6% PA gel. First three lanes show control BACs carrying all RGAs followed by two lanes with PC control leaves, two lanes with RGA-infiltrated leaves and last two lanes with isolated RGA8 plasmid, respectively. In PC-control leaves the expression of RGA1 and RGA9 is also indicated.

Table 5.4: Summary of optimized parameters for GUS transient expression in roses

Parameters	Rose Petals	Rose Leaves
Host genotypes	PC and Marvel	PC and 91/ 100-5
Host culture method	Greenhouse	Under plastic tunnels in high temperature and humidity
Properties of tissues	Petals of inner whirl of just opened flowers	light green leaves with dark green prominent veins and red edges
Agrobacterium strain	GV3101::pMP90	GV3101::pMP90
pBINPLUS plasmid	35S: GUS-Intron	35S: GUS-Intron
Growth of bacteria	Overnight in YEP	Overnight in YEP
Bacterial density (OD_{600})	0.4-0.5	1.5-2.0
Addition of acetosyringone	No	No
Addition of surfactant	No	No
Infiltration method	Syringe without a needle	Syringe without a needle
Incubation temperature	22ºC	22ºC
Incubation time	3-days	3-days

5.3.3. Optimization of transient disease assay in rose petals and leaves

To study the interaction of isolated single CGs and black spot, the petals and leaves of Pariser Charme were infiltrated with single RGAs and were challenged with different spore concentrations of *Diplocarpon rosae*. According to preliminary experiments in contrast to some biotrophs as powdery mildew, when *D. rosae* was infiltrated in petals or leaves, it was able to grow inter-cellularly showing its typical pattern of growth. Therefore in all subsequent experiments, leaves were infiltrated with spore suspensions to assess the activity of CGs. Whereas *D. rosae* was not able to grow in rose petals when co-infiltrated with *Agrobacterium* so *D. rosae* was inoculated by spotting spores suspension 24 hours post-agro-infiltrations.

5.3.3.1. Rose petals

Although the rose petals were found very feasible system to carry out transient expression analysis these were not suitable for disease assay as have short lifespan and limited seasonal supply. To shorten the phyto-patho interaction study period the petals were co-infiltrated using agro-suspension of CGs (OD_{600} = 0.5) and spores of *D. rosae* ($5x10^5$ spores/ ml), co-infiltration of spores and bacterial suspension did not reveal any

fungal growth even after 5-7 days of infiltrations. Microscopic examination of such samples revealed the presence of non-germinated conidia within intercellular spaces. In addition to that microscopy of spore infiltrated petals also revealed the sieving effect of spores, accumulating at the place of infiltration (Figure 5.11). Therefore, all spore inoculations were done by dropping 3-5 spots of spore suspension per petal after 24 hours of agro-infiltrations of CGs. When petals were infiltrated without *Agrobacterium* or after 24 hours of agro-infiltrations these displayed the type 1 interaction (Blechert and Debener, 2005) against Dort E 4 characterized by haustoria, long and short distance hyphae and conidia. Although higher concentrations of bacteria (OD_{600}= 1.5-4) promoted the fungal growth, the optimal concentration of bacterial that displayed the optimal GUS expression without effecting fungal growth was OD_{600}= 0.5. The bacterial concentration at OD_{600}= 0.5 and spore concentration at $5x10^5$ spores/ ml did not reveal significant differences in fungal growth. Due to limited seasonal supply of flowers the concentration of spores could not be optimized to reveal significant effect of infiltrated CGs in terms of reduction in fungal growth. Although off season flowers were maintained in the greenhouse and were used to optimize the system, they showed the presence of contaminating fungi on microscopy and in some cases displayed senescence within 24 hours of agro-infiltrations.

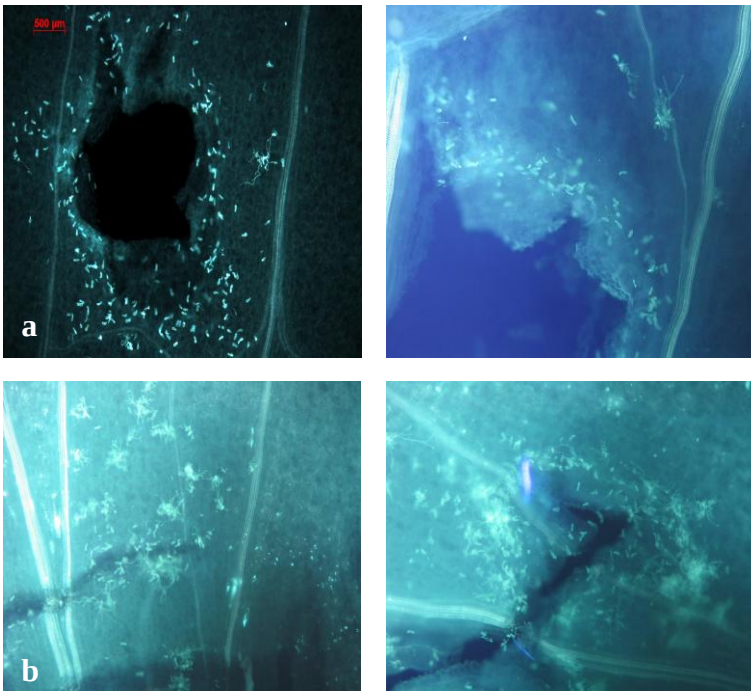


Figure 5.11: *Agrobacterium* and spore infiltrations in the petals of PC. a- Sieving effect and no spore germination when spores co-infiltrated with *Agrobacterium;* b- Fungal growth when spores inoculated by spotting 24 hours after agro-infiltrations.

5.3.3.2. Rose leaves

To study the interaction of isolated single CGs against black spot, the type B leaves of Pariser Charme (light green leaves with dark green prominent veins and red edges) were co-infiltrated with single RGAs in GV3101 together with conidia of the isolate Dort E4. Spore concentrations starting from $5x10^2$ to $5x10^5$conidia/ml of *Diplocarpon rosae* was tested. Based on the observation that the transient expression in rose leaves is lower than in *Nicotiana* due to a lower number of transformed cells; a low concentration of conidia was chosen to obtain a higher ratio of transformed cells to conidia. The spore concentration of $5x10^2$ conidia/ml was found the optimum; revealing significant affects of RGAs activity on fungal growth and non-significantly affecting fungal growth in the presence *Agrobacterium* (OD_{600}= 1.5), when compared to negative control samples infiltrated with spores only (Kruskal Wallis Test p= 0.1806). Therefore, PC leaves were co-infiltrated with single RGAs and $5x10^2$ spores/ml together in all subsequent experiments and the activity of RGAs against Dort E4 was evaluated by counting fungal colonies. As described in section 4.2.8.1 a fungal colony is the fungal growth showing different developmental levels of prolonged hyphae and/ or formed acervuli within a spot representing the circular area (diameter: 0.3-0.5mm) where bacterial suspension and spores were co-infiltrated. The microscopy of RGAs and Dort E 4 infiltrated spots displayed a range of developing (short, long, branched and unbranched) hyphae with or without acervuli that cannot be easily related to the presence of a particular RGA due to the fact that infiltrated areas in leaves did not contain all genetically complemented cells but represent a mixture of transformed (resistant; transiently complimented) and non-transformed cells (susceptible; not complemented). Moreover spore suspensions also contained some dead spores that of course could not be germinated on inoculations representing type 8 interaction as described by Blechert and Debener (2005). Although it was not possible to relate a specific morphology of fungal colony to the presence of a particular RGA the reduction in colony number was very obvious and significant when RGA8 or RGA1 were infiltrated in PC leaves and challenged by Dort E 4. In six independent experiments (Figure 5.13) RGA1 and RGA8 significantly reduced the growth of fungus (Kruskal Wallis Test p = 1.175 e^{-09}; Figure 5.12). Two out of six experiments were found to be non-significant on the basis Kruskal Wallis Test using original data of colony numbers (Figure 5.13 c and d). Although RGA8 (Wilcoxon Test p= 2.798 e^{-08}) and RGA1 (Wilcoxon Test p= 5.274 e^{-05}) were found significantly reducing fungal growth when compared to controls (spores in presence of GUS

Agrobacterium and/ or spores only), RGA8 restricted fungal growth in 4 out of 6, whereas RGA1 reduced fungal growth in 2 out of six independent experiments in total (Figure 13). When compared to RGA8, RGA1 only showed significant reduction in 1 out of 6-independent experiments (Figure 5.13 b) and on average RGA8 reduced the fungal growth significantly to 41% in contrast RGA1 revealed 26% reduction in fungal growth (Figure 5.12). It is very clear from the Figure 5.13 that the RGA8 consistently reduced the fungal growth followed by RGA1. These observations make RGA8 the most favorable candidate for *Rdr1*.

However, on the basis of RGA expression profiles in the homologous system, RGA2, 5 and 6 were excluded from the list of *Rdr1* candidates. Two independent experiments were performed to validate their effect on fungal growth in the transient disease assay (Figure 5.14) and results confirmed their non- functionality against race 5 (Wilcoxson Test p values = RGA2-0.003 and 0.07; RGA5-5.6 e^{-6} and 5.5 e^{-4}; RGA6-1.2 e^{-4} and 0.002 when compared to RGA8 and RGA1 respectively). In addition to that independent experiments were carried out to evaluate the interaction of CGs and race 6 and all CGs were found to be non-active against race 6 as it was expected (Kruskal Wallis Test p = 0.5679; Figure 5.15; 5.16a). Interestingly, when GUS *Agrobacterium* were infiltrated in the leaves of PC and 91/ 100-5 together with race 6 conidia the fungal growth was more profound (~ 1.5 x more) relative to negative control samples (leaves infiltrated by spore suspension only; Figure 5.16 b).

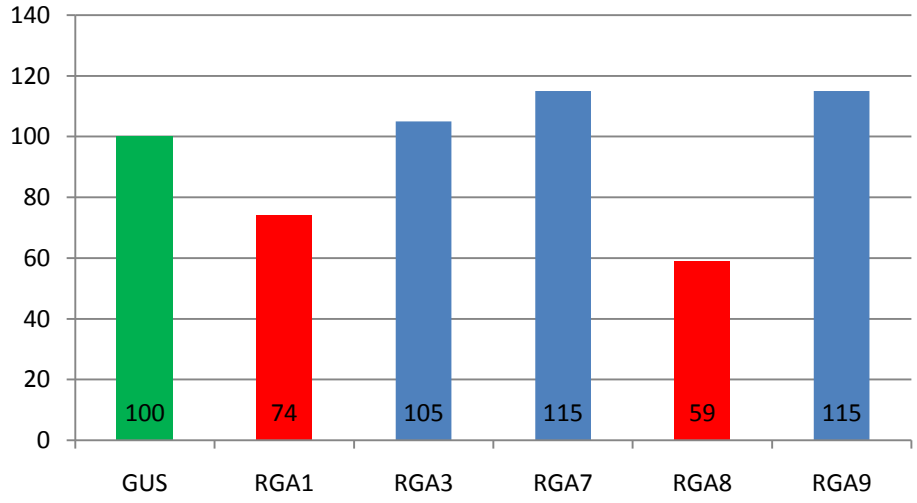


Figure 5.12: Transient complementation disease assay with individual CGs and DortE4. This graph represents average data of five independent experiments. The y-axis indicates the percent colony number; the x-axis indicates different constructs infiltrated in PC leaves in corresponding *Agrobacterium* suspensions together with Dort E 4. Red highlighted columns indicate different mean

populations based on original data i.e. number of colonies tested with Kruskal Wallis Test at P< 0.05. For this graph Kruskal Wallis Test p= 1.175 e^{-9}.

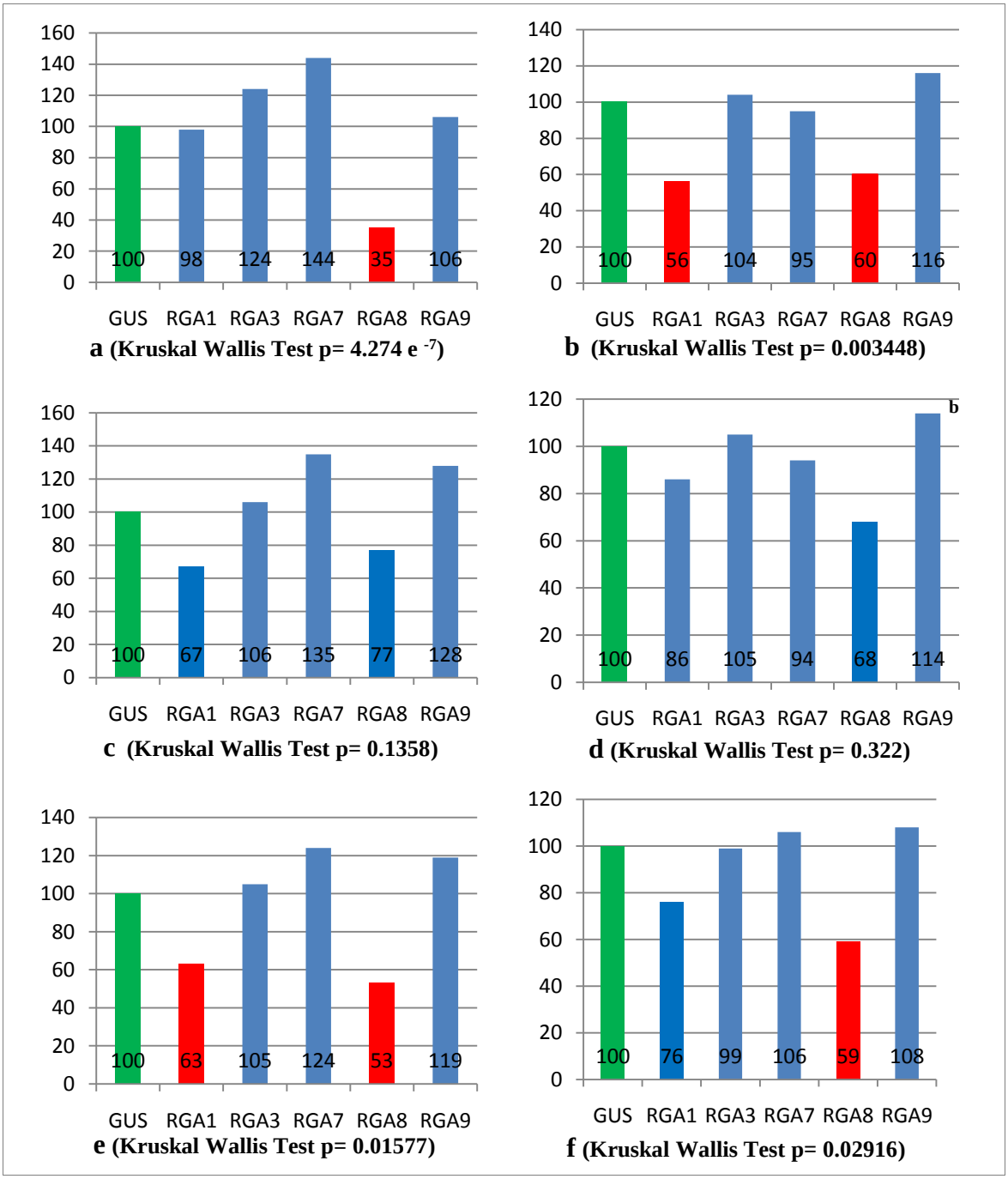


Figure 5.13: Transient complementation disease assay with individual CGs and DortE4. These graphs represent the data of six independent experiments. The y-axis indicates the percent colony number; the x-axis indicates different constructs infiltrated in PC leaves in corresponding *Agrobacterium* suspensions together with Dort E 4. Red highlighted columns indicate different mean populations based on original data i.e. number of colonies tested with Kruskal Wallis Test at P< 0.05. For these graphs Kruskal Wallis Test p- values are indicated below the corresponding graphs. Graph b and c show RGA1 as the most effective gene to reduce the fungal growth and graphs e and f also represent its effect whereas RGA8 is consistently showing its functionality against Dort E 4.

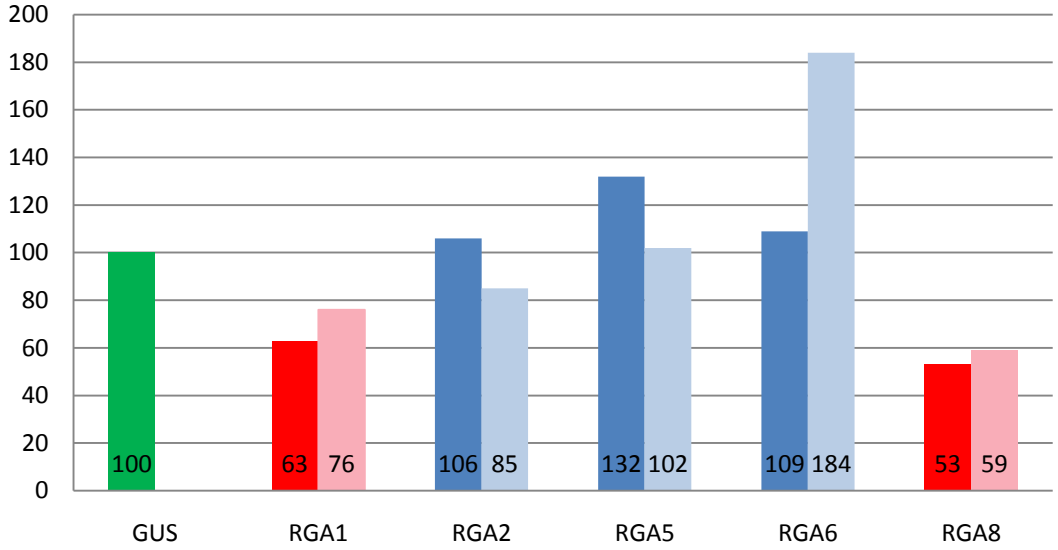


Figure 5.14: Transient complementation disease assay with individual CGs and DortE4. The graph represents the data of two independent experiments. The y-axis indicates the percent colony number; the x-axis indicates different constructs infiltrated in PC leaves in corresponding *Agrobacterium* suspensions together with Dort E 4. Red highlighted columns indicate significantly different mean populations based on original data i.e. number of colonies tested with Kruskal Wallis Test at P< 0.05. For this graph Kruskal Wallis Test p- values are 0.006 and 0.008 respectively. RGA2, 5 and 6 were found non-active genes against Dort E 4 as compared to RGA1 and 8.

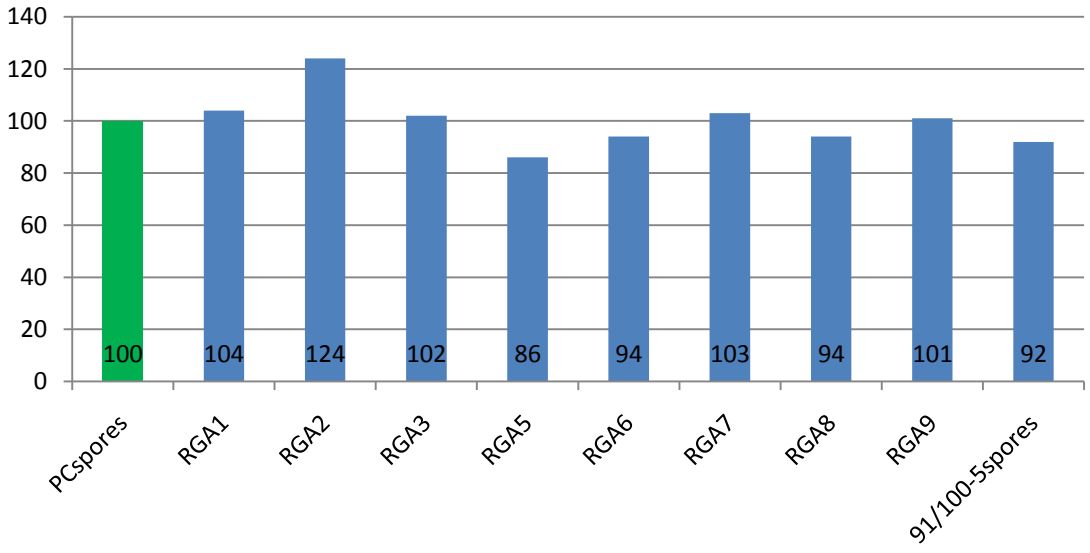


Figure 5.15: Transient complementation disease assay with individual CGs and Race6. The y-axis indicates the percent colony number; the x-axis indicates different constructs infiltrated in PC leaves in corresponding *Agrobacterium* suspensions together with race 6. Last column in graph represents percent of fungal colonies in 91/ 100-5 rose genotype infiltrated with race 6 conidia. For this graph Kruskal Wallis Test p= 0.5679.

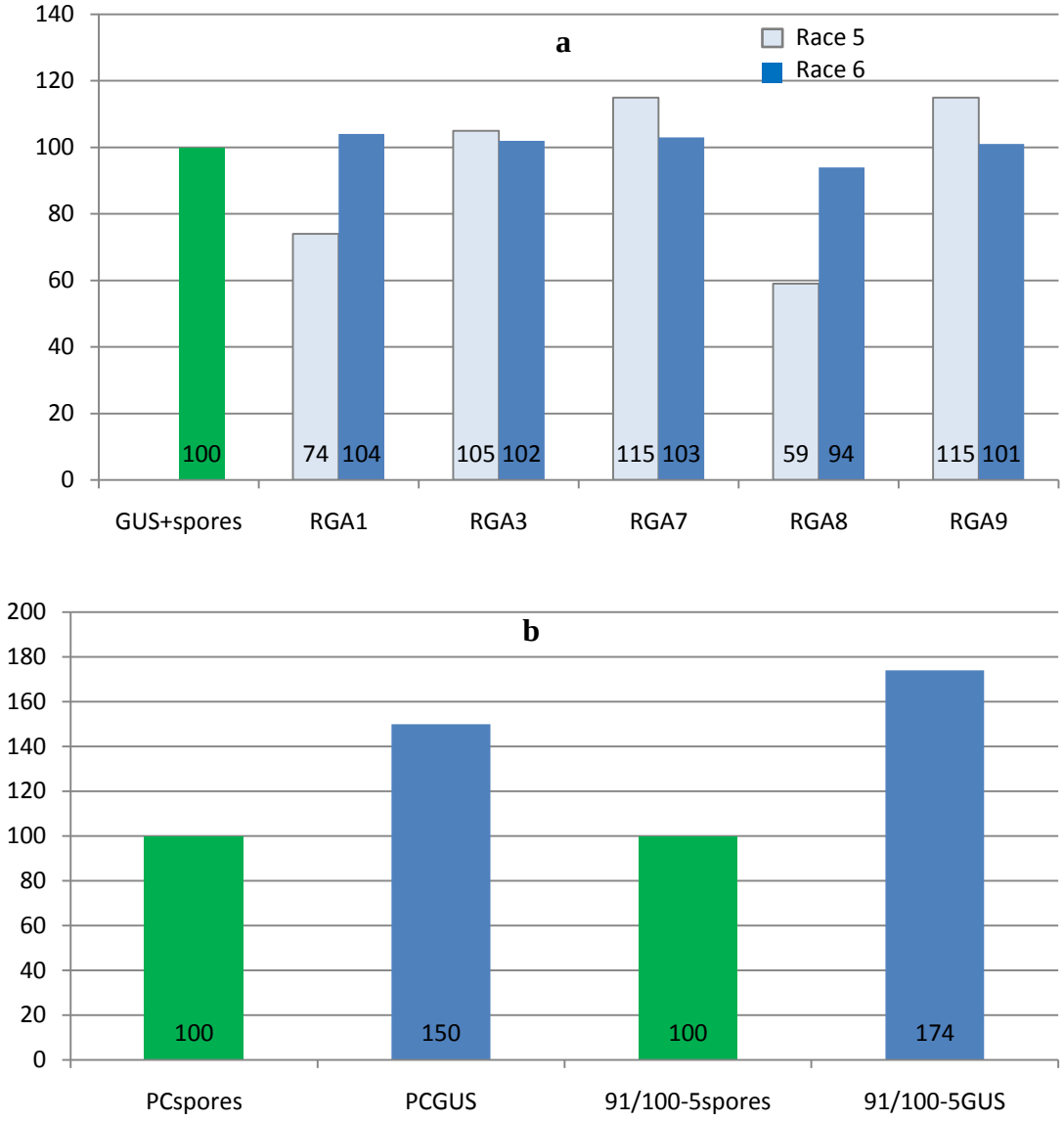


Figure 5.16: Transient complementation disease assay with individual CGs and Race 5 and 6. The y-axis indicates the percent colony number; the x-axis indicates different constructs infiltrated in leaves of PC or 91/ 100-5 in corresponding *Agrobacterium* suspensions together with Race 5 and Race6; a- comparison of transiently complementated PC leaves with single CGs as indicated and challenged by race 5 (first column) and race 6 (2nd column); b- Effect of transiently expressed GUS construct in leaves of PC or 91/ 100-5 on fungal growth (race 6) compared to negative control (infiltrated by spores only).

5.4. Transient silencing of RGA8 gene

RGA8 was knocked out in resistant rose genotype 91/100-5 using RNAi construct (RGA8-exon2) to verify the effect of RGA8 in restricting growth of Dort E4. The silencing construct used in this study was derived from exon 2 of RGA8 gene that encode NBS region of functional protein, it has a similarity of 87-99 % to exon 2 of other paralogs of *Rdr1* family. Due to high similarity of construct to other homologos of *Rdr1* family it was expected that the construct could silence other members of the

family along with RGA8. In addition to resistant 91/ 100-5, PC a susceptible rose genotype was also infiltrated with silencing construct and challenged by Dort E 4 as a part of this experiment. RNAi experiments resulted in a significantly high fungal growth in resistant (Krukskal Wallis Test $p < 2.2e\text{-}^{16}$; Figure 5.17) and susceptible rose genotypes (Krukskal Wallis Test p value = 9.99 $e\text{-}^{7}$; Figure 5.17) after infiltrating the RNAi construct with a helper plasmid VIP1 and fungal spores at a high concentration ranging between $2.5x10^5$ - $5x10^5$ spores/ ml. However, the Dort E 4 is not able to germinate in 91/ 100-5 as displayed in control samples it showed about 4x more growth after silencing *Rdr1* family compared to control samples. In contrast to 91/ 100-5, PC is a susceptible genotype with a defective *Rdr1* locus; even the use of same silencing construct in this genotype resulted double the number of fungal colonies (Figure 5.17) suggesting the silencing of *Rdr1* family because this genotype does not contain RGA8 and the effect of other homologos in restricting fungal growth. Figure 5.18 presents the growth of Dort E 4 in 91/ 100-5 resistant genotype in absence of RNAi construct and *Agrobacterium* (1^{st} control), in the presence of GUS and VIP1-*Agrobacterium* (2^{nd} control) and in presence of RNAi construct and VIP1 (silenced samples). Observations related to morphology of fungal colony in control samples revealed the typical type 7 interaction (Blechert and Debener 2005) of 91/ 100-5 with Dort E 4 characterized by penetration hyphae with very little further development of hyphae (Figure 5.18 a). In the presence of VIP and GUS constructs some samples displayed well developed hyphal growth without any indication of acervuli development however most of the samples showed penetration hyphae with short, long and poorly branched hyphae, moreover in some samples fungal growth was limited by host produced cell-wall appositions (Figure 5.18 b and c). In addition samples infiltrated by RNAi and VIP construct displayed well developed hyphal growth with short or long hyphal branching at the site of inoculation but none of the colony showed mature or developing acervuli. According to Blechert and Debener (2005) such interaction can be designated as weakly susceptible (interaction types 3, 4 and 5). In contrast, PC displayed the typical type 1 interaction characterized by well developed hyphae and acervuli.

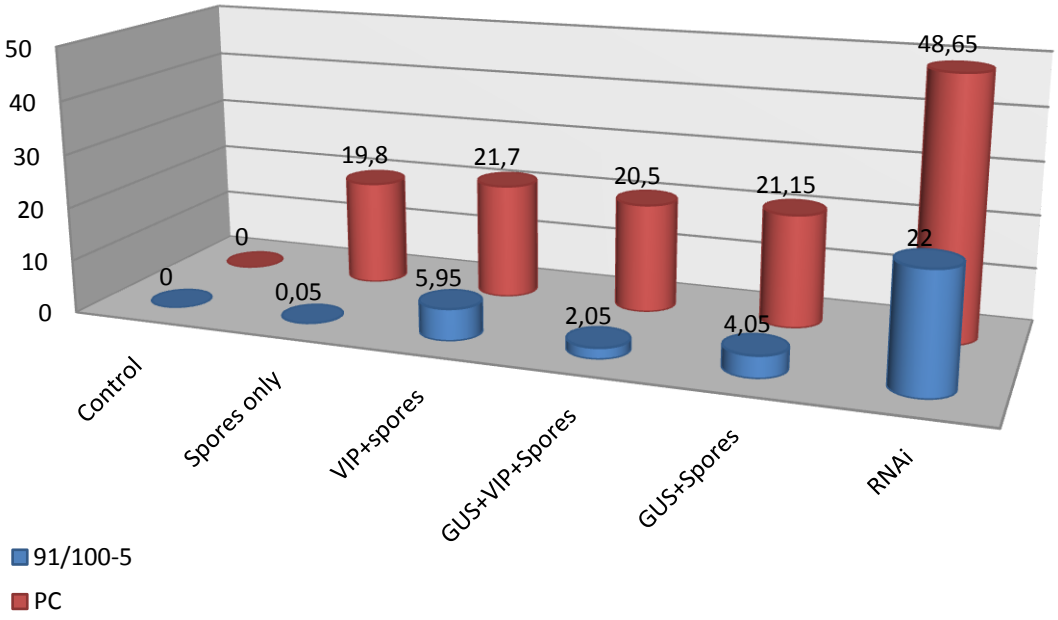
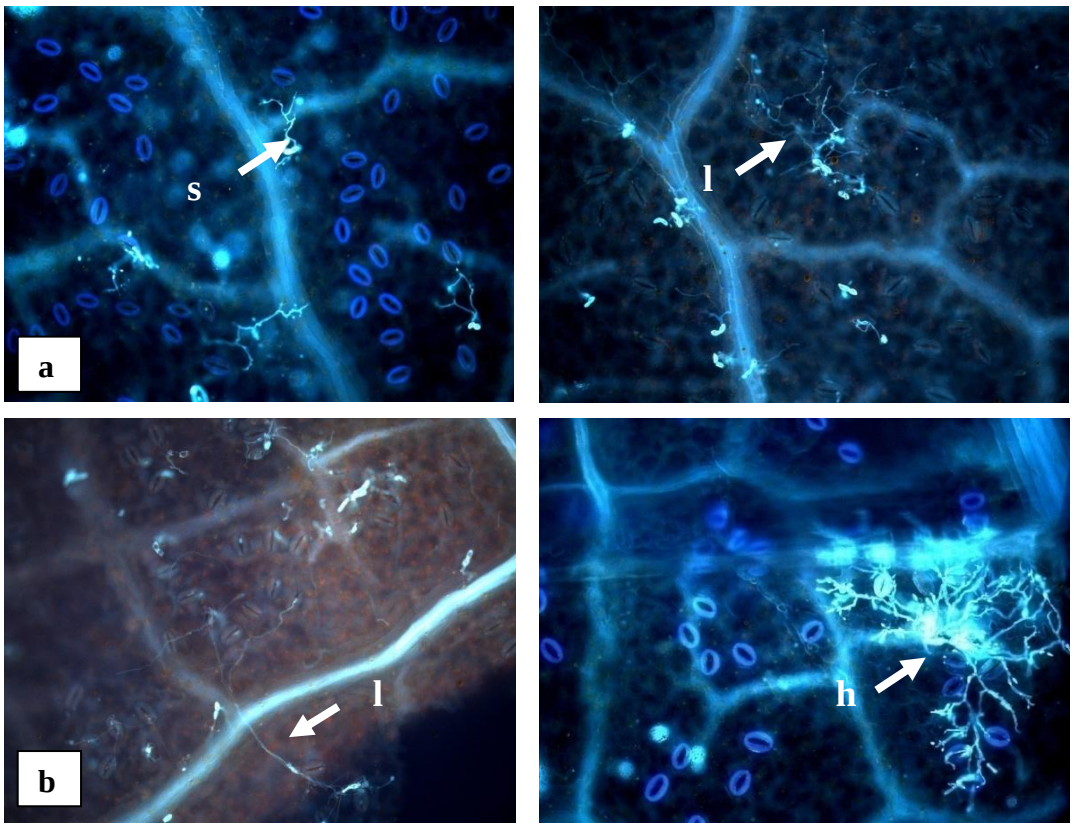


Figure 5.17: Effect of Rdr1 family knockout in PC and 91/ 100-5 rose genotypes on fungal growth (Dort E 4). The RNAi construct was infiltrated in resistant (91/ 100-5) and susceptible rose genotype PC, its activity was evaluated in terms of increase in fungal growth (race 5).



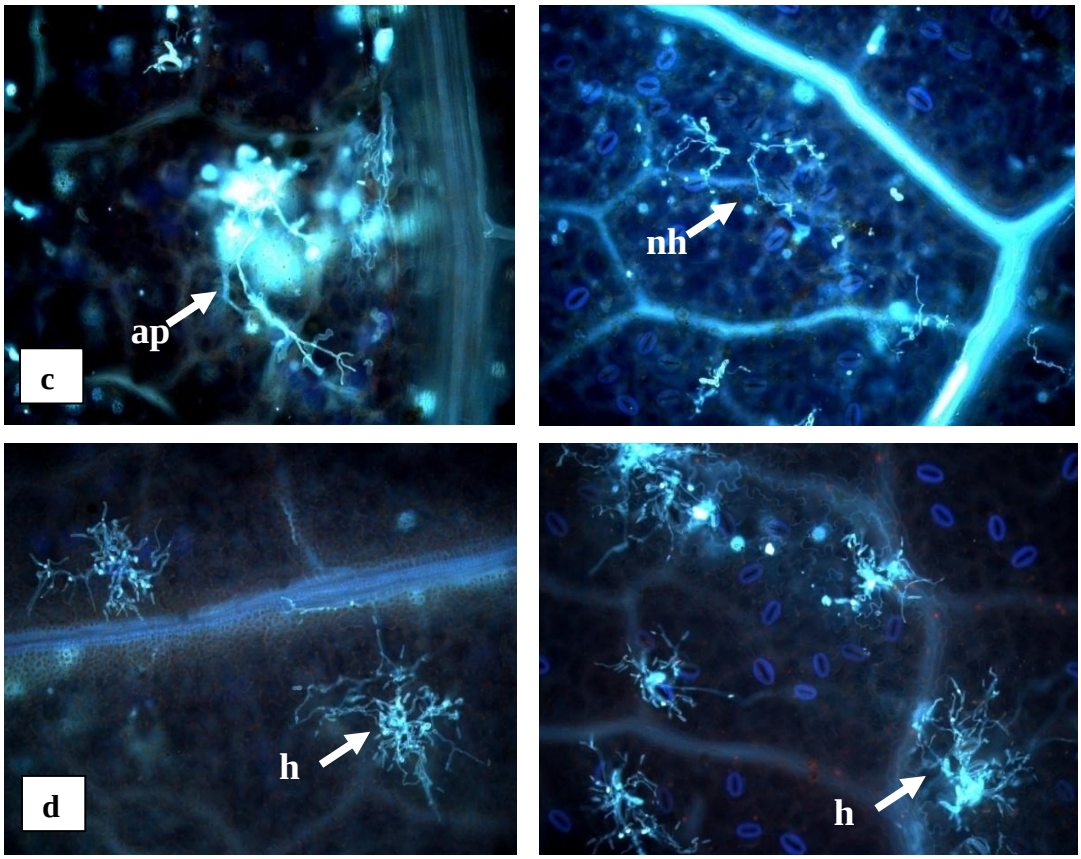


Figure 5.18: Growth pattern of Dort E4 in leaves of resistant rose genotype 91/ 100-5. a-samples infiltrated with Dort E4 spores only; b-samples co-infiltrated with Dort E4 and GV3101 harbouring VIP1 helper plasmid; c- samples co-infiltration with Dort E4 and GV3101 harbouring GUS Intron; d- samples co-infiltration using Dort E4 and GV3101 harbouring VIP1 & RNAi constructs in 1:1 ratio. s- short hyphal strands that did not develop in long distance hyphae; l- long very thin hyphae; ap- cell wall depositions visible around the area of fungal invasion; h- well developed hyphal network at the site of inoculation; nh-short distance hyphae poorly branched.

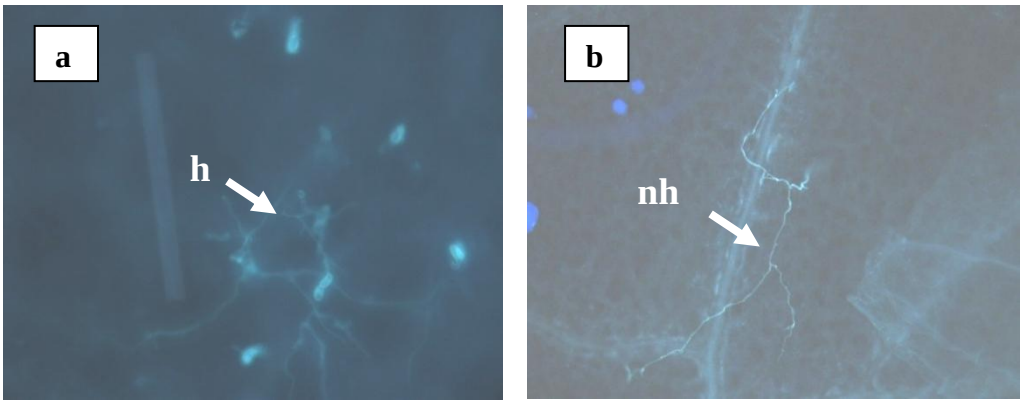


Figure 5.19: The growth pattern of Dort E4 infiltrated in *Arabidopsis* varieties. a- Columbia; b- PEN2-1 Mutant; h- well developed hyphal network at the site of inoculation; nh-short distance poorly branched hyphae.

5.5. Generation of *Arabidopsis* lines homozygous for single CGs

As Dort E4 was able to grow in *Arabidopsis* (Figure 5.19), this heterologous system was used to generate homozygous lines for GUS, pGJ28 and all RGAs. This attempt

resulted the generation of homozygous lines for GUS (Columbia and PEN-2 mutant), pGJ28 (Columbia only), RGA3 (Columbia only) and RGA8 (Columbia only) following the scheme presented in Table 5.5. The generated stable transformants have to be utilized for the confirmation and evaluation of the interaction data of RGA8 and Dort E4 obtained through transient complementation assays. Due to time limitations further experimental findings could not be added in this thesis.

Table 5.5: The generation of *Arabidopsis* stable homozygous lines

Practical steps carried out	Generation No.	Resultant genotype
Floral parts of plants were dipped in agro-suspension followed by seed collection and *in vitro* selection of transformants.	T 0	All Rr
Transformants were rescued and transferred to green house from *in vitro* culture. Seeds were collected after selfing, separately from each transformant and designated as single line.	T 1	Cross: Rr x Rr Result: 1:3 (RR: Rr: Rr: rr)
From each line 30 plants were selfed and seed were collected.	T2	RR x RR = all RR Rr x Rr = 1:3 rr x rr = all rr
Seeds of all 30 plants of each transformant line were screened *in vitro* and homozygous lines were selected.	T3	All RR

5.6. Gene structure and corresponding proteins of some *Rdr1* candidates

5.6.1. RGA8- black spot resistance gene

As mentioned before the RGA8 was isolated from BAC 20F5 by complete enzymatic digestion (*Xba I*) and cloned in binary vector pBINPLUS. Clone 2B7 harbour RGA8 with an insert size of 11065bp. The isolated RGA8 fragment was located on the available contig for *Rdr1* locus at positions 180729-191793 by comparing end sequences of genomic insert in subclone 2B7. According to the available cDNA sequence and genomic sequence of RGA8 it has a gene structure of four exons and three introns; all *Rdr1* candidates share this basic gene structure. The first two exons encode TIR and NBS domains respectively, while third and fourth exons codes LRR domain (Kaufmann et al,. 2010). The isolated RGA8 clone was infiltrated in tobacco and total mRNA was isolated 3-days post-infiltration and processed to determine the 5' and 3' end sequences of RGA8-cDNA experimentally (Section 4.2.4). The determined sequences were aligned to the genomic sequence of RGA8 (Figure 5.20) indicating the presence of two alternative transcripts. It is important to report that no truncated

transcript was found during these experiments and alternative transcripts resulted in the same ORF as described below.

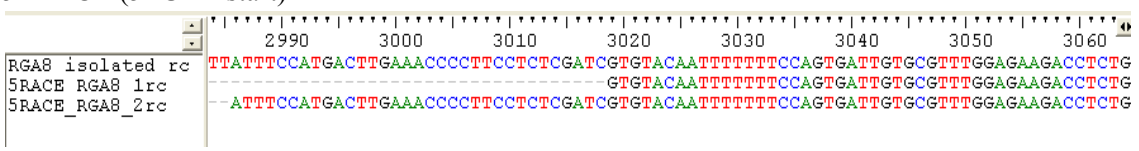
5.6.1.1. 5' RACE

RACE identified two 5' cDNA fragments of 68 and 101 nucleotides as 5' UTR upstream to ATG (Figure 5.20 a, b and c). Four gene specific primer pairs, one outer and three inner (Table A10 in appendix), were designed from 1st exon (498 bp) of RGA8 gene. However, outer primer PCR did not result in any product, it displayed single bands in 1% agarose gel when diluted as 1:20 and used as template for other three inner primers. The product of farthest inner primer RGA8_54 (234 bp downstream to ATG) was purified and cloned for further analysis as described in section 4.2.4. Sequencing of positive clones revealed the presence of two 5' RACE UTR of 68 and 101 nucleotides upstream to ATG.

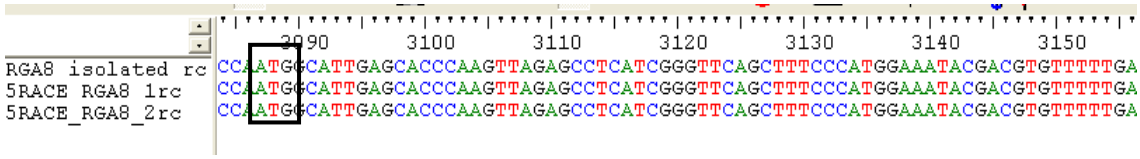
5.6.1.2. 3' RACE

Sequence alignments of 3' RACE fragments to isolated RGA8 genomic sequence revealed the presence of two alternative transcripts for RGA8 gene representing 3' UTR of 30 and 3517 nucleotides downstream to TGA (Figure 5.20 d and e). Seven gene specific primer pairs were developed from exons 2, 3 and 4 of RGA8 (Table A10 in appendix). Primer pairs 2GSP-2 and 2GSP-nested developed from exon 4 resulted in specific single bands in 1% agarose gels. The bands were purified and cloned for further analysis as described in section 4.2.4. No truncated versions of RGA8 transcripts were observed, as it was predicted that sometimes the presence of full length and truncated proteins is necessary for the function of resistance genes (Mestre and Baulcombe, 2006; Jordan *et al.*, 2002).

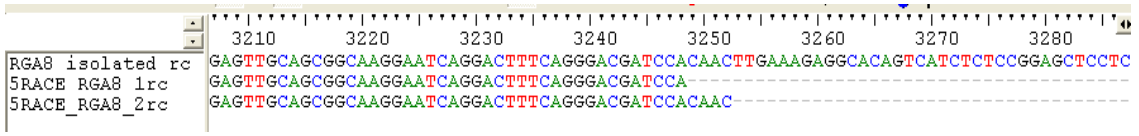
a- 5' RACE (5' UTR start)



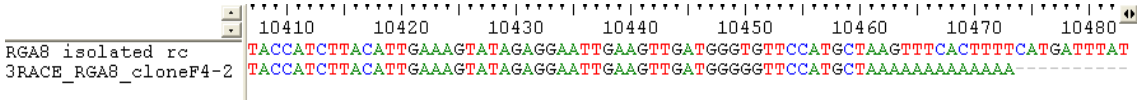
b- 5' RACE (ATG overlap)



c- 5‘ RACE (overlap end)



d- 3‘ RACE (poly A-tail) clone F4-2



e- 3‘ RACE (poly A-tail) clone F1-2

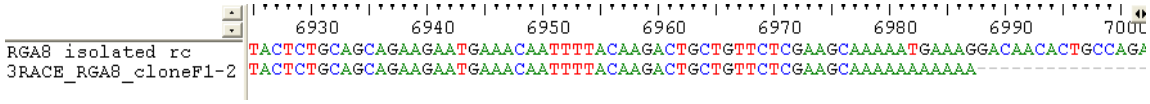


Figure 5.20: RGA8 5’ and 3’ RACE ends aligned to genomic clone 2B7 harbouring isolated RGA8.

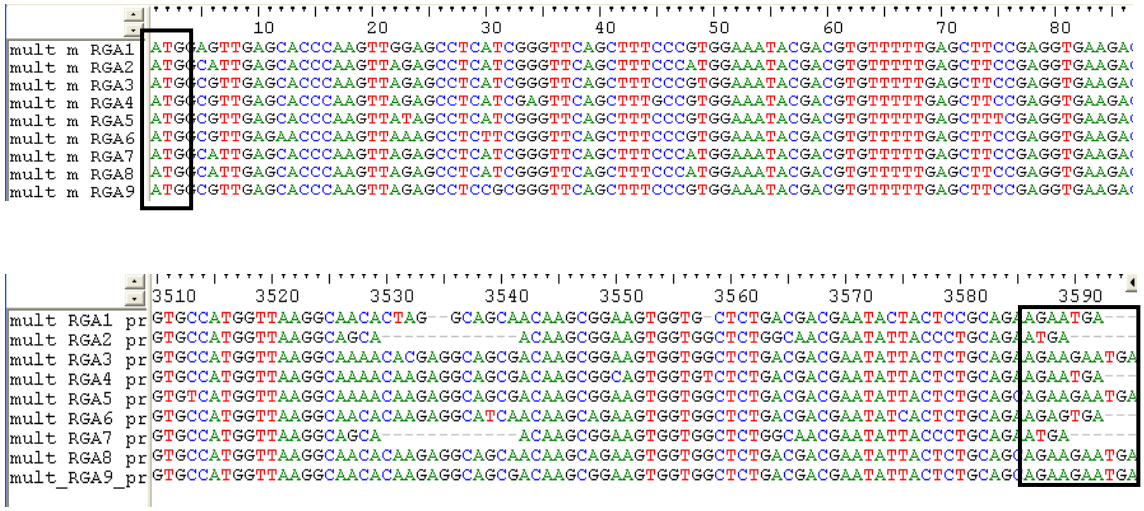
Although, these results predict the presence of atleast four different mRNAs for RGA8, only one ORF was found for different full length cDNAs comprising of 3369 bp (NCBI ORF-Finder). The predicted ORF encodes a predicted polypeptide of 1122 amino acids (aa) with an average molecular weight of 123.4 kDa. The deduced aa sequence show homology to TIR, NB and LRR domains (PROFILESCAN and NCBI-conserved domain search). The conserved motifs of these domains are presented in Table 5.6 according to Lukasik and Takken (2009) and Meyers and colleagues (1999). The LRR domain contains 10-11 irregular LRR repeats, 5 of which show little variation whereas the remaining strictly follow the consensus sequence of xLxxLxLxx, where L= leucine and x= any amino acid (Table 5.6; Table 7.12 in appendix). Exon 2 showed similarity to *Borrelia* protein repeats within a region of 14-nucleotides at the end of NBS domain; *Borrelia* proteins are not characterized yet, but contain repeated regions. In addition to that at the end of exon four, it has two regions similar to Nebulin *repeat (41-nucleotides) and* Apopolysialoglyco *protein (PSGP 12-nucleotides)* as predicted by PROFILESCAN. When the complete *Rdr1* contig was assessed for the presence of different genes (Fgenesh, Softberry), software predicted the presence of a dehydroquinate dehydratase-AAS90325 (6.00E-31) gene in front of RGA8 at the 5’end with predicted 690 bp cDNA and 229 amino acids. This gene is present on isolated RGA8 subclone 2B7 with some damage to upstream sequences as according to software prediction 3953bp are required in total to get full upstream and downstream sequences for this gene, whereas isolated RGA8 sub clone has 3146 bp upstream sequences only. This enzyme is a member of lyases enzyme family (hydro-lyases) and can cleave

carbon-oxygen bonds. The only known role of this enzyme is its participation in the biosynthesis of phenylalanine, tyrosine and tryptophan.

5.6.2. RGA7- inactive gene against black spot

The subclone 1C7 harbour an insert of 7995 bp representing RGA7. End sequences of this insert located it on *Rdr1* contig positioning from 172735 to 180729 bp. The 3' terminal structure of RGA7 was determined experimentally. The comparison of available genomic sequences and RACE identified 3' cDNA fragments resulted 3' UTR regions of 733 and 737 nucleotides downstream to TGA (Figure 5.23). 3' UTR region has an intron of 168 nt long which is positioned 90 nt downstream of TGA codon of the gene. Although 5' RACE end for RGA7 gene was not isolated experimentally, when available genomic sequences of all *Rdr1* candidates converted to cDNA and aligned they nicely aligned at ATG (Figure 5.21). In addition to that experimentally isolated 5' RACE ends available for RGA3 and 8 revealed the presence of 145/ 172 and 68/ 101 nucleotides as 5' UTR before ATG, respectively. Keeping in view these observations, a 5' UTR of 200 nucleotides was added in front of ATG (RGA7-cDNA) and ORF was determined by ORF-Finder (NCBI). The deduced ORF of RGA7 encodes a predicted polypeptide of 944 amino acids. The N-terminal of the RGA7 protein contains conserved motifs for TIR and NBS domains (Figure 5.24). The C-terminal consists of 11 LRRs that follow the consensus sequence LxxLxLxx (Figure 5.24; Table 7.12 in appendix).

Figure 5.21: Alignment of software predicted cDNA of *Rdr1* candidates. All RGAs are perfectly aligned at ATG (start codon) and TGA (stop codon).



5.6.3. Bioinformatics of RGA 8 protein sequence

To identify the homologues of RGA8 protein BLASTx searches were carried out (Altschul *et al.,* 1997) against the GenBank non-redundant database (http://blast.ncbi.nlm.nih.gov). The RGA8 protein shares the highest identity (41%) to TIR-NBS-LRR-resistance protein of *Populus trichocarpa* (ACCESSION XP_002329162). It also shows identity to hypothetical proteins of Vitis vinifera (39-44%), to TIR of Medicago truncatula (40%; ACCESSION ABD28703), to CMR1 of Phaseolus vulgaris (40%; ACCESSION ABH07384) and to *N*-like protein of *N. tabacum* (39%; ACCESSION BAF95888 for resistance to Tobacco Mosaic Virus; Whitham et al. 1994; Figure 6.1). The comparison of three proteins (RGA 8, 3 and 7) revealed high similarity ranging between 58-80% (Table 6.1) and presence of 119 unique amino acids in RGA8 protein (Figure 5.24; 5.25); 2 aa in TIR, 40 aa in NBS and 77 aa in LRR domains. According to the occurrence of specific amino acids LRR domain is the most divergent followed by NBS domain. K_a and K_s values (Table 6.1) show that the N-terminal halves of these proteins display higher degree of sequence homology than the C-terminal halves of proteins. The type of selection pressure exerted on different domains of three genes, ratio of synonymous (K_s) and non-synonymous (K_a) substitutions rates per synonymous / non-synonymous site was calculated (Table 6.1). K_a / K_s ratio equal to 1 suggests random mutation or no selection pressure on gene; whereas $K_a / K_s > 1$ and $K_a / K_s < 1$ reflect sequence diversification and conservation, respectively (Hughes and Nei, 1988). The overall trend of selection operating on gene data presented here is sequence conservation however; the N-terminal halves of the genes suggest more sequence conservation when compared to C-terminal region of genes.

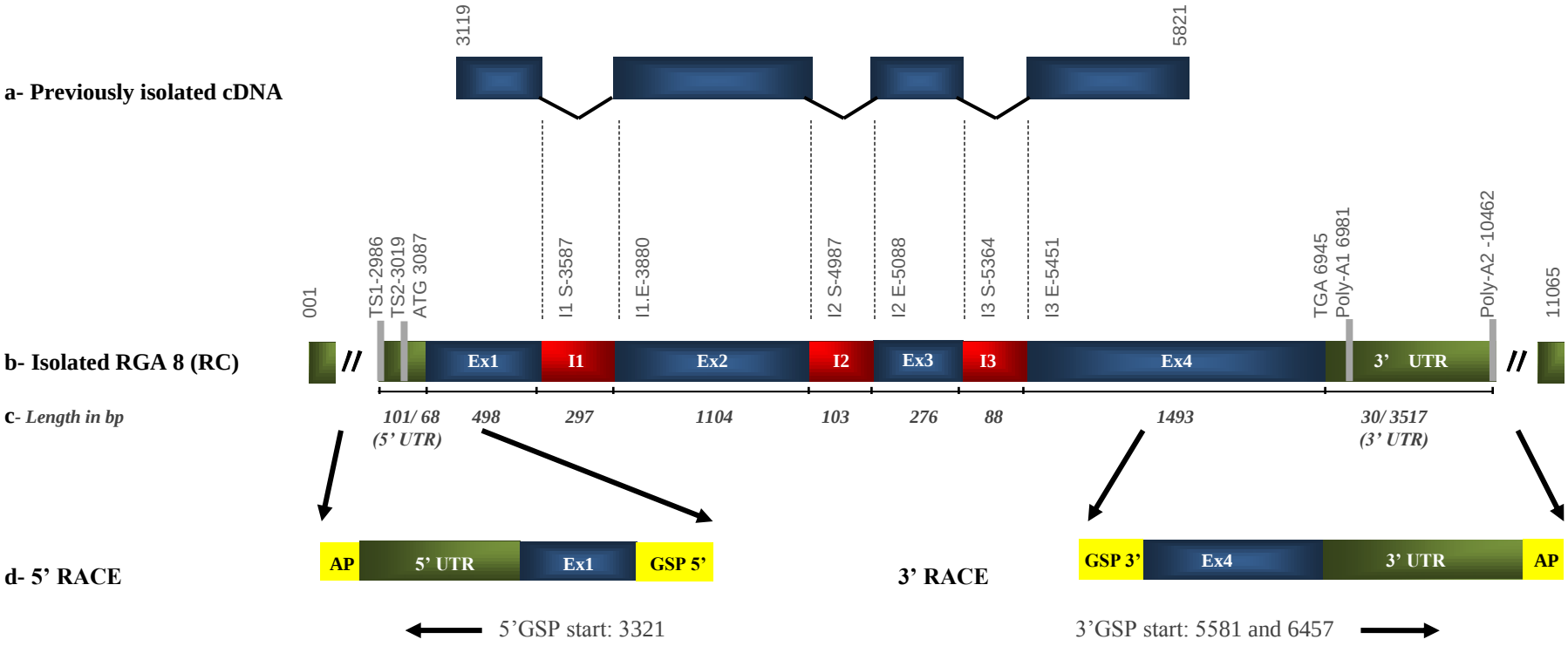


Figure 5.22: Schematic illustration of full length cDNA of RGA 8.
a- previously isolated cDNA of RGA8; b- Genomic subclone of RGA8-2B7 that was used to isolate 5'and 3' RACE sequences and all positions mentioned here refer to the same clone; c- length of different exons, introns and UTRs in bp; d- isolated 3' and 5' RACE products with start positions of GSP primers as indicated below the illustration.
This illustration does not represent the actual distances and sizes of the DNA sequences.

AP: Adaptor Primer
TS: Transcription Start
I : Intron
Ex: Exon
GSP: Gene Specific Primer
RC: Reverse Complement
E: End
S: Start

Domain	Sequence	Exons
TIR (17-157)	0001 MALSTQVRASSGSAFPWKYDVFLSFRGEDTRKGFTGFLYHELQRQGIRTFRDDPQLERGTVISPELLTAIEQSRFAIVVLSP 0082 *TIR-1* 0083 NFASSTWCLLELSKILECMEERGRILPIFYEVDPSHVRHQRGSFAEAFQEHEEKFGVGNKKVEGWRDALTKVAGLAGWTSKD 0164 *TIR-2* *TIR-3* 0165 YRY	1 (1-167)
NBS-ARC (199-469)	0168 ETELIREIVQALWSKLHPSLTVFGSSEKLFGMDSKLEEIDVLLDKEANEVRFIGIWGMGGIGKTTLARLVYQKISHQFEVCI 0249 *Pre-P-loop* *Walker A/P loop(kinase1a)* 0250 FLDNVREVSKTTHGLVDLQKKILSQIFKEENVQVLDVYSGMTMIKRCVCNKAVLLVLDDMDQSEQLENLVGEKDCFGLRSRI **0331** *RNBS-A* *Walker B(kinase2)* *RNBS-B/* **0332** IITTRDRHVLVTHGVEKPYELNGLNKNEALQLFSWKAFRKCEPEEDFAELCKSFVTYAGGLPLALKILGSFLKGRTPDEWNS 0413 *Sensor1(kinase3a)* *RNBS-C* *GLPL(ARC1)* 0414 ALAKLQQTPDITVFKILKMSFDGLDEMEKKIFLDIACFFWLYRKEFMIELVDSSDPCNRITRSVLAEKSLLTISSDNQVHVH 0495 *RNBS-D(ARC2)* **Borrelia repeat protein** 0496 HDLIHEMGCEIVRQENKEPGGRSRLCLRDDIFHVFTKNTGTE	2 (168-536)
LRR (597-872)	0537 AIEGILLDLAELEEADWNLEAFSKMCKLKLLYIHNLRLSVGPRLLPNSLRFLSWSWYPSK 0596 0597 SLPPCFQPDELAEISLVHSN 1 0617 IDHLWNGIKYLVNLKSIDLSYSINLT 2 0643 RTPDFTGIPNLEKLVLEGCTN 3 0664 LVKIHPSIALLKRLRIWN 4 0682 LRNCKSIRS-LPSEVNMEFLETFDVSGCSKLKM 5 0713 ISEFVMQMKRLSKLYLGGTAVEKL 6 0737 PSSIEHLSESLVVLDLSG 7 0755 IVIREQPYSRLLKQNLIASSFGLFPRKSPHPLIP 8 0789 LLASLKHFSCLRTLKLNDCNLCEGE 9 0814 IPNDIGSLSSLQRLELRGNNFVSLPASIHLLEDV 10 0848 DVENCKRLQQLPELPDLPNLCRLR 11 0873 ANFWLNCINCLSMVGNQDASYFLYSVLKRWIEIEALSRCDMMIRQETHCSFEYFRFVIPGSEIPEWFNNQSVGDTVTEKLPW 0953 0954 WDACNSKWIGFAVCALIVPHDNPSAVPEKSHLDPDTCCIWCFWNDYGIDVIGVGTNNVKQIVSDHLYLLVLPSPFRKPENYL 1035 1036 EVNFVFKIARAVGSNRGMKVKKCGVRALYEHDTEELISKMNQSKTSSISLYEEAMDEQEGAMVKATQEAATSRSGGSDDEYY 1117 1118 SAAEE **Nebulin repeat profile** **Apopolysialoglycoprotein (PSGP)**	3 (537-629) 4 (630-1122)

Table 5.6: Deduced RGA8 protein sequence and conserved motifs of different domains. The amino acid sequence obtained from cDNA sequence of RGA8 is divided into three domains i.e. TIR, NBS and LRR. Conserved structural motifs in R-proteins are underlined and unmatched aa are marked red. The LRR subunit LxxLxLxxxx is framed. L Residues matching the consensus LRR sequence are indicated in bold.

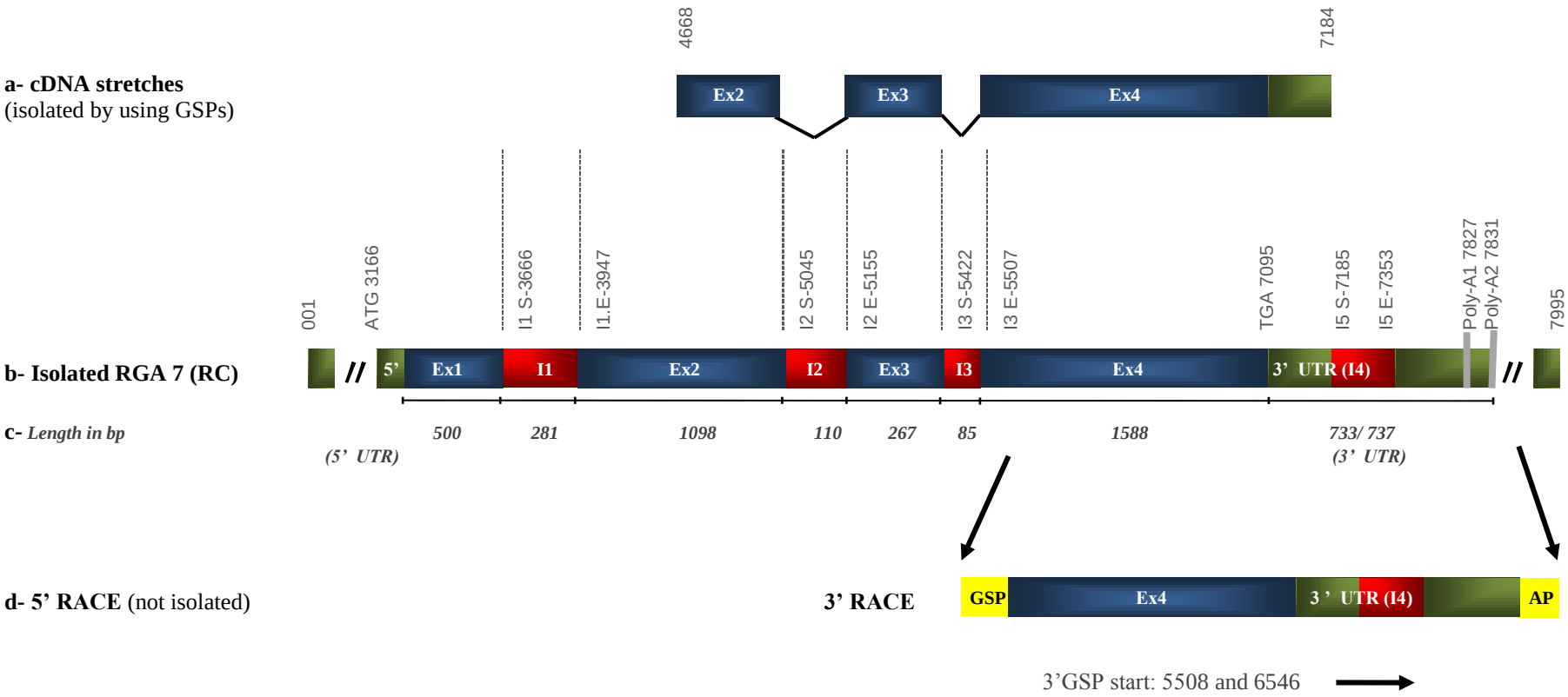


Figure 5.23: Schematic illustration of full length cDNA of RGA 7.
a- cDNA of RGA7 isolated by different GSPs used in this study and previously;
b- Genomic subclone of RGA7-1C7 that was used to isolate 3' RACE sequences
and all positions mentioned here refer to the same clone; c- length of different exons,
introns and UTR in bp; d- isolated 3' RACE products with start positions of GSP primers
as indicated below the illustration.This illustration does not represent the actual distances and sizes of the DNA sequences.

AP: Adaptor Primer
TS: Transcription Start
I : Intron
Ex: Exon
GSP: Gene Specific Primer
RC: Reverse Complement
E: End
S: Start

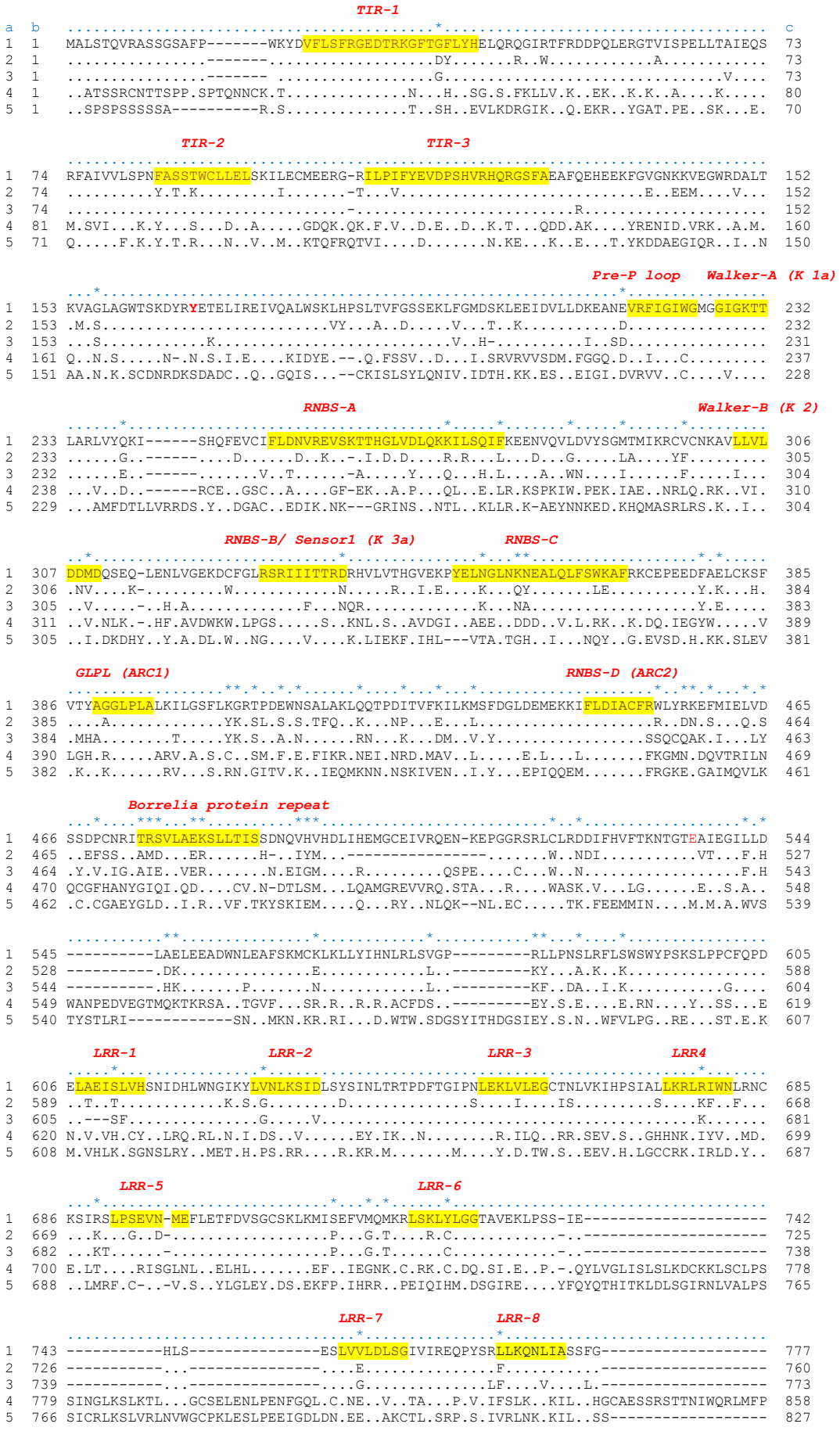

TIR-1
TIR-2
TIR-3
Pre-P loop
Walker-A (K 1a)
RNBS-A
Walker-B (K 2)
RNBS-B/ Sensor1 (K 3a)
RNBS-C
GLPL (ARC1)
RNBS-D (ARC2)
Borrelia protein repeat
LRR-1
LRR-2
LRR-3
LRR4
LRR-5
LRR-6
LRR-7
LRR-8

Figure 5.24: Alignment of the proteins of RGA 8, 3, 7 of roses, TIR-NBS-LRR resistance protein of *Populus trichocarpa* and N like protein of *N. tabacum*. The RGA8 protein and its predicted TIR, NBS and LRR domains with conserved motifs are high-lighted. Blue stars on RGA8 protein sequence represent unique amino acids of this protein when compared to proteins of RGA 3 and 7. Within RGA8 protein sequence the end amino acid of each domain is marked in red. a- Numbers in first column corresponds to RGA8 (1), RGA3 (2), RGA7 (3), resistance protein of *Populus trichocarpa* (4) and *N* like protein of *N. tabacum* (5); b and c- sequence positions; Dashes indicate gaps inserted to maintain optimal alignment.

```
a  b                                                                                                                  c
1  545 ----------LAELEEADWNLEAFSKMCKLKLLYIHNLRLSVGP---------RLLPNSLRFLSWSWYPSKSLPPCFQPD  605
2  528 ----------LDKLEEADWNLEAFSKMCELKLLYIHNLRLSLGP---------KYLPNALKFLKWSWYPSKSLPPCFQPD  588
3  544 ----------LHKLEEADWNPEAFSKMCNLKLLYIHNLRLSLGP---------KFLPDALRILKWSWYPSKSLPPGFQPD  604
Con.   ----------*..*******.*******.************.**---------..**..*..*.***********.****

1  606 ELAEISLVHSNIDHLWNGIKYLVNLKSIDLSYSINLTRTPDFTGIPNLEKLVLEGCTNLVKIHPSIALLKRLRIWNLRNC  685
2  589 ELTELTLVHSNIDHLWNGKKSLGNLKSIDLSDSINLTRTPDFTGIPSLEKLILEGCISLVKIHPSIASLKRLKFWNFRNC  668
3  605 EL---SFVHSNIDHLWNGIKYLGNLKSIVLSYSINLIRTPDFTGIPNLEKLVLEGCTNLVKIHPSIALLKRLKIWNFRNC  681
Con.   **.....***********.*.*.********.****.*********.****.****..*********.****..******

1  686 KSIRSLPSEVN-MEFLETFDVSGCSKLKMISEFVMQMKRLSKLYLGGTAVEKLPSS-IE----------------------  742
2  669 KSIKSLPGEVD-MEFLETFDVSGCSKLKMIPEFVGQTKRLSRLCLGGTAVEKLPSS-IE----------------------  725
3  682 KSIKTLPSEVN-MEFLETFDVSGCSKLKMIPEFVGQTKRLSKLCLGGTAVEKLPSS-IE----------------------  738
Con.   ***..**.**.-******************.***.*.****.*.************-**

1  743 -----------HLS---------------ESLVVLDLSGIVIREQPYSRLLKQNLIASSFG-------------------  777
2  726 -----------HLS---------------ESLVELDLSGIVIREQPYSRFLKQNLIASSFG-------------------  760
3  739 -----------HLS---------------ESLVGLDLSGIVIREQPYSLFLKQNVIASSLG-------------------  773
Con.   -----------***---------------****.**************..****.****.*--------------------

1  778 LFPRKSPHPLIPLLASLKHFSCLRTLKLNDCNLCEGEIPNDIGSLSSLQRLELRGNNFVSLPASIHLLEDVD---VENCK  854
2  761 LFPRKSPHPLLPLLASLKHFSSLRTLKLNDCNLCEGEIPNDIGSLSSLKRLELRGNNFVSLPASIHLLSKLTYFGVENCT  840
3  774 LFPRKSHHPLIPVLASLKHFSSLKELNLNDCNLCEGEIPNDIGSLSSLECLELGGNNFVSLPASIHLLCRLGSINVENCK  853
Con.   ******.***.*.**********..*.*********************..***.**************.......*****

1  855 RLQQLPELP-------------------D-LPNLCRLRANFWLNCINC---LSMVGNQDA-SYFLYSVLKRWIEIEALS-  909
2  841 KLQQLPALPVSDYLNVLTNNCTSLQVFPD-PPDLSRL-SEFFLDCSNC---LSC---QDS-SYFLYSVLKRWIEIQVLS-  910
3  854 RLQQLPELPVSGSLRVTTVNCTSLQVFPELPPDLCRL-SAFSLNSVNC---LSTIGNQDA-SFFLYSVINRLLEVISLSL  928
Con.   *****.**.....................*.*.**.....*...**---**....**.-*.*****..*..*...**.

1  910 RCDMMIR-QETHCS-FEYFRFVIPGSEIPEWFNNQSVGDTVTEKLPWDAC-NSKWIGFAVCALIVPHDNPSAVPEKSHLD  986
2  911 RCDMMVHMQETNRRPLEFVDFVIPGSEIPEWFNNQSVGDRVTEKLPSDAC-NSKWIGFAVCALIVPQDNPSALLERPFLD  989
3  929 SLSLSLSLSLSLSRSL-------------------------------------------------------------------  944
Con.   .................*...******************.******.***-***************.*****..*...**

1  987 PDTCCIWCFWNDYGIDVIGVGTNNVK--QIVSDHLYLLVLPSPFRKPENYLEVNFVFKIARAVGSNRGMKVKKCGVRALY 1064
2  990 PDTYGIECYWNDYGIGFVGLVVP-VK--QFVSDHLWLLVLLSPFRKPENCLEVNFVFEITRAVGNNRGMKVKKCGVRALY 1066
3      ----------------------------------------------------------------------------------
Con.   ***..*.*.******...*.....**--*.*****.****.********.*******.*.****.***************


1  1065  EHDTEELISKMNQSKTSSISLYEEAMDEQEGAMVKA---TQEAATSRSGGSDDEYYSAAEE  1122
2  1067  EHDVEELISKMNQSKSSSISLYEEGMDEQEGAMVKA---KHEAATSGSGGSDDEYYSAEEE  1124
3        ------------------------------------------------------------
Con.     ***.***********.********.***********---..*****.************.**
```

Figure 5.25: Comparison of protein sequences encoded by 3-members of *Rdr1* family. Protein sequences RGA8 (1), RGA3 (2) and RGA7 (3) were aligned to check amino acid similarities and divergence. Red stars represent consensus sequence and red dots show differences in amino acids. a- RGA 8, 3 and 7 proteins as described above; b and c- sequence positions. Dashes indicate gaps inserted to maintain optimal alignment.

6. Discussion

The present study was focused on the identification and molecular characterization of major dominant resistance gene to black spot of roses introgressed from *R. multiflora*. Isolation of such genes from wild species followed by transformation of already well established cultivars is a quicker method of exploiting resistance. Many resistance genes, the *Pto* gene (Rommens *et al.,* 1995) and the *Cf-9* gene (Hammond-Kosack *et al.,* 1998) of tomato, the *N* gene of tobacco (Whitham *et al.,* 1994), the *Bs2* gene of pepper (Tai *et al.,* 1999), have been transferred to other related species as trans-genes. Although in the current study through functional characterization of *Rdr1* candidates, RGA8 and RGA1 (TIR-NBS-LRR genes) were identified as active resistance genes against black spot race 5 (Dort E4), RGA8 showed more consistent and profound effect in restricting fungal growth as compared to RGA1.

The *Rdr1* resistance locus of *R. multiflora* confers vertical resistance to five races of *D. rosae* (Von Malek and Debener, 1998). In order to clone the *Rdr1* gene a classical map based cloning strategy was applied. Several genes involved in disease resistance process have been cloned and isolated from different plant species by positional cloning (Lamb *et al.,* 1989; Staskawicz *et al.,* 1995; Bent, 1996; Hammond-Kosack and Jones, 1996; and 1997; Gebhardt, 1997; Ballvora *et al.,* 1995). To fulfill the first prerequisite for this process the *Rdr1* gene has been introgressed in diploid and tetraploid rose populations segregating for black spot resistance from *R. multiflora* (Drewes-Alvarez, 1992; Von Malek and Debener, 1998; Kaufmann *et al.,* 2003) followed by the development of linked molecular marker for the gene (Von Malek *et al.,* 2000) and the construction of two large insert BAC libraries for *R. rugosa* (Kaufmann *et al.,* 2003) and *R. multiflora* resulting in the *R. multiflora* BAC contig of 220kb of black spot resistance *Rdr1* locus (Biber *et al.,* 2009). The map based cloning of *Rdr1* took quite long time due to the high genomic heterozygosity in roses and presence of numerous repetitive elements on the BAC contig (Kaufmann *et al.,* 2003). In parallel to map based cloning the candidate gene approach was exploited to isolate RGAs using degenerate primers to speed up the process of *Rdr1* cloning and some of the isolated RGAs were located in the genomic region containing the *Rdr1* locus at the telomeric end of linkage group 1 (Hattendorf and Debener, 2007). This isolation of RGAs was based on degenerate primers developed from conserved amino acid motifs of NBS, TIR and non-TIR domains. The same technique was also utilized successfully to isolate RGAs from different plant

species (Potato- Paal *et al.,* 2004; tomato- Pan *et al.,* 2000; pepper- Pflieger *et al.,* 1999; lettuce- Woo *et al.,* 1998 and Shen *et al.,* 1998; common bean- Geffroy *et al.,* 1999a and Rivkin *et al.,* 1999; soybean- Kanazin *et al.,* 1996 and Yu *et al.,* 1996; Rosaceae family- Samuelian *et al.,* 2008; Martinez Zamora *et al.,* 2004; Lee *et al.,* 2003; Baldi *et al.,* 2004; Lalli *et al.,* 2005; Soriano *et al.,* 2005; Xu *et al.,* 2005). Further sequence analysis of *Rdr1* locus revealed the presence of nine RGAs of TIR-NBS-LRR type as putative candidates of the *Rdr1* resistance gene (Terefe *et al.,* 2010). It is usual that R-genes are mostly organized in clusters (Michelmore and Meyers, 1998; Edwin *et al.,* 2000). The *Gro1* resistance locus in potato has 15 members (Paal *et al.,* 2004); the *Hero* resistance locus in tomato has 14 homologous genes (Ernst *et al.,* 2002); the *RGC2* family in lettuce contains 14 to 40 paralogous sequences (Wroblewski *et al.,* 2007); the *Gpa2* locus in potato harbour 4 homologous genes (Edwin *et al.,* 2000). It is suggested that the organization of R-genes as a tightly linked multigene families favors their rapid evolution as generation of novel genes by recombination, duplication and partial deletion (Leister, 2004; Collins *et al.,* 1999; Meyers *et al.,* 1998; Dixon *et al.,* 1998).

After estimating the number of CGs the next step was their validation through complementing assays. Performing genetic complementation assays in roses was not an easy task, primarily because roses are polyploidy with high heterozygosity that effect transformation efficiency and it need several months to get real transformants. In addition, isolation of functionally active 8 single CGs was also technically difficult and time consuming process. The potato (*Solanum tuberosum*) also show comparable degree of genomic complexicity as described for roses; It is a tetraploid, non-inbred, although annual plant species its polyploidy and inbreeding depression prevent the generation of homozygous lines (Ballvora *et al.,* 2007). The *Gro1* locus, active against all pathotypes of *G. rostochiensis*, a nematode, was located on potato chromosome VII (Barone *et al.,* 1990) and high resolution mapping restricted *Gro1* locus to an interval of 1.4 cM (Ballvora *et al.,* 1995). Several RGAs were then amplified from the potato genome using conserved sequences of tobacco *N* gene (Whitham *et al.,* 1994) and *Arabidopsis RPS2* gene (Bent *et al.,* 1994). The RGAs *St332* and *St334* identified the *Gro1* locus harboring a cluster of 15 similar genes (Leister *et al.,* 1996). Further analysis of inheritance, linkage mapping and sequencing reduced the number of CGs to three, which were subcloned into binary vector pCLD04541 and used to complement a susceptible potato cv. *Desiree* by *Agrobacterium* mediated transformation (Paal *et al.,*

2004). The subclones of above mentioned three CGs (2, 4 and 5) of *Gro1* have 2.3, 4.0 and 2.7 kb extra sequence upstream of the putative start codon and 3.4, 1.1 and 3.0 kb sequence downstream to putative stop codon respectively. The complementation analysis of stable transformants showed that the *Gro1-4* gene conferred resistance to *G. rostochiensis* pathotype *Ro1*. In the current study the nine CGs of *Rdr1* are about 4 kb in size with a common gene structure of four exons and three introns and share a sequence similarity ranging between 85-99% (Kaufmann *et al.,* 2010). According to sequence analysis all RGAs found to encode an ORF of about 1100 amino acids except RGA 4 and 9. RGA9 has a stop codon in third exon that reduces the ORF to 794 amino acids with some part of LRR region in putative protein, making it a candidate for *Rdr1* whereas; RGA4 is considered a pseudogene because of the presence of a 7kb retrotransposon in the non-coding region of its first intron (Kaufmann *et al.,* 2010). In view this information the *Rdr1* candidates were reduced to eight i.e. RGA1 to 3 and 5 to 9. These eight CGs were isolated and sub-cloned as single genes under the control of their endogenous promoter into the plant transformation binary vector pBINPLUS. The isolated single CGs, on average, included a 5 kb sequence upstream of the putative start site and a 3 kb sequence downstream of the putative stop site (Figure 5.1). The isolated CGs were further analyzed for their expression and activity against Dort E4 in heterologous and homologous system to reduce the number of candidates. The RGA expression profiling in heterologous system did not lead any reduction to number of candidates as all CGs were expressed in contrast RGAs 1, 3, 7, 8 and 9 were found to be expressed in the homologous system.

On the basis of sequence and expression analysis the number of CGs was reduced to five (RGAs 1, 3, 7, 8 and 9) that were used to carry out complementation assays. In case of potato, generation of stable transformants and their testing for resistance against nematodes needed 6-months (Paal *et al.,* 2004). Even the transformation of model plants as *Arabidopsis thaliana* or *Nicotiana species* (Zhang *et al.,* 2006: Clemente 2006) require several months to produce transgenics ready for analysis. In addition to the lengthy process, stable transformants also vary in their level of transgene expression due to the gene silencing and/ or gene position in genome (reviewed by Vaucheret *et al.,* 1998). In contrast to this minimum 9-12 months are required to obtain stable rose transformants ready for resistance testing (Dohm *et al.,* 2001 2002; Marchant *et al.,* 1998a & b). The *Agrobacterium* mediated rose transformations were carried out using

five candidates to generate stable transformants; putative transformants are in process of screening and due to time limitations these results could not be included here. To solve the problem of the lengthy process and the low efficiency of the rose stable transformation system, *Arabidopsis* heterologous system was used to generate homozygous lines carrying single CGs, in addition transient complementation disease assays were also optimized in homologous system as an alternative. Transient expression assay have already been employed to study host and pathogen interactions for example: grapevine-downy mildew interaction (Santos-Rosa *et al.,* 2008), wheat-powdery mildew interaction (Schweizer *et al.,* 1999) and barley- powdery mildew interaction (Nelson and Bushnell 1997; Shirasu *et al.,* 1999). In the present investigation transient expression assay was optimized in rose petals and leaves followed by its use in the transient disease assay. On the basis of transient disease assay the number of CGs was reduced to 2; RGA8 as a major active gene and RGA1 as a partially active gene against race 5 of *D. rosae*.

6.1. Expression Analysis of CGs

N. benthamiana, the model plant for heterologous expression studies, was used to elucidate the function and activity of CGs. The use of different heterologous systems is common in transient assays due to over expression of the target gene and lack of related sequences making the expression validation of the target easier as compared to expression in the homologous system (Goodin *et al.,* 2008; Sparkes *et al.,* 2006; Wroblewski *et al.,* 2005). The *Agrobacterium* suspensions containing single CGs in pBINPLUS were infiltrated into *N. benthamiana* leaves (Schob *et al.,* 1997; Wroblewski *et al.,* 2005) and samples were collected 4-days post-infiltration. The ectopic expression of all CGs was validated through RT-PCR with different general primers for RGAs. The results revealed the expression of all RGAs showing not only the presence of all regulatory elements on the isolated clone necessary for the gene expression, also the conserved nature of promoter activity in dicot plants. 24 hours post-infiltration of CGs the intact plants were challenged by the black spot isolate Dort E4. None of the CGs responded to Dort E4 in a form of hypersensitive response (HR) or necrosis and microscopy revealed the presence of spores but without germination. Even in control experiments, without transgene infiltrations, Dort E4 was not found to infect tobacco representing it a non-host species and this kind of resistance is referred as non-host resistance (Mysore and Ryu, 2004 Kamoun, 2001; Heath, 2000; Dangl *et al.,*

1996). However, the mechanisms and the signaling components affecting this phenomenon have to be explored. It could be concluded that the propagation of Dort E4 is not possible on the model plant tobacco as spores do not germinate and do not form mycelia; Avr gene products are not most probably expressed to interact with R-genes and therefore no HR or necrosis was observed.

The expression profile of all CGs in homologous system was also analyzed. Two black spot resistant rose genotypes 91/ 100-5 and 88/ 124-46 (*R. multiflora*) were subjected to RT-PCR analysis using a set of specific primers. As the R genes are often expressed constitutively at low levels (Tan *et al.,* 2007) the results revealed the expression of six CGs out of nine. RGA4, 5 and 6 were never found to be expressed in different tissues (leaves, petals and roots), whereas RGA2 was found to be expressed only in the leaves of above mentioned rose genotypes and the petals of these genotypes were already found resistant against Dort E4 in disease assay that excluded the RGA2 as a candidate for *Rdr1*. Technically it was a difficult task to develop one specific primer pair for each single RGA that demonstrate the expression of corresponding single RGA among the others as all RGAs were expressed in the homologous system sharing very high DNA sequence similarities (85-99%; Kaufmann, 2010) that's why for each RGA several specific primer pairs were developed to detect its expression with maximum surety (Table A 11 in appendix).

All RGAs were found to be expressed in tobacco whereas only RGAs 1, 2, 3, 7, 8 and 9 were found to be expressed in the resistant rose genotypes. The differences in heterologous and homologous expression could be due to the transcriptional and/ or post-transcriptional regulation in homologous system as described in *Arabidopsis* for *RPP5* resistance locus (Hankuil and Richards, 2007). The *RPP5* locus R genes were demonstrated to be targeted by small RNA species in the wild type plants restricting the fitness cost related to constitutive activation of defense genes. Authors also suggested that the pathogen attack disturbs this RNA silencing of R genes and activate defense pathways. Padmanabhan *et al.,* (2009) have reviewed the endogenous small RNA-mediated regulation as a general molecular mechanism against a wide range of pathogens and insects. As the heterologous system lack the regulatory elements specific for rose RGAs, all RGAs were found to be expressed. In summary, on the basis of

above mentioned observations the number of candidates was reduced to five i.e. RGAs 1, 3, 7, 8 and 9.

6.2. Validation of CGs

The ultimate way to validate a CG is genetic complementation by transformation of a deficient phenotype or transforming a non-deficient plant using a silencing construct (Pflieger S. *et al.,* 2001). The complexity of the technique depends on the mono or polygenic nature of the target trait. The genetic transformation could be stable or transient. In stable transformations the expression of CG depends on its insertion site and the number of inserted copies in genome. Whereas transient transformation can measure non biased expression of a gene within a very short time period after agro-infections independent of the position of insertion because non-integrated copies of T-DNA present in the nucleus of the host can also be expressed (Kapila *et al.,* 1997). Therefore, transiently expressed genes express up to 1000 fold higher than they express in the stable transformants (Janssen and Gardner 1989). In the current study primarily the susceptible rose genotype Pariser Charme (a susceptible plant not expressing the *Rdr1* function) was transiently and stably complemented using CGs to check the expression and activity of these genes. Secondly, 91/ 100-5 (a CGs non-deficient plant) was transiently silenced for all members of *Rdr1* family and challenged by Dort E 4. In addition to these *Arabidopsis* (Columbia and PEN2 mutant) was stably complemented using CGs.

6.2.1. Stable genetic complementation

Stable genetic complementation of a susceptible rose variety using *Rdr1* candidates can demonstrate conclusively if the complementing CG is the functional *Rdr1* gene, determining the resistance trait. Stable rose transformants ready for functional analyses need minimum 9-12 months (Dohm *et al.,* 2001; Marchant *et al.,* 1998a). In addition to lengthy process the low efficiency of transformation system has also hampered the study of gene function in roses.

6.2.1.1. Production of stable rose transformants

To fulfill the criterion of CGs validation by stable transformation, Pariser Charme (PC)- a susceptible rose genotype, was initially subjected to the biolistic genetic complementations using complete BACs harboring *Rdr1* locus (155F3, 29O3, 94G8 and

20F5); due to technical problems, use of old somatic embryos and fungal contaminations these experiments were not successful. Although all BAC inserts were cloned in the binary vector pCLD04541 (Jones *et al.,* 1992), that was designed for *Agrobacterium*-mediated plant transformation and is capable of stable maintenance of large DNA inserts (Tao and Zhang 1998) available previous data showed its incompatibility to transform rose somatic embryos via *Agrobacterium* mediated transformations. This may be due to large size of DNA inserts in BACs as four BAC spanning *Rdr1* has an average inset of 75kb (BACs 155F3, 29O3, 94G8 and 20F5; section 4.1.4). According to Ercolano and colleagues (2004) the success of stable plant transformations with DNA fragments of about 20 kb is easier as compared to DNA fragments larger than 50 kb. To validate the function of three CGs (22-, 14.5- and 10.5-kb) of the *Gro1* family, these were subcloned in the binary vector pCLD04541 and were used to complement a susceptible potato cv. Desiree by *Agrobacterium* mediated transformation successfully (Paal *et al.,* 2004). Although Hamilton *et al.,* (1996) has reported the genetic complementation of tobacco using 150 kb of human DNA by *Agrobacterium*-mediated transformation with a binary BAC vector, biolistic transformation with whole BAC DNA is an alternative method for transferring large DNA fragments into plant genomes. The potato transgenic plants have been obtained after biolistic transformation with up to 106 kb of potato DNA cloned in the binary vector pCLD04541 (Ercolano *et al.,* 2004). As part of this study, *Agrobacterium* mediated transformations of PC using single CGs in binary vector pBINPLUS were carried out and the putative transformants are in process of screening. Due to time limitations the final disease resistance evaluation could not be included here.

6.2.1.2. Production of stable *Arabidopsis* transformants

In addition to rose genotype PC, two varieties of *Arabidopsis*, Columbia (an accession) and PEN2-1 (a mutant; Lipka *et al.,* 2005) were used to generate stable transformants of CGs by *Agrobacterium* mediated floral dip method (Zhang *et al.,* 2006). Preliminary spore (Dort E4 $5x10^2$ spores/ ml) infiltrations in leaves of both *Arabidopsis* varieties resulted in germination of fungus. Therefore to study the interaction of black spot and stably expressed CGs, *Arabidopsis* was selected to generate stable transformants carrying single CGs.

The PEN2-1 mutant was selected to facilitate the penetration of black spot spores to leaf cells as in non host plant species the first defense barrier is the plant cell wall that cannot be penetrated by pathogenic fungi (Collin *et al.,* 2003). According to the previous reports *Arabidopsis* plant is a non-host species for the barley powdery mildew fungus *Blumeria graminis* and several *Arabidopsis* PEN mutants have been isolated that permit this fungal entry into leaf cells; the corresponding wild type genes confer penetration resistance and known as PEN genes. These PEN genes encode secretion-associated and efflux-associated proteins. The PEN1 syntaxin is plasma membrane-resident, the PEN2 glycosyl hydrolase is associated to peroxisome and the PEN3 ABC transporter is found in plasma membrane (Stein *et al.,* 2006; Lipka *et al.,* 2005; Collins *et al.,* 2003). The PEN2 was isolated by map-based cloning and encodes glycosyl hydrolases. The PEN2 mutant alleles, pen2-1 and pen2-3 both were chemically induced and were characterized by point mutations showing the presence of stop codons that led to truncated peptide (Lipka *et al.,* 2005).

Although both varieties were subjected to generate stable transformants for all CGs, the homozygous lines ready for further analysis were generated only for RGA3, 8, GUS and pJG-28 in case of Columbia and only for GUS in case of the PEN2-1 mutant. The large DNA fragment size of isolated CGs and already stressed form of the PEN2-1 mutant could be the reasons for not getting transformants for all CGs. The pGJ-28 construct encodes a ribosome inhibiting protein that when secreted into the apoplast of transformed rose plants reduced their susceptibility against black spot to 60 % (Dohm *et al.,* 2001a). The Columbia homozygous lines for pGJ-28 will be utilized as positive control in subsequent detailed studies using the remaining generated lines and their response to Dort E4. Due to time limitations these studies could not be included here. It is expected that when these homozygous lines expressing RGAs stably will be inoculated with Dort E 4 conidia will show some antifungal effects (HR) or some specific type of interaction with tested fungal isolates. If this could be demonstrated then these generated lines can be used as parents (stocks) to generate different progeny combinations through controlled breeding strategies in very short time as within 6-months and generated lines then could be tested against different isolates of *D. rosae*, challenged by taxonomically different pathogens or even evaluated in different stress conditions. This will not only provide quick insights to the expression and functionality of single RGAs but also generate a wealth of data for the *Rdr1* locus.

6.2.2. Transient genetic complementation assay via agroinfiltration

In the current study the *Agrobacterium* mediated transient assay was utilized as an alternative to stable transformations because the generation of rose stable transformants is a lengthy process that requires several months to confirm the function of a CG. The transient assays are faster and simpler compared to this conventional approach especially when several paralogous CGs have to be screened. The successful utilization of the technique is demonstrated for genetic complementation (Zottini *et al.,* 2008; Shao *et al.,* 2003; Van der Hoorn *et al.,* 2000), RNAi experiments (Schöb *et al.,* 1997), assessment of resistance genes (Santos-Rosa *et al.,* 2008; Nelson and Bushnell 1997; Shirasu *et al.,* 1999; Schweizer *et al.,* 1999), Protein trafficking (Batoko *et al.,* 2000) and recombinant protein production (Vaqucro *et al.,* 1999; Stoger *et al.,* 2002; Galeffi *et al.,* 2005; Sheludko *et al.,* 2007). Thus, a rapid and cost effective functional gene assay was optimized in the leaves and petals of rose genotype Pariser Charme.

6.2.2.1. Optimization of GUS expression assay

The transient expression assay was optimized in the rose petals and leaves using the GUS (β-glucuronidase) reporter gene (Jefferson *et al.,* 1987) aiming at its future use to characterize rose resistance genes and to assess their activity against different fungal pathogens. Among the most important factors identified so far are the genotype of the host plant, the *Agrobacterium* strain, the pre-culture of the host plant and of the agrobacteria, the temperature at which the cocultivation of *Agrobacterium* and host was conducted (Wroblewski *et al.,* 2005, Joh *et al.,* 2005, Zottini *et al.,* 2008). The agro-infiltration was optimized and data revealed its dependence on the host genotypes and their pre-culture, flower age, petal position, leaf type, infiltration method, bacterial density and temperature. It is reported that the genetic background of a plant influences the efficiency of transient expression significantly (Wroblewski *et al.,* 2005; Santos-Rosa *et al.,* 2008; Zottini *et al.,* 2008). In case of petals it was observed that two rose genotypes (Pariser Charme and Marvel) susceptible for fungal infections were significantly susceptible to *Agrobacterium* infections as compared to the other three genotypes (Heckenzauber, 91/ 100-5 and 88/ 124-46) whereas in case of leaves two genotypes (Pariser Charme and 91/ 100-5) out of three (88/ 124-46) were found susceptible to agro-infection. The different pre-cultures of host plants were found to affect the expression significantly in leaves. The fresh shoots of Pariser Charme and 91/ 100-5 on their cuttings growing in plastic tunnels with high humidity and high

temperature displayed significantly higher levels of GUS expression as compared to greenhouse or in vitro grown plants which showed almost no GUS expression. There are many reports demonstrating the pre-culture of host plants with high humidity improve the transient expression positively (Kim *et al.,* 2009; Zottini *et al.,* 2008). However, conditions for growing cuttings in plastic tunnels were not the only important factor for successful assay. The genetic background was also determining factor as in all subsequent treatments 88/ 124-46 leaves always found resistance to agro-infection. It is likely that there are some genetic factors affecting agro-infections (Santos-Rosa *et al.,* 2008). The number and nature of genetic factors influencing the agro-infection has to be addressed and a strategy to answer this question could be analyzing segregating progeny from defined crosses between susceptible and resistant genotypes. The phenomenon of being recalcitrant to genetic transformation using *Agrobacterium* is not specific for plant transient expression assays but also common in stable plant transformations. Different plant species as rice, maize, legumes, cucurbits, *Pinus* species, tomato, *Arabidopsis* and grape differ in their susceptibility to agro-infection; in addition, even different tissues, organs and cell types within a plant show different tendency towards agro-infection (reviewed by Gelvin, 2000). The involvement of a specific LRR receptor kinase is reported in *Arabidopsis* that make some species recalcitrant to agro-infection (Zipfel *et al.,* 2006). Tzfira and Citovsky (2006) have reviewed the key host proteins necessary for the initial *Agrobacterium*-host interaction and T-DNA import, chromatin targeting, uncoating and its integration to host genome in detail suggesting their over-expression to increase the transformation efficiency. During these studies considerable variation was observed in the expression of GUS in different leaf types, flowers of different ages and within a flower from petals to petal. Similar levels of variations in expression are reported within a single plant or in plants of different ages or even in tissues of different developmental stages of a single plant of *Arabidopsis*, *Nicotiana*, pepper, cotton, tomato and lettuce (Wroblewski *et al.,* 2005; Lawrence *et al.,* 2005). Middle petals of a half bloomed rose flower and young light green leaves with dark green prominent veins and red edges were found optimal for the transient expression studies carried out here.

It was demonstrated earlier that the use of different infiltration media (Mclntosh *et al.,* 2004), addition of acetosyringone (Kapila *et al.,* 1997) or surfactants (Lawrence *et al.,* 2005) improve the end expression of foreign genes significantly. Acetosyringone is

known for its ability to artificially induce virulence genes of *Agrobacterium* necessary to transfer T-DNA (McCullen and Binns 2006). In the present study neither the addition of acetosyringone nor the addition of surfactant improved the transient expression in rose petals. This is in agreement to Wroblewski and colleagues (2005), who investigated the same factors in *Arabidopsis* and lettuce. Temperature is also considered a determinant for *Agrobacterium* mediated gene transfer in plants (Dillen *et al.*, 1997; Riker, 1926). Rose petals after agro-infiltration were incubated at 19ºC, 22ºC, 25ºC, and 28ºC for 4 days post-infiltration. The highest GUS expression was observed at 22 ˚C. It suggests that the regulation of T-DNA transfer through *vir* genes is temperature dependent as already demonstrated by Dillen and colleagues (1997) in detail.

In case of petals the density of *Agrobacterium* suspensions had no significant effect over a broad range of OD values from 0.5 to 4.0. Densities < 0.5 in petals and < 1.5 in leaves did not lead to visible GUS-expression. This is in contrast to the results from several other studies as for example conducted by Santos Rosa *et al.,* (2008) and Kim *et al.,* (2009) who found at least weak expression down to densities of OD 0.1. One explanation for this could be due to physiological and genomic differences between plant species. Another difference to published reports lies in the time from which onwards GUS-expression is visible. According to the previous studies at least weak GUS-expression was visible after 24 hours of infiltration of agrobacteria (Kim *et al.,* 2009) during the current study the GUS-expression was detected at significant levels only from day three on and only weak signals at day two after infiltration. The reasons for this difference are as elusive as for the lack of expression at low densities. Because only a small number of the available genetic variants on both the host plant and the *Agrobacterium* side were screened, there is also a great potential to further optimize the system by including additional rose and *Agrobacterium* genotypes.

6.2.2.2. Transient homologous disease assay

The optimized transient expression assay was used to characterize and assess the activity of rose resistance genes against two fungal isolates race 5 (Dort E 4) and race 6 in the leaves of Pariser Charme, susceptible to both isolates. Rose leaves were found to be very suitable systems to study the interaction of single CGs and black spot. In agreement to Blechert and Debener (2005) during control disease assays when fungal spores were infiltrated in leaves of PC displayed type 1 colonization pattern of Dort E 4

characterized by germination hyphae, haustoria with penetration necks, long and/ or short hyphae, round cellular hypae, psedudoplectenchymatic hypae, conidiogenic hypae and conidia whereas *R. multiflora* and 91/ 100-5 displayed type 7 interaction against infiltrated Dort E 4 where fungus germinated and penetrated cuticle followed by plant produced cell-wall appositions that restricted further fungal growth. The major prerequisite for a successful transient disease assay apart from the transformation is the fact that black spot conidia germinate, infect and produce new conidia when infiltrated to intercellular space in the presence of *Agrobacterium*. As it is not possible for powdery mildew, *Colletotrichum* species and leaf rust to germinate and form appressoria without solid surface contact (Feuillet *et al.,,* 2003; Kim *et al.,,* 1998; Podila *et al.,,* 1993; Liu and Kolattukudy, 1998). In case of powdery mildew since the fungus attack epidermal cell layer, it is necessary to target these tissues for transformation to study the plant-patho-interactions (Schweizer *et al.,* 1999). The described transient disease assay was tested with different CGs (RGA1, 3, 7, 8 and 9) in six independent experiments (Figure 5.13) and produced reproducible data, revealing the significant differences between active and inactive CGs. CGs 3, 7 and 9 did not restrict the fungal growth of race 5 compared to controls whereas after infiltrating CG-RGA8 (belongs to *R. multiflora*) and Dort E-4 spores in PC resulted in reduced growth of the pathogen as found in interaction types 3, 4 and 5 (Blechert and Debener, 2005). On average, results revealed RGA8 as an active and functional gene that reduced fungal growth (race 5 of *D. rosae*) to 41%. Meanwhile RGA1 was also found to have some effect in restricting fungal growth (26%) with some in-constancies (Figure 5.13). To study the effect of genes in more meaningful manner the original data, collected as number of colonies, was converted to percent and in all six independent experiments RGA8 consistently reduced the fungal growth whereas RGA1 reduced fungal growth in 4 experiments out of six (Figure 5.13). It is possible that in combination, these both RGAs restrict fungal growth more profoundly. The degree of protection provided by RGA8 is 41% that is in agreement with the fact that transient expression in rose leaves is not as efficient as in *Nicotiana* due to inefficient agro-infiltration resulting in lower number of transformed cells. The lower transient transformation efficiency of the system did not indicate a clear cut answer of resistant or susceptible genotype, as usually stable transformants show, but presented significantly higher reduction in fungal growth (Figure 5.12). That was the reason why a low concentration of conidia was chosen to maintain an effective ratio between transformed cells and conidia; $5x10^2$ spores/ ml concentration of spores was

found optimal to maintain a higher ratio of transformed cells to conidia that revealed significant differences in antifungal activity of the tested CGs. In agreement to our data transient disease assays established to study the wheat- powdery mildew interaction (degree of protection: 73%; 61%; 48%) and potato- late blight interaction (degree of protection: 80%) always showed the protection efficiencies of transgenes less than 100% and authors consider it enough to claim their significant antifungal effect (Pel *et al.,* 2009; Yahiaoui *et al.,* 2009; Schweizer *et al.,* 1999). To study the wheat and powdery mildew interaction authors (Yahiaoui *et al.,* 2009; Schweizer *et al.,* 1999) co-bombarded the corresponding R-genes along with GUS-reporter gene into leaf epidermis cells of susceptible genotypes and challenged with powdery mildew isolates followed by observing compatible reactions. On the basis of different morphology of fungal colonies within a single cell they were able to demonstrate the presence of weaker and stronger resistance alleles and genes; in contrast Dort E 4 growing on the RGA8 or RGA1 infiltrated leaf areas displayed almost all types of compatible and incompatible interactions pointing to the fact that the rose leaves have a low transformation efficiency and the infiltrated areas contained a mixture of transformed and non-transformed cells so on the basis of morphology of fungal colonies in transiently complemented rose leaf system, it was not easy to conclude the presence of a weakly or strongly effecting R-gene. However this could be further explored by infiltrating RGA8 and 1 together with Dort E 4 in combination and studying their effect on fungal growth and morphology. In a transient expression assay genes are over expressed and it is already demonstrated for different functional R-genes that the over-expression of such genes using 35S promoter can lead to cell death even in the absence of pathogen displaying HR artifacts (reviewed by Pel *et al.,* 2009). In the current study initially the expression of all RGAs was demonstrated in the heterologous system followed by their transient over-expression and disease bioassay in susceptible genotype; none of these RGAs revealed any HR like interaction and RGA8 and RGA1 were found to restrict fungal growth significantly. To study the potato- late blight interaction in a transient disease assay Pel *et al.,* (2009) initially used truncated R-genes (*Rpi* genes) in tobacco that displayed HR and infection efficiency of 40-60% when challenged by fungus, these same genes were then used to stably complement a susceptible potato genotype resulting in fully susceptible genotype revealing the fact that the used genes were truncated and needed additional 42-nt insertion in the 5' upstream region for their full function; later on with complete genes authors again

performed transient (infection efficiency 10-30%) and stable transformations resulting in expected spectrum of resistance and demonstrating the importance of stable transformants. As part of the current study stable transformations were already carried out in PC using *Rdr1* candidates (RGA1, 3, 7, 8 and 9) and putative transformants are under screening process. It is highly recommended that the very next focus of this project should be the generation of stable transformants of RGA8 and 1 and their testing in a disease bioassay using different fungal isolates of *D. rosae*.

Three CGs (RGAs 2, 5 and 6) were excluded from the list of *Rdr1* candidates on the basis of homologous expression analysis in resistant rose genotypes their antifungal activity against race 5 was tested in two independent transient disease assays (Figure 5.14). Three of the tested CGs were found non functional against race 5 isolate Dort E 4; in general, showing no significant reduction in fungal growth compared to GUS control. Resistance provided by RGA8 and RGA1 was checked for race specificity using race 6 that is virulent to *R. multiflora*- the source of *Rdr1* candidates. When checked in transient disease assay none of the RGAs showed resistance to this isolate including RGA8 and RGA1 (Figure 5.15; 5.16). This demonstrates the race specific functionality of RGA8.

There is also an option to check the activity of remaining expressed RGAs (1, 2, 3, 7 and 9) in the disease assay against different isolates of *D. rosae* while *Rdr1* resistance locus confers resistance to five races of *D. rosae* (Von Malek and Debener, 1998). It is possible that the other members of this family are also functional; may be against other races of *D. rosae* or against some taxonomically different pathogen. In addition, their non- functionality is also possible. In most cases when many members of an R- gene family actively transcribed it suggests their functionality (*Rp1*, Collins *et al.*, 1999; *Dm3*, Meyers *et al.*, 1998; *Mi*, Milligan *et al.*, 1998). It is also reported that when one or more members of an R-family are functionally active then they usually recognize different effectors of the same pathogen species (Takken *et al.*, 1999). In *Arabidopsis*, three homologs of *RPP1* and two of *RPP4/ 5* resistance loci are able to recognize different avirulence factors of *Hyaloperonospora parasitica* (van der Biezen *et al.*, 2002; Botella *et al.*, 1998). The *Mla* locus of barely with four members is active against different specificities of fungus *Blumeria graminis* (Halterman and Wise, 2004; Zhou *et al.*, 2001). In contrast there are also some reports demonstrating the role of homologs

within a family conferring resistance against taxonomically different pathogens. *Gpa2* resistance locus of potato harbor four members, two of which confer resistance to unrelated pathogens; a virus and a nematode (Edwin *et al.,* 2000). *Mi* locus in tomato confers dual resistance to root knot nematodes, potato aphids and whitefly (Nombela *et al.,* 2003; Rossi *et al.,* 1998; Vos *et al.,* 1998) in addition; within the syntenic region of *Mi* locus in tomato, potato has a close homolog, *Rpi-blb2* that is active against late blight (Van der Vossen *et al.,* 2005).

Although in the current study the rose petals proved to be very efficient system to carry out transient expression studies, even better than tobacco; this system was evaluated for its suitability to study the interaction of rose and black spot. Rose petals were not found optimal system for disease assay because of their short lifespan and limited seasonal supply. To overcome the lifespan problem of petals, these were co-infiltrated with *Agrobacterium* suspensions and Dort E 4 conidia together to save about 24 hours, this attempt did not display any fungal growth even after 5 days of post-infiltrations (Figure 5.11). Presence of *Agrobacterium* together with conidia somehow stopped the conidial germination and growth, due to this reason in all subsequent experiments initially petals were infiltrated by CGs and 24 hours post infiltration these were inoculated with fungal conidia. However, this phenomenon was not observed in rose leaves when these were co-infiltrated with *Agrobacterium* together with fungal spores. In addition, it was not possible to infiltrate fungal spores in rose petals as they could not be able to evenly distribute in whole petals but stay at the place of infiltration revealing sieving effect (Figure 5.11). Moreover higher concentrations of bacteria (OD_{600}= 1.5-4) promoted the fungal growth; the bacterial density that displayed the optimal GUS expression without effecting fungal growth was OD_{600}= 0.5. The bacterial concentration at OD_{600}= 0.5 and spore concentration of $5x10^5$ spores/ ml did not revealed antifungal effects of CGs. The spore concentration that show significant effect of infiltrated CGs could not be optimized due to limited seasonal supply of flowers. Although it is possible to bloom roses in controlled conditions of greenhouse but off season flowers showed early senescence and presence of the contaminating fungi on microscopy. During the senescent process of a cell there is degradation of macromolecules due to autophagic process in the vacuole, protein degradation in the mitochondria, nuclei and cytoplasm, fatty acid breakdown in peroxisomes and degradation of nucleic acids in the nuclei (reviewed by van Doorn and Woltering, 2008). Since *Diplocarpon rosae* is a

hemibiotrophic fungi, it may germinate upon inoculation / infiltration into rose petal and commence its growth in susceptible genotypes and sometimes in senesced resistant genotypes. Several putative genes encoding enzymes that are putatively involved in pathogen defense were up-regulated exceptionally during petal senescence (van Doorn *et al.,* 2003; Hoeberichts *et al.,* 2007; van Doorn and Woltering, 2008) in similar way as in leaf senescence (Bhalerao *et al.,* 2003). For instance, Avr9/Cf-9 rapidly elicited protein 146 and Class III peroxidase in carnation (Hoeberichts *et al.,* 2007), Chitinase IIa in *Alstroemeria pelegrina* (Breeze *et al.,* 2004), Ribosome-inactivating proteins in *Iris hollandica* (van Doorn *et al.,* 2003) show the same phenomenon. The rapid senescence in petal may lead to quick accumulation of up-regulated defense related genes which could interfere with fungal pathogens that have already initiated growth in petal. However, the data presented here demonstrate the utility of rose petals as a suitable system to carry out transient expression studies and allows characterizing both petal specific and constitutively expressed rose genes.

Interestingly, race 6 showed almost 1.5x higher growth rate in the presence of GUS bacteria than in control samples not treated with *Agrobacterium* (Figure 5.16). It is difficult to speculate that how the presence of GUS bacteria is supporting fungal growth of race 6 especially in rose system where race 5 did not show such tendency. *Agrobacterium* is a plant pathogen that infects a plant by transferring its T-DNA to the host genome (Gelvin 2003; McCullen and Binns 2006). Although it lacks the *hrp* gene cluster present in most of the plant pathogenic bacteria (Goodner *et al.,* 2001; Wood *et al.,* 2001) it induces plant defenses in *Arabidopsis* (Ditt *et al.,* 2006), *N. tabacum* (Pruss *et al.,* 2008), grapevine (Santos-Rosa *et al.,* 2008) and suppresses plant defenses in tobacco cell cultures (Veena *et al.,* 2003), *Arabidopsis* (Gasper *et al.,* 2004; reviewed by Dafny-Yelin *et al.,* 2008). To normalize this phenomenon a control was added in the present study where leaves and petals were infiltrated with GUS bacteria and challenged by the fungus. In petals the plant defense is suppressed by infiltrating high bacterial densities (OD_{600}: 1.5- 4), such samples showed very vigorous growth of fungus as compared to control (samples without or infiltrating low concentration of *Agrobacterium* OD_{600}: 0.5-1). In contrast, leaves did not show such defense suppression in presence of *Agrobacterium* at the tested OD_{600} ranging between 0.5-2.0. The transient transformation efficiency of rose leaves was low as compared to rose petals due their incomplete infiltration and hard waxy structure. However, to overcome this problem the

spore concentration was significantly reduced to $5x10^2$ spores/ ml at 1.5 OD_{600} of agrobacteria for rose leaves that revealed the significant differences for the activity of CGs against Dort E 4.

In summary a rapid method was optimized in rose leaves to assess the functionality of CGs encoding putatively active antifungal proteins that produced reproducible results. This transient disease assay can be used to pre-screen different resistance candidate genes of rose species against black spot (or may be some other fungi that can effectively germinate intercellularly) in a short time with functional insights.

6.2.3. Transient silencing of RGA8

Reverse genetics is an important tool to evaluate the functionality of a gene through silencing it (Baulcombe, 2004). Transient transformation of 91/ 100-5 (a CGs non-deficient plant) and PC (deficient for some CGs), using RGA8-exon2 silencing construct in p9U10 was carried out to validate the expression and activity of *Rdr1* family. The silencing construct was derived from NBS encoding region (exon 2- 1104 bp) of RGA8 having a similarity of 87-99 % to exon 2 of other paralogs within the *Rdr1* locus. Use of dsRNA sharing similarity to multiple genes of a family triggers post-transcriptional gene silencing of many members simultaneously (Wroblewski etal., 2007; Miki *et al.*, 2005). In case of using small interfering RNAs the minimum sequence similarity of 19 bp is required to trigger the silencing of a gene (Vanitharani *et al.*, 2003) and in our case the exon 2 of all RGAs has three stretches of consensus DNA sequence within 1104 bp ranging between 22-33 bp. When exon 2 of RGA8 is compared one by one to the exon 2 of other RGAs the number of DNA stretches showing similar DNA sequences is increased as for example the exon 2 of RGA8 and RGA1 revealed 7 different DNA stretches showing a sequence similarity ranging between 26-100 bp. On the basis of this observation we can speculate that if not all, majority of the *Rdr1* family members should be silenced through this strategy. Although preliminary experiments revealed incomplete silencing of *Rdr1* family when RNAi construct treated samples were evaluated by RT PCR and qRT-PCR it needs further experimental proofs.

In both rose genotypes the RNAi-based silencing construct was agro-infiltrated and challenged by Dort E-4 to validate the effect of RGA8 and other members of *Rdr1*

family on fungal growth. Two preliminary independent experiments were performed using *Agrobacterium* harboring RNAi-construct (optical density 1.5) and challenged by Dort E 4 (spore concentration $5x10^2$ spore/ ml) resulting non-significant differences. Next repeat was performed by infiltrating RNAi-construct in presence of VIP1 in 1:1 ratio (optical density 1.5) and challenged by Dort E 4 (spore concentration $2.5x10^5$ spore/ ml) resulting in a significant effect on fungal growth in terms of increase. As it is described earlier that the efficiency of infiltration and transient transformation of the rose leaves is low as compared to model plants of tobacco or *Arabidopsis* resulting in low number of transformed cells; to target these transformed cells during disease assay in susceptible genotype (PC) a lower spore concentration was used to maintain a balanced ratio between low transformed cells and conidia whereas in transient silencing assay a higher concentration of spores was used to target the low number of transformed cells by conidia in a resistant genotype (91/ 100-5). The samples infiltrated by RNAi and VIP construct displayed weakly susceptible interaction (interaction types 3, 4 and 5; Blechert and Debener, 2005) characterized by well developed hyphal growth with short or long hyphal branching but interestingly none of the colony showed mature or developing acervuli after 5 days of infiltration. Further studies are required to proof this phenomenon by increasing the incubation time and observing the samples microscopically and macroscopically. VIP1- VirE2 interacting protein 1 was found to facilitate *Agrobacterium* mediated transformation as reported by Tzfira *et al.,* (2006) for tobacco and Li *et al.,* (2005) for *Arabidopsis* systems. It is predicted that the VIP1 protein makes super-complex with T-DNA, interacts with host histones and targets T-DNA to host chromatin (Li *et al.,* 2005a; Lee *et al.,* 2008). In addition to VIP1 Tenea and colleagues (2009) have demonstrated that the over-expression of many histone genes can also increase *Agrobacterium* mediated transformation and transgene expression in plants by protecting and expressing its DNA at a higher rate resulting in a higher chances of stable transformation.

RT PCR and qRT-PCR evaluation of RNAi constructs treated samples revealed incomplete silencing of *Rdr1* family in preliminary experiment. Although this is in agreement to silencing NBS-LRR family-*RGC2* in lettuce (Wroblewski etal., 2007), this needs further experimental proof. According to Miki *et al.,* (2005) highly expressed genes are easier to silence than low level expressed genes because of higher or lower concentration of the target, respectively. The *Rdr1* family is constitutively low-

expressed multigene family that showed eightfold higher expression after black spot inoculations as compared to wild type plants (Hattendorf and Debener, 2007a). However, incomplete silencing of *Rdr1* family was sufficient to show an effect on phenotypic level suggesting their optimal expression level necessary to activate fungal resistance as reported by Wroblewski *et al.,* (2007) for silencing the *RGC2* family of lettuce.

It is interesting to report that the incomplete silencing of *Rdr1* locus in PC resulted almost double the intensity of fungal growth displaying the typical type 1 interaction characterized by well developed hyphae and acervuli when compared to different controls as described in section 5.4. This observation could be explained by the presence of some background resistance of RGAs present in PC, a black spot susceptible genotype that has to be subjected for further studies. The RGA expression profile for PC leaf was created using Rd1LRR primer pair in 6% PA gels that revealed the expression of RGAs 1, 3, 7 and 9 (Table 5.3; Figure 5.6; Figure 5.10). Although this resistance is not enough to restrict the growth of Dort E 4, it is able to reduce the fungal destruction by at least 50 %. This again points to the fact that other RGAs may have functionality to some other races of *D. rosae* and/ or some genes as for example RGA1 in combination with RGA8 could have more drastic effects on fungal growth. In addition, further studies are required to repeat and refine the silencing assay for *Rdr1* candidates. Although the refinement in terms to target individual family members will increase the possibility to pin-point active genes ready for functional complementation assays technically this will be challenging and difficult task because of very high sequence similarity among these paralogs (85-99%; Kaufmann *et al.,* 2010). The use of empty vector or GUS silencing vector as a control is also recommended to check for artifacts.

6.3. Protein sequences analysis of three members of *Rdr1* resistance locus

Rdr1 resistance locus in *R. multiflora* consists of a cluster of nine paralogous disease resistance genes that play important roles in innate immunity against black spot. The protein sequences of three members; RGA8, RGA7 and RGA3 were deduced and subjected to sequence variability, nucleotide substitution rates and amino acid homology check. According to the current investigation, among the nine R genes in this locus RGA8 confers resistance to race 5 of the fungal pathogen *Diplocarpon rosae*.

Comparison of genomic sequence of isolated RGA8 clone and its 5' and 3' RACE products revealed the presence of two alternatively spliced transcripts for this gene when expressed transiently in the heterologous tobacco system. One transcript is shorter with expected length of 3995 bp representing four exons and second transcript is about 3 kb longer than the first one representing the same four exons with extra 3' UTR (Figure 5.12). Such alternative splicing is also reported for *N* gene conferring resistance to Tobacco Mosaic Virus (Whitham *et al.,* 1994). However, in case of RGA8 both transcripts resulted in the same ORF of 1122 amino acids (ORF finder-NCBI). It is reported that the alternative transcripts encode proteins without or truncated LRR domain and in case of *N* and *RPS4* genes such transcripts are necessary for the complete resistance (Schornack *et al.,* 2004; Dinesh-Kumar and Baker, 2000). Such variant proteins are predicted to interact and form TIR mediated oligomers that are active signaling complexes (reviewed by Padmanabhan *et al.,* 2009). In addition to the RGA8 race products, 3' RACE of RGA7 was also determined experimentally and 5' RACE was assumed to be 200 nucleotides upstream of ATG (Figure 5.13) and the deduced protein of RGA7 has 944 amino acids. The full length cDNA sequence of RGA3 was already available (Kaufmann *et al.,* 2010) and the predicted protein contains 1139 aa. All three proteins show the presence of TIR, NBS and LRR domains with all conserved motifs as suggested by Lukasik and Takken (2009) and Meyers and colleagues (1999; Table 5.6; Figure 5.24).

6.3.1. RGA 8 protein similarities

According to BLASTx searches RGA8 protein shares the highest 41% identity to TIR-NBS-LRR-resistance proteins of *Populus trichocarpa* (ACCESSION XP_002329162), both genes belong to the same class of TIR-NBS-LRR resistance genes, followed by 39-44% to hypothetical proteins of *Vitis vinifera*, 40% to TIR of *Medicago truncatula* (ACCESSION ABD28703), 40% to *CMR1* of Phaseolus vulgaris (ACCESSION ABH07384) and 39% identity to *N*-like protein of *N. tabacum* (ACCESSION BAF95888) for resistance to the tobacco Mosaic Virus (Whitham *et al.,* 1994). Figure 6.1 shows phylogenetic tree of different amino acid sequences showing considerable identity to RGA 8 protein, these full length aligned protein sequences were downloaded from NCBI and tree was constructed based on the bootstrap neignbor-joining (NJ) method with the Kimura2-parameter model by MEGA4 (Tamura *et al.,* 2007; Kimura, 1980). It is interesting to code here that the *CMR1* is a viral resistance gene from

common bean that functions across plant families (Seo *et al.*, 2006). This protein sequence shows 40% identity to the RGA8 protein that found to be active against black spot fungus, which suggests that the predicted NB-LRR structures and recognition of certain pathogen type lack correlation.

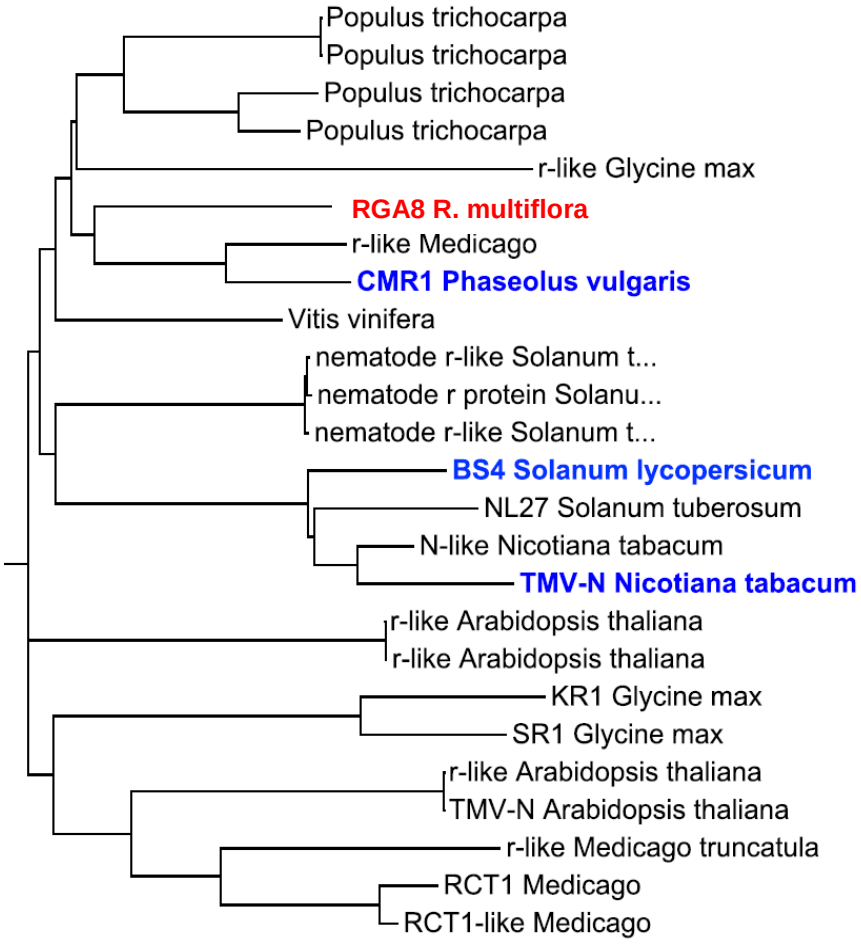


Figure 6.1: NJ analysis of protein sequence showing considerable similarities to RGA8. The full length aligned protein sequences were downloaded from NCBI and analyzed in MEGA 4.0.

To date there are more than 50 well characterized R-genes conferring resistance to bacteria, fungi, oomycetes, viruses, nematodes or insects and classified on structural differences regardless of pathogen specificity, as in *Arabidopsis* members of both classes of NB-LRR family confer resistance to the *Peronospora parasitica* (oomycete) and *Pseudomonas syringae* (bacterium) in addition to that one member of the CC-NBS-LRR class is active against virus invasion (Bittner-Eddy *et al.*, 2000; Cooley *et al.*, 2000; Parker *et al.*, 2000). This also suggests the involvement of TIR or CC domains in activation of defense pathways through downstream processing as the presence of TIR and CC domain cannot be correlated with specific pathogen type. However, the LRR

and TIR domains of flax *L* alleles participate in pathogen recognition (Luck *et al.,* 2000). The finding that the highly homologous genes can confer resistance to unrelated species emphasize the use of such genes in breeding to engineer new resistance specificities against plant pathogens.

6.3.2. Protein sequence homology

Comparison of RGA8 protein sequence with other two (RGA3 and 7) members of the same *Rdr1* family revealed 119 RGA8 specific amino acids residues (119/ 1122; 10.6%), which are part of TIR (2 aa; 0.002%), NBS (40 aa; 3.6%), and LRR (77 aa; 6.8%) domains. For RAG8, as the majority of unique amino acids are located in LRR region followed by NBS region suggesting some important role of these domains in functionality of RGA8. The deduced *Gro1-4* protein (resistance gene) showed only 16 non-conserved differences in amino acids sequence when compared to non-functional members of the *Gro1* gene family (Paal J. *et al.,* 2004). Mutagenesis analysis of the RGA8 protein or comparison of functional and non-functional orthologs could determine the essential residues necessary for pathogen recognition and/ or downstream signaling. The present data is generated by interaction of *Rdr1* candidates against one patho-type, race 5, of *D. rosae*. In contrast *Rdr1* resistance locus confers resistance to five races of *D. rosae* (Von Malek and Debener, 1998). It is possible that the other members of this family are also functionally active against other races of *D. rosae*. This is subject for further studies. Although the amino acid sequence identity of these three paralogues of *Rdr1* resistance gene cluster ranges between 58% and 80% and RGA8 shares the highest overall amino acid sequence homology to RGA3 (80%; Table 6.1) the functionality of RGA3 and 7 has to be explored against different isolates of *D. rosae* and/ or some other taxonomically different pathogens. Alignment of deduced amino acids of RGA 8, 3 and 7 show that the N-terminal halves of proteins that harbour putative effector domains show higher degree of sequence homology than the C-terminal halves of proteins (Table 6.1) suggesting the LRR domain under selection as demonstrated for other closely related NBS-LRR proteins (Yahiaoui *et al.,* 2006; Shen *et al.,* 2003; Kuang *et al.,* 2004; Van der Vossen *et al.,* 2000). This kind of selection in LRR domain is exerted by single base changes, insertions, deletions and unequal exchange of meiotic recombination events for the evolution of new pathogen specificities within R-genes or between closely linked R-genes in a cluster (Ellis *et al.,* 2000).

6.3.3. Evolution of the RGA8 gene based on nucleotide substitution pattern in three paralogs

To determine the type of selection pressure exerted on different parts of the genes, ratio of synonymous (K_s) and non-synonymous (K_a) substitutions rates per synonymous/ non-synonymous site were calculated for the different regions of the ORF of three paralogs (RGA8, 7 and 3). When K_a / K_s ratio is 1 it represents random mutation or no selection pressure operating on sequences may be for diversification ($K_a / K_s > 1$) or sequence conservation ($K_a / K_s < 1$) (Hughes and Nei, 1988). Estimation of the number of synonyms and non-synonyms nucleotide per site showed that the three genes are under conservation selection and all domains (TIR, NBS and LRR domain) are not under positive selection compared to N- and C-flanking regions to conserved LRR motifs which showed K_a / K_s ratio > 1 (Table 6.1). The comparison of complete LRR domains of RGA8 and 3 resulted in higher K_a values than K_s suggesting diversification in this region, further insights to data revealed that this diversification is actually the result of lower K_s for the N- and C- flanking region of xxLxLxx motifs which always shows a $K_a / K_s \geq 1$ (Table 6.1). Although three genes show conserved type of selection, LRR domains show the highest frequency of non-synonymous substitutions followed by NBS domain. Calculations of K_a / K_s for C-terminal region of genes supported the hypothesis that diversifying selection is exerted on those parts of proteins which participate in pathogen recognition. This pattern is in agreement to previous studies that show diversifying selection acts on the LRR encoding domain of various plant disease resistance genes (Jiang *et al.*, 2007).

Here only three homologs of *Rdr1* family are compared, where sequence similarity and conservation was already expected. Comparison of additional orthologs and/ or alleles isolated from wild relative of *Rosa* and RGA8 will help to understand real selection operating on the RGA8 gene and/ or *Rdr1* locus. Complementation assays using these genes directly or after deleting or exchanging different domains between different genes and generation of mutants can provide information to define the role and significance of different domains in resistance specificities. It is demonstrated for different R-genes that the loss of resistance because of lacking some part of gene is possible. LRR region in wheat (*Lr10*) needs the last five LRRs for its function (Feuillet *et al.*, 2003), the flax resistance gene *M* needs 426 bp of coding region of LRR domain for its function

(Anderson *et al.,* 1997). In addition to that the importance of P-loop motif for the functionality of resistance genes of tobacco-*N* (Dinesh-Kumar *et al.,* 2000) and of *Arabidopsis-RPM1* (Tornero *et al.,* 2002) genes is proved by mutational analysis.

Table 6.1: Sequence variability and nucleotide diversity in different regions of three paralogs of *Rdr1* family

R-genes compared	Analyzed regions[1]	Nucleotide substitutions[2]			
		aa[3] (%)	K_a	K_s	K_a/K_s
RGA8 vs. RGA3	Complete CDS	80	0.0977	0.1041	0.92
	TIR domain	90	0.0484	0.0836	0.58
	NBS domain	76	0.1168	0.1196	0.98
	Complete LRR domain	79	0.1034	0.0983*	1.05
	N-terminal flanking	-	0.1146	0.1108*	1.03
	xxLxLxx motif	-	0.0722	0.0935	0.77
	C-terminal flanking	-	0.1679	0.1156*	1.45
RGA8 vs. RGA7	Complete CDS	75	0.0991	0.1354	0.73
	TIR domain	98	0.0079	0.0356	0.22
	NBS domain	78	0.1184	0.1853	0.64
	Complete LRR domain	66	0.1209	0.1429	0.85
	N-terminal flanking	-	0.1148	0.0530*	2.17
	xxLxLxx motif	-	0.0717	0.1142	0.63
	C-terminal flanking	-	0.5067	0.5738	0.88
RGA7 vs. RGA3	Complete CDS	64	0.1180	0.1497	0.79
	TIR domain	90	0.0512	0.1134	0.45
	NBS domain	71	0.1439	0.1737	0.83
	Complete LRR domain	52	0.1267	0.1508	0.84
	N-terminal flanking	-	0.0740	0.1132	0.65
	xxLxLxx motif	-	0.0793	0.1249	0.64
	C-terminal flanking	-	0.5657	0.5029*	1.13
RGA8 vs. RGA3, 7	Complete CDS	58	0.09228	0.11032	0.83
	TIR domain	89	0.02736	0.05688	0.47
	NBS domain	67	0.10896	0.13689	0.78
	Complete LRR domain	49	0.10421	0.11089	0.94
	N-terminal flanking	-	0.10613	0.07759*	1.40
	xxLxLxx motif	-	0.06861	0.09678	0.69
	C-terminal flanking	-	0.25922	0.25760*	1.01

[1] Different region of R-genes is analyzed for nucleotide variability as presented in Figure 5.12.
[2] The ratio of synonymous (K_s) and non-synonymous (K_a) substitutions rates per synonymous/ non-synonymous site were calculated using software DnaSP v 5.
[3] Amino acid (aa) homology in percentage.
[*] High-lighted numbers represent the exceeded value of synonymous (K_s) compared to non-synonymous (K_a) substitutions for specified region

Typically, R-genes activate a HR/PCD to restrict the pathogenic growth (Goehre and Robatzek, 2008). Although the resistance mechanism of RGA8 is not yet investigated, preliminary disease assays suggest that it is not associated with localized necrosis

response or HR. Keeping in view the proposed resistance activation mechanism models of TIR-NBS-LRR genes (Lukasik and Takken, 2009; 2006; Bent and Mackey, 2007), it could be speculated that the direct binding of pathogen effectors to the C-terminal half of RGA8 protein may lead to conformational changes in the N-terminal half of the protein that trigger defense responses. The speculated model requires detailed experiments. According to one of the original models for gene-for-gene model the elicitors are recognized directly by LRR domain, whereas according to the guard or decoy model these elicitors are detected indirectly (Hoorn and Kamoun, 2009). However, TIR or CC domains are predicted to participate in indirect recognition of pathogen elicitors (reviewed in Padmanabhan *et al.,* 2009).

6.4. Outlook

The main objective of this investigation was the identification of the major gene active against black spot of roses by molecular and functional characterization of the *Rdr1* locus harboring nine candidate genes. When RGA8 was infiltrated into the black spot susceptible rose genotype (PC) it was alone able to reduce the fungal growth significantly. Therefore RGA8 was found as a major gene active against black spot. Meanwhile a second gene RGA1 also showed some effect on fungal growth in transient assays. Further studies focusing on the generation of stable transformants carrying CGs in a deficient rose phenotype and their testing in a disease bioassay are highly recommended as the *Agrobacterium* mediated stable transformations in PC were completed and putative transgenics are already in process of screening. In addition, homozygous lines of *Arabidopsis* carrying RGA8 and RGA3, generated in the current study, are ready for the evaluation of the expression and activity of CGs against black spot.

Breeding for disease resistant plants armed with effective, long-term and broad spectrum resistance against numerous pathogenic races of a particular pathogen needs complete information of genes responsible for a particular resistance. In the current study RGA8, (consistently as a major active gene) and RGA1 are found to participate in disease resistance against race 5 of *D. rosae*. The effect of different RGAs, in combinations, on fungal growth should be next focus as there is quite high probability that the RGA8 in combination with RGA1 can restrict the fungal growth more profoundly.

Previous studies have proved the functionality of the *Rdr1* locus against five races of *D. rosae*, whereas in the current study only one race (race 5- Dort E 4) of those was used to evaluate the functionality of this locus resulting RGA8 and RGA1 out of 5 CGs active against race 5. Therefore, it is possible that the remaining CGs (RGA3, 7 and 9) could be functional against other races of black spot fungus; this has to be proofed by extensive phytopathological studies using different races of *D. rosae*. To facilitate this evaluation and screening of other fungal isolates, a homologous disease assay in leaves of PC and a transient gene silencing assay in leaves of resistant rose genotype (91/100-5) were optimized (Section 5.3.3.2; 5.4). There is also great potential to refine these assays by screening new rose genotypes and *Agrobacterium* strains. Schulz and colleagues (2009) have identified some rose genotypes highly resistant to black spot as tetraploid *R. bella* 00-57-04, *R. californica* v. *plena* 00-33-01, *R. majalis* 93-09-03 and *R. nanothamnus* 00-56-01. According to Whitaker *et al.,* (2010) there is no universally susceptible or resistant rose genotype against black spot. PC represents narrow acting alleles against black spot since race 1 (isolate- HSN) is the only race of *D. rosae* out of 11 races that cannot infect it and *R. majalis* represents broadly acting alleles against black spot. The optimized transient disease assays can be utilized to screen a large number of R-genes against different fungal isolates. In addition, refinement and further optimization for RNAi experiments is highly recommended as in case of targeting single R- family members can help to clone various R-genes in a short time and using a construct for a common RGA motif could knockout many RGA families simultaneously and screening against different pathogens. This will not only provide insights to functionality of individual RGAs but also narrow down specific RGA families active against different pathogens and such RNAi constructs can be used as probes to screen genomic libraries of the relevant genotypes to dig corresponding candidates as suggested by Wroblewski *et al.,* (2007).

To exploit full potential of R genes against black spot needs information about the evolution of *Rdr1* locus in rosa gene pool, its addition in natural habitats of rosa, selection pressure operating on this locus, resistance mechanism and function of effectors that activate this locus or if this locus could also be activated through multiple signaling components, as demonstrated for *RPP4* and *RPP5* of *Arabidopsis* (Van der Biezen *et al.,* 2002). Application of mutation analysis to demonstrate functional domains and motifs of active genes could also be a subject for future studies.

According to Noel and colleagues (1999) the allelic variation at resistance locus is thought to generate spatial and genetic diversity to reduce the selection pressure for pathogens to be virulent (frequency dependent selection). To study the allelic variation for the *Rdr1* locus is highly recommended to characterize different functional alleles of *Rdr1* from different rose species. The flax *L* alleles against rust races, barley *Mla* alleles against powdery mildew races and tomato *Cf* alleles against leaf mould races show allelic variation, a common phenomenon, at loci that specify resistance to different races of the same fungal species. In 2000 Bittner-Eddy and colleagues cloned two single copy alleles of *RPP13* gene of *Arabidopsis* that encode CC-NB-LRR proteins active against different races of *P. parasitica*.

In the current study expression profiles for RGAs were created utilizing heterologous and homologous systems. In homologous system the expression of RGA 4, 5, and 6 was not detected whereas all RGAs were expressed in heterologous system of tobacco. According to recent findings this difference could be due to the regulatory machinery of homologous system (resistant rose genotypes) i.e. miRNAs (Padmanabhan *et al.,* 2009). This may be evaluated through northern blotting of isolated miRNAs from resistant rose genotype using RGA general and specific probes.

Although RGA4 is excluded from the list of candidate CGs for *Rdr1* because of the insertion of a 7 kb retrotransposon in intron I and its expression in homologous system was never detected, there are reports demonstrating the expression and functionality of genes harboring retrotransposons in introns for example *Gro1-4* of potato (Paal *et al.,* 2004) and *SBEIIb* of barley (Sun *et al.,* 1998). RGA4 should be isolated for further detailed analysis.

Isolation of additional rose resistance genes active against different fungal diseases from different wild genotypes and their functional characterization using same strategies optimized in this study are recommended. This characterization will provide better understanding of molecular basis of resistance genes and will allow the generation of transgenic rose varieties, ultimately improving resistance of roses against fungal diseases.

Appendix

Table 7.1: Enzymes

Enzymes	Source
Restriction endonucleases and their buffers	
*Bam*HI (10U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
Eco RI (10U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
*Hind*III (10U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
*Kpn*I (10U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
Sau 3A1 (4U/µl)	New England Biolabs Inc.
*Bfu*C1 (4U/µl)	New England Biolabs Inc.
Xba I (10U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
Restriction endonuclease (R^+, B^+, O^+, G) buffers	MBI Fermentas GmbH Leon-Rot, Germany
Polymerases, ligases and other enzymes	
Taq DNA polymerase (5U/µl) and 10x Buffer	Bioline USA Inc.
TaKaRa DNA polymerase (2.5U/µl) and 10x Buffer	TaKaRa, Shiga, Japan
T4 DNA ligase (5U/µl) and Buffer	MBI Fermentas GmbH Leon-Rot, Germany
Shrimp Alkaline phosphatase (SAP-1U/µl)	MBI Fermentas GmbH Leon-Rot, Germany
T4 DNA polymerase (5U/µl) and Buffer	MBI Fermentas GmbH Leon-Rot, Germany
T4 polynucleotide kinase (10U/µl) and Buffer	MBI Fermentas GmbH Leon-Rot, Germany

Table 7.2: Kits and other consumables

Product	Purpose	Source
1 kb-DNA-Ladder (250 µg)	DNA-size standard	Invitrogen Karlsruhe, Germany
λ-Phage DNA (500 µg)	Quantification standard	Invitrogen Karlsruhe, Germany
Ambion® DNAfree™ Kit	DNA digestion to get pure RNA	Ambion Cambridgeshire, UK
High-Capacity cDNA Reverse Transcription Kit	cDNA Synthesis	Applied Biosystems (see invitrogen)
Invisorb® Spin Plant RNA Mini Kit	RNA isolation	Invitek Berlin, Germany
Mini Elute Gel Extraction Kit	DNA extraction (< 4kb) from agarose gel	Qiagen GmbH Hilden, Germany
NucleoSpin® Plasmid	Plasmid-DNA isolation	Macherey-Nagel GmbH & Co. KG Dueren, Germany
2´-deoxynucleoside 5'-triphosphates (dNTPs)	PCR consumables	Invitrogen Karlsruhe, Germany
FirstChoice RLM-RACE	Isolation of 5' RACE products	Ambion Cambridgeshire, UK
QIAquick Gel Extraction KIT	Isolation of DNA fragments (> 4kb) from agarose gel	Qiagen GmbH Hilden, Germany
Quick Blunting KIT	Polishing blunt ends of PCR fragments	New England Biolabs GmbH Frankfurt, Germany
pGEM® T- Easy Vector	Cloning of PCR products	Promega Madison, Wisconsin, USA

Table 7.3: Chemicals

The chemicals were purchased from the companies listed below their corresponding numbers are indicated in front of chemicals.

a. AplliChem GmbH Darmstadt Germany
b. Becton Dickinson GmbH Heidelberg, Germany
c. BIO-RAD laboratories GmbH Munich, Germany
d. Boehringer Mannheim GmbH Lenzkirch, Germany
e. Carl Roth GmbH & Co. KG Karlsruhe, Germany
f. Devender, J. T. Baker
g. Duchefa, The Netherlands
h. Fluka Sigma-Aldrich St. Louis, Missouri, USA
i. Merck KGaA Darmstadt Germany
j. PeqLab Biotechnologie GmbH Erlangen, Germ.
k. Sigma-Aldrich St. Louis, Missouri, USA
l. GIBCO BRL Karlsruhe, Germany

Consumables	Consumables
5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid g	Kalium hydrogen phosphate e
3,5-Dimethoxy-4-hydoxyacetophenon k	Kalium hexacyanoferrrat(II) i
2-(N-Morpholino) ethansulfon acid g	Kanamycin sulfate a
4-Methylumbelliferyl-ß-Dglucuronid g	Lithium chloride e
Acetic Acid e	Lysozyme d
Agar-Agar e	Magnesium chloride e
Agarose j	Magnesium sulfate k
Ampicillin e	2-Mercaptoethanol h
Ammonium sulfat i	Natrium chloride 99,8 % e
Ammonium persulphate c	Natrium citrate e
Bacto®-Tryptose b	Natrium dodecylsulfate e
Boric acid e	N,N-Dimethyl formamid k
Bromophenol blue i	Poly acrylamide solution 30% e
Calcium chloride e	Polyvinylpyrrolidon 40 k
Chloroform/Trichlor methane 99 % e	Roti-Phenol e
Di-ethylen pyro-carbonate a	Sodium hydroxide i
DMSO (Di-methyl sulfoxide) e	Sodium hydrogen phosphate i
DTT (1,4-Dithiotreitol) k	Sodium dihydrogen phosphate e
Ethanol abs. f	Streptomycin l
Ethidiumbromide 1% (10 ng/µL) e	Sucrose a
Ethylen-diamin-tetra-acetate e	Tetracyclin k
EDTA- Di-natrium salt Dihydrate e	TEMED (N,N,N,N–tetramethyldiamine) e
Glycerin 99,5 % e	Tris(hydroxymethyl)-aminomethan e
Isopropanol (2-Propanol) 99,5 % e	Triton-X-100 e
Isopropyl-β-G-thiogalctopyranoside e	Tween-20 e
Kalium acetate e	Trypton e
Kalium di-hydrogen phosphate i	X-Glc-A-Cyclo-hexyl-ammonium salt g
Kalium hydrogen phosphate e	Yeast Extract e

Table 7.4: Equipments and instruments

Equipments / Instruments	Firm
Autoclave: Sanoclav Tischautoklav	Wolff
Gel Jet Imager Version 2004	Intas Goettingen, Germany
DNA Analyzer 4200 und 4300	LI-COR Biosciences Lincoln, USA
Electroblotter and power assembly	BioRad
Electrophoresis stand and clips	Eurofins MWG Operon Ebersberg, Germany G. Kisker GbR Steinfurt, Germany Peqlab Biotechnologie GmbH Erlangen, Germany Hybaid Ltd Ulm, Germany
Electroporator: MicroPulser	BIO-RAD laboratories GmbH Munich, Germany
Fine scale: Delta Range®AE 260	Mettler-Toledo GmbH Giessen, Germany
Heating block: Thermo Stat plus	Eppendorf AG Hamburg, Germany
Incubator	Memmert GmbH + Co. KG Schwabach, Germany
Incubation shaker	Labortechnik Edmund Bühler GmbH Hechingen, Germany
Plastic disposable material (96-well plate, 6-well plates, 12-well plates and etc.)	Sarstedt Nürnbrecht, Germany Eppendorf AG Hamburg, Germany
Magnet stirrer	Ikamag Staufen, Germany
Minispin C1301	Neolab Heidelberg, Germany
Simple & Multipipette® plus	Eppendorf AG Hamburg, Germany
Petri dishes (Ø 8,5 cm)	Fisher Scientific GmbH Schwerte, Germany

pH-Meter: pH 211	Hanna Instruments GmbH Kehl am Rhein, Germany
Photometer: GENOVA	Jenway Tirana, Albana
Photometer	Safas LAT Monaco, France
Pipettes	Labmate Langenfeld, Germany
Water purification system: Arium 611	Sartorius AG Goettingen, Germany
qRT-PCR machine	Applied Biosystems, USA
Scanner Infrared Imaging System Odyssey®	LI-COR Biosciences GmbH Hamburg, Germany
Swing grinder	Retsch GmbH Haan, Germany
Thermocycler: Primus 96 advanced	Peqlab Biotechnologie GmbH Erlangen, Germany
Table centrifuge, cooling centrigfuge: Universal 32R, Mikro200, Rotina420R	Hettich GmbH & Co.KG Tuttlingen, Germany
Vertical Electrophoresis system Maxi gel	Biometra GmbH Goettingen, Germany
Vortexer	VWR International GmbH Darmstadt Germany
Waterbath: Typ W16	LHG
Rocking dish	Biometra GmbH Goettingen, Germany

Table 7.5: Media

Product	Recipes	Purpose
FB (freezing broth)	1 % (w/v) Peptone 0.5 (w/v) Yeast extract 85.5 mM NaCl 36.2 mM K_2HPO_4 13.2 mM KH_2PO_4 1.5 mM Natrium acetate 6.8 mM Ammonium sulfate 365 µM $MgSO_4$ 4.4 (v/v) Glycerin autoclave and use after addition of specific antibiotic	Long-term storage of bacteria at -80 °C
LB (Luria Bertani) Liquid	1 % (w/v) Peptone 0.5 % (w/v) Yeast extract 171 mM NaCl pH 7.0 with 5 M NaOH	Growth medium for *E. coli*
LB (Luria Bertani) Solid	First prepare LB liquid + 1.5 % (w/v) Bacto- Agar pH 7.0 autoclave and use after addition of specific antibiotic 16 µM IPTG and 3 mM X-Gal	Media for growing bacteria and for blue white screening of bacterial transformants
YEP liquid medium	10.00 g Peptone 10.00 g Yeast extract 5.00 g NaCl pH 7.5 autoclave and use after addition of specific antibiotics	Growth medium for *Agrobacterium*
YEP solid medium	10.00 g Peptone 10.00 g Yeast extract 5.00 g NaCl 10.00 g Bacto-Agar autoclave and use after addition of specific antibiotics	Growth medium for *Agrobacterium*
SOB	20 g/l Peptone 5 g/l Yeast extract 10 ml 1 M NaCl- solution 10 ml 250 mM KCl- solution	Stock solution for the preparation of fresh SOC
SOC	10 ml SOB 100 µl 2 M Glucose 100 µl 1 M $MgCl_2$ 100 µl 1M $MgSO_4$	After electroporation, bacteria are to be transferred to this media for stabilization

Table 7.6: Solutions and buffers

Solution	Recipes	Purpose
Alkaline lysis solution 1	50 mM Glucose 25 mM Tris/HCl, pH 8.0 10 mM EDTA, pH 8.0 Autoclave and store at 4 °C. Before use add 3 mg/ml Lysozyme	BAC-Plasmid isolation
Alkaline lysis solution 2	0.2 N NaOH 1 % SDS Do not autoclave and use within 5-days after preparation	BAC-Plasmid isolation
Alkaline lysis solution 3	3 M Kalium acetat 11.5 % Acetic acid Store at 4 °C	BAC-Plasmid isolation
Loading dye	0.25 % (w/v) Bromphenol blue 30 % (v/v) Glycerin 1 mM EDTA (pH 8.0)	Mixed in DNA samples to track their position in a running agarose gel
TAE-buffer	0.04 M Tris 1 mM EDTA pH 8.0 store at RT	Gel electrophoresis
Tris-EDTA-buffer (TE)	10 mM Tris- HCl (pH 8.0) 1 mM EDTA (pH 8.0)	DNA-stabilization
10 x Williams buffer	100 mM TrisHCl (pH 8.3) 500 mM KCl 20 mM $MgCl_2$ 0.01 % Gelatin	PCR-Buffer
1x TBE	89 mM Tris-Base 89 mM Boric acid 2 mM EDTA pH 8.0	Running Buffer for PA gels
5x Takara buffer	50 mM Tris-HCl 100 mM NaCl 5 mM Mg2+ pH 8.2	RACE PCRs
AFLP-Loading buffer (stop buffer for PA-Gels)	98 % (v/v) Formamid 0.025 % (v/v) Bromphenol blue 0.025 % (v/v) Xylencyanol 10 mM EDTA	Samples loading buffer for PA gels
Fixing solution for SSCP gels	7.5 % (v/v) Acetic acid	Silver staining of SSCP gels
Developing solution for SSCP gels	9.4 mM Na_2CO_3 0.15 % (v/v) Formaldehyde 6.4 µM Natrium thiosulfat	Silver staining of SSCP gels
Staining solution for SSCP gels	5.9 mM $AgNO_3$ 0.15 % (v/v) Formaldehyd	Silver staining of SSCP gels
Methacryloxypropyltrimethoxy silan solution	0.3 % (v/v) Methacryloxypropyltrimethoxy silan 0.3 % (v/v) Acetic acid in 100 % Ethanol	To fix SSCP gel to rare plate
SSCP-Loading buffer	95 % (v/v) Formamid 0.01 M NaOH 0.05 % (w/v) Xylen cyanol 0.05 % (w/v) Bromphenol blue	Denature samples before loading on SSCP gel

Table 7.7: Real time PCR primer sequences

Primer	Sequences (5´-3')	Tm °C/ product size	Purpose
RGA8_1a-f	AGCGCTTTCTTTCTTCTCCAC	For all primer pairs the Tm is 60 °C / 80-120 bp	qRT-PCR primer to evaluate the expression of RGA8 after infiltrating RGA8 gene silencing construct
RGA8_1b-r	ATGACGTCCTTTCAGCTACCA		
RGA8_2a-f	CAGAGGCATGAAGGTGAAGAA		
RGA8_2b-r	AGAGAAATGCTGCTGGTCTTG		
RGA8_3a-f	GATTGGGTTTGCTGTGTGTG		
RGA8_3b-r	CCAAATGCAACAGGTATCAGG		

Table 7.8: General PCR primer sequences

Primer	Sequences (5´-3')	Tm °C/ product size	Purpose
Actin-f	CGA GGA AGA TCT GGC ATC A	60/ 600 bp	Internal control for RT-PCR
Actin-r	AGG AGC TGC TCT TGG CAG T		
consP1-F	TGA GCA CCC AAG TTA GAG CC	56-60/ 2500 bp	Final PCR screening of transformed *E. coli* for single RGA positive clones
consP1-B	TCC ACT CAA ATC AAG CTC CA		
M13-f	GTA AAA CGA CGG CCA GT	52-56	Insert-PCR
M13-r	CAG GAA ACA GCT ATG AC		
Kuehr3-f	GCT GGW TGG ACT TCA AAG G	60/ gDNA-1550bp, cDNA-1180bp	Specific for RGAs 1, 3, 7, 8, 9-Can differentiate b/w gDNA and cDNA
Kuehr3-r	ATT WTA GAG AAG GCT TCA AGA TTC C		
Rd1LRR_R	GGA ATT TCA CTT CCA GGA	62/ NA	Able to differentiate b/w all RGAs on the basis of different PCR product sizes in acrylamide sequencing gels
Rd1LRR_F	AGG CTT CAA CAA TTG CC		
Cons cDNA-B	TCC ATT CCA GAG GTG ATC AAT	56/ 1.7 kb	Initial PCR screening of transformed *E. coli* for single RGA positive clones
Cons cDNA-F1	GCT GGA TGG ACT TCA AAG G		
Aneela 1F	GCC TCA AAT TAT GAA TGT AGA GCA G	60/ gDNA-1950, cDNA-1600	Specific for RGAs 1, 2, 3, 5, 6, 7, 8, 9--Can differentiate b/w gDNA and cDNA
Aneela 1R	ATC AGG ACT TTC AGG GAC GA		
GUS_F	GGT GGG AAA GCG CGT TAC AAG	60/ 1200 bp	To check the presence of GUS construct
GUS_R	GTT TAC GCG TTG CTT CCG CCA		
NTPII_1	GAG GCT ATT CGG CTA TGA CTG	60/ 600 bp	To check the presence of constructs with Kanamycin resistamce
NPTII_2	ATC GGG AGC GGC GAT ACC GTA		

Table 7.9: 5' and 3' RACE primer sequences

Primer	**Sequences (5´-3')**	**Tm °C/ product size**	**Purpose**
1GSP-1	TTC CGG CAA GGC AGA GTT TA	63 °C for all primer pairs	Gene specific primers to isolate 3'-RACE products for RGA1
1GSR-2	GCG GTC GGT AAT CAA GAT GC		
1GSP-nested	AGC GGA AGT GGT GCT CTG AC		
2GSP-1	CCA TCC ATT GCA TTG CTG AA		
2GSP-2	TTC CGG CAA GGC AGA GTT TA		
2GSP-3	GCG GTC GGT AAT CAA GAT GC		
2GSP-nested	TGG ATT GGG TTT GCT GTG TG		
1GSP-1	TGG CGG GAG AAA AAG ACT GG	64	Gene specific primers to isolate 3'-RACE products for RGA7
1GSR-2	TGC ATG GAA TTC TGC ATT GG	63	
1GSP-nested	GAA TTT GTG GGG CAA ACG AA	63	
1GSP-1	GAG CCT GGT GGA CGT AGT CG	63	Gene specific primers to isolate 3'-RACE products for RGA8
1GSR-2	TCT GTT GGC CCA AGA TTG CT	63	
1GSP-nested	CTC CCA CCG TGT TTT CAA CC	63	
2GSP-1	CCA TCC ATT GCA TTG CTG AA	63	
2GSP-2	AGG CTT CAA CAA TTG CCA GA	61	
2GSP-3	ACC TCA CGA CAA TCC GTC TG	63	
2GSP-nested	TGG ATT GGG TTT GCT GTG TG	61	
1GSR-1	TTT GTG GGG CAA ATG GAA AG	63 °C for all primer pairs	Gene specific primers to isolate 3'-RACE products for RGA9
1GSP-nested	ATG GGA CTG CCG TTG AGA AA		
2GSP-1	TTG GTG GAG CTT GAT TTG AGT G		
2GSP-2	GGT TGG GAC TCA GAG GAA ACAA		
2GSP-3	AAT CCG TCT GCC ATT CCT GA		
2GSP-nested	TCA CTG CCG TCA ATT GCT TG		
AP_XmaI	CCG GAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TV	NA	3'RACE
AUAP XmaI	CCG GAC GCG TCG ACT AGT AC	60	3'RACE
RGA8_51	GTCTTCACCTCGGAAGCTCAAA		5' RACE primers for RGA8
RGA8_52	CGCTGCAACTCGTGGTATAAGA		
RGA8_53	GTTGTGGATCGTCCCTGAAA		
RGA8_54	CAACGATCGCAAACCTCGAT		

Table 7.10: Primers for homologous expression analysis of single RGAs in resistant rose genotypes

Primer	Sequences (5´-3')	Tm °C/ product size	Expression revealed of
Rd1LRR_R	GGA ATT TCA CTT CCA GGA	62/ NA	RGAs 1, 2, 3, 7, 8, 9
Rd1LRR_F	AGG CTT CAA CAA TTG CC		
MSM13-RGA 5 F1	GTAAAACGACGGCCAGTCAGAACTTTCGGCAAAGGAC	60/ 231bp	RGA 5
MS-RGA 5 REV 1	GGGTTTCCTGCATATGAACC		
MSM13-RGA 6 F1	GTAAAACGACGGCCAGTAATTTTGGCTGTCTGGCATC	60/ 201bp	RGA 6
MS-RGA 6 REV 1	ACAAAGACCAAGGGGTTTCC		
MSM13-RGA 2 F1	GTAAAACGACGGCCAGTATTATCGGCTTTTTCGCTCA	60/ 245bp	RGA 2
MS-RGA 2 REV 1	CACTTCCAGGAATTAGAAAATTGA		
MSM13-RGA 7 F1	GTAAAACGACGGCCAGTGGCTTTTTCGCTCAATTCTG	60/ 184bp	RGA 7
MS-RGA 7 REV 1	AAACTCAAAAGAGAGATGGGTTTC		
RGA 1 FWD (04)	TGAGCTCTTGCCTGAGAGTTTG	65 °C for all primer pairs / 80-120bp	RGA 1
RGA 1 REV (04)	GAGTGTGGCTGCTCTCTTATAACAGTT		
RGA 1 FWD (02)	GAAGCTTGGGCAAGGTAATAAGG		RGA 1
RGA 1 REV (02)	GAGACTGGCCGCTTTGGTT		
RGA 3 FWD (01a)	ACATGTAGAAAGTTAGGCCATAGACACT		RGA 3
RGA 3 FWD (01b)	TGCACATGTAGAAAGTTAGGCCATA		
RGA 3 FWD (01c)	TTGCACATGTAGAAAGTTAGGCCATA		RGA 3
RGA 3 REV (01)	AAAATGTGCACAGATCGAGAGAAA		
RGA 8 FWD (03)	TCCTCCTTACAGCGGTTGGA		RGA 8
RGA 8 REV (03)	GCAAATGAATGCTTGCAGGAA		
RGA 8 FWD (01a)	AAGATTGCTTCCTAATTCCTTGAGAT		RGA 8
RGA 8 REV (01)	ACACGGTGGGAGAGATTTTGA		
RGA 9 FWD (02)	GAGCTTATCAGAGAGATTGTGCAAGT		RGA 9
RGA 9 REV (02)	GAGGAACCGAATACTGTGAGACAAG		
RGA 8 FWD (03)	TCCTCCTTACAGCGGTTGGA		RGA 8
RGA 8 REV (03)	GCAAATGAATGCTTGCAGGAA		

Table 7.11: Gel recipes for DNA separation

Gel	Recipes	Purpose
Agarose gel	1 % / 1.5 % / 2 % Agarose in 1 x TAE warm to get a homogenized solution, pour in assembly after adding 0.5 µg/mL EtBr	Separation of DNA fragments on the basis of their size
0.5 x MDE-Gel for SSCP-Analysis (0,4 mm thick)	0.5x MDR-Gel Stammlösung 0.6x TBE 5 % Glycerin 2.2 µM Ammonium persulfat (APS) 0.06 % Tetramethylethylendiamin (TEMED)	Polyacrylamide gel for SSCP
6 % Polyacrylamid Sequence gel (0,25 mm thick)	40 % Acrylamid stock solution (Acrylamid/Bisacrylamid = 19:1) 1x TBE long run buffer 5.8 M Urea Ultrapure 0.03 % (v/v) Dimethyl sulfoxid (DMSO) 2.6 µM Ammonium persulfat (APS) 0.09 % Tetramethlethylendiamin (TEMED)	Polyacrylamide gel for Sequencing or SSRs

Table 7.12: LRR domain representing different options for LRR repeats of RGA 8, 7 and 3.

RGA8-10 LRR repeats following xxLxxLxL pattern.

```
0537  AIEGILLDLAELEEADWNLEAFSKMCKLKLLYIHNLRLSVGPRLLPNSLRFLSWSWYPSK  0596
0597  SLPPCFQPDELAEISLVHSN                                          1
0617  IDHLWNGIKYLVNLKSIDLSYSINLT                                    2
0643  RTPDFTGIPNLEKLVLEGCTN                                         3
0664  LVKIHPSIALLKRLRIWN                                            4
0682  LRNCKSIRS-LPSEVNMEFLETFDVSGCSKLKM
0713  ISEFVMQMKRLSKLYLGGTAVEKL                                      5
0737  PSSIEHLSESLVVLDLSG                                            6
0755  IVIREQPYSRLLKQNLIASSFGLFPRKSPHPLIP                            7
0789  LLASLKHFSCLRTLKLNDCNLCEGE                                     8
0814  IPNDIGSLSSLQRLELRGNNFVSLPASIHLLEDV                            9
0848  DVENCKRLQQLPELPDLPNLCRLR                                     10
0873  ANFWLNCINCLSMVGNQDASYFLYSVLKRWIEIEALSRCDMMIRQETHCSFEYFRFVIPGSEIPEWFNNQSVGD  0945
0946  TVTEKLPWWDACNSKWIGFAVCALIVPHDNPSAVPEKSHLDPDTCCIWCFWNDYGIDVIGVGTNNVKQIVSDHL  1019
1020  YLLVLPSPFRKPENYLEVNFVFKIARAVGSNRGMKVKKCGVRALYEHDTEELISKMNQSKTSSISLYEEAMDEQ  1093
1094  EGAMVKATQEAATSRSGGSDDEYYSAAEE
```

RGA8-10 LRR repeats following xxLxxLxL pattern.

```
--AIEGILLDLAELEEADWNLEAF                                           1
SKMCKLKLLYIHNLRLSVG                                                2
PRLLPNSLRFLSWSWYPSKSLPPC
FQPDELAEISLVHSN
IDHLWNGIKYLVNLKSIDLSYSINLT                                         3
RTPDFTGIPNLEKLVLEGCTN                                              4
LVKIHPSIALLKRLRIWN                                                 5
LRNCKSIRS-LPSEVNMEFLETFDVSGCSKLKM
ISEFVMQMKRLSKLYLGGTAVEKL                                           6
PSSIEHLSESLVVLDLSGIVIRE                                            7
QPYSRLLKQNLIASSFGLFPRKSPHPLIP
LLASLKHFSCLRTLKLNDCNLCEGE                                          8
IPNDIGSLSSLQRLELRGNNFVSLPASIHLLEDV                                 9
DVENCKRLQQLPELPDLPNLCRLR                                          10
ANFWLNCINCLSMVGNQDASYFLYSVLKRWIEIEALSRCDMMIRQETHCSFEYFRFVIPGSEIPEWFNNQSVGD
TVTEKLPWWDACNSKWIGFAVCALIVPHDNPSAVPEKSHLDPDTCCIWCFWNDYGIDVIGVGTNNVKQIVSDHL
YLLVLPSPFRKPENYLEVNFVFKIARAVGSNRGMKVKKCGVRALYEHDTEELISKMNQSKTSSISLYEEAMDEQ
EGAMVKATQEAATSRSGGSDDEYYSAAEE
```

RGA8-11 LRR repeats following LxxLxxLxxLxLxxCxx--LxxxPxx pattern. In the pattern followed here C can be replaced by T or N and the numbers of amino acids following this aa can varies.

```
------------------T
------------------Nx(x)
----LxxLxxLxxLxLxxCxx--LxxxPxx
-----AIEGILLDLAELEEADWNLEAFS                                       1
-KMCKLKLLYIHNLRLSVGPRL-LPNSLRFLSWSWYPSK
SLPPCFQPDELAEISLVHSNIDHLWNGI                                       2
-----KYLVNLKSIDLSYSIN--LTRTPDFT                                    3
------GIPNLEKLVLEGCTN--LVKIHPSIAL                                  4
-------LKRLRIWNLRNCKS--IRSLPSEVNME                                 5
---------FLETFDVSGCSK--LKMISEFVMQ
-------MKRLSKLYLGGTAVEKLPSSIEH                                     6
------LSESLVVLDLSGIVIREQPYSR-                                      7
----------LLKQNLIASSFG-LFPRKSPHPLIP                               8
LLASLKHFSCLRTLKLNDCN---LCEGEIPNDIG                                 9
------SLSSLQRLELRGNNFVSLPASIHLLEDVDVEN                           10
----CKRLQQLPELPDLPNLCR-LRANFWL                                    11
--NCINCLSMVGNQDASYFLYSVLKRWIEIEAL
SRCDMMIRQETHCSFEYFRFVIPGSEIPEWFNNQSVGDTVTEKLPWDACNSKWIGFAVCALIVPHDNPSAVPEKSHL
DPDTCCIWCFWNDYGIDVIGVGTNNVKQIVSDHLYLLVLPSPFRKPENYLEVNFVFKIARAVGSNRGMKVKKCGVRA
LYEHDTEELISKMNQSKTSSISLYEEAMDEQEGAMVKATQEAATSRSGGSDDEYYSAAEE
```

RGA7-11 LRR repeats following LxxLxxLxxLxLxxCxx—LxxxPxx pattern. In the pattern followed here C can be replaced by T or N and the numbers of amino acids following this aa can varies.

```
                  T
                  Nx(x)
----LxxLxxLxxLxLxxCxx---LxxxPxx
536-AIEGIFLHLHKLEEADWNPEAFSKM                                  1
-----CNLKLLYIHNLRLSLGPKFLPDAL                                  2
---RILKWSWYPSKSLPPGFQPDELSFVHSNIDH-LWNG
----IKYLGNLKSIVLSYSIN---LIRTPDFTGI                            3
--------PNLEKLVLEGCtN---LVKIHPSIA                             4
------LLKRLKIWNFRNCKSIKTLPSEVNM                               5
--------EFLETFDVSgCSK---LKMIPEFV
-----GQTKRLSKLCLGGTAVEK-LPSSI                                  6
----EhLSESLVGLDLSGIVIREQPYSL                                   7
---FLKQNVIASsLgLFPRKSHHP
---LIPVLASLKHFSSLKELNDCNLCEGEIPND                              8
----IGSLSSLECLELGGNNFVS-LPAS                                   9
-------IHLLCRLGSINVENCKRLQQLP                                 10
---ELPVSGSLRVTTVNCTSLQVFPELP
--------PDLCRLSAFSLNSVNCLSTIGNQDASFF                          11
LYSVINRLLEVISLSLSLSLSLSLSLSRSL  944 (LRR region ranges between 536- 944 amino acids)
```

RGA3-13 LRR repeats following LxxLxxLxxLxLxxCxx—LxxxPxx pattern. In the pattern followed here C can be replaced by T or N and the numbers of amino acids following this aa can varies.

```
--------------------T
--------------------Nx(x)
-------LxxLxxLxxLxLxxCxx--------LxxxPxx
520--VTEGIFLHLDKLEEADWN---------LEAFSKM                        1
--------CELKLLYIHNLRLSLGPKY-----LPNALKFLKWSWYP                 2
-SKSLPPCFQPDELTELTLVHSNIDH------LWNGKK                         3
---------SLGNLKSIDLSDSIN--------LTRTPDF                       4
--------tgIPSLEKLILEGCIS--------LVKIHPSI                      5
-----------ASLKRLKFWNFRNCKSIKS--LPG                           6
------EVDM-EFLETFDVSGCSK--------LKMIPEFVGQ
----------TKRLSRLCLGGTAVEK------LPSSI                         7
-------EHLSESLVELDLSGIVIREQPYSRFLKQN                          8
---------LIASSFGLFPRKSPHPL------LP-
---LLasLkhfssLRTLKLNDCNLCegE----IPNDI                         9
--------GSLSSLKRLELRGNNFVS------LPAS                         10
----------IHLLSKLTYFGVENcTKLQQ--LP                            11
------aLPVSDYLNVLTNNCTSLQVFPD---PPD                           12
----------LSRLSEFFLDCSNCLSCQDSS                               13
YFLYSVLKRWIEIQVLSRCDMMVHMQETNRRPLEFVDFVIPGSEIPEWFNNQSVGDRVTEKLPSDACNSKWIGFAVCALIVPQDN
PSALLERPFLDPDTYGIECYWNDYGIGFVGLVVP-VK—QFVSDHLWLLVLLSPFRKPENCLEVNFVFEITRAVGNNRGMKVKKCG
VRALYEHDVEELISKMNQSKSSSISLYEEGMDEQEGAMVKAKHEAATSGSGGSDDEYYSAEEE  1124
(LRR region ranges between 520- 1124 amino acids)
```

Note: The LRR repeats motifs for RGA 3, 7 and 8 proteins were identified by conseved domain database at NCBI and then manually aligned to get above patterns.

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Publications

Terefe-Ayana D, **Yasmin A**, Le T L, Kaufmann H, Biber A, Kühr A, Schmidt N, Debener T (2010) In defence of beauty: mining disease resistance genes in roses. (Submitted)

Yasmin A and Debener T (2010) Transient gene expression in rose petals via *Agrobacterium* infiltration. Plant Cell Tissue and Organ Culture. DOI: 10.1007/s11240-010-9728-2

Kaufmann H, Terefe D, **Yasmin A**, Biber A, Kuehr A and Debener T (2010) Cloning and analysis of *Rdr1*, a black spot resistance gene from roses. Acta Hort. (ISHS) 870: 191-196

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Khan S, Zafar Y, **Yasmin A**, and Saeed B (2001) An efficient and economical method of mass multiplication of virus and disease free banana using plant tissue culture techniques. Pakistan Journal of Biological Sciences 4(5):562-563.

Posters presented

Khan S, **Yasmin A**, Saeed B and Tariq S (2000) The exploitation of tissue culture technology for large scale multiplication of virus and disease free plants. International seminar on biotechnology for development Islamabad, Pakistan.

Khan S, **Yasmin A**, and Saeed B (2000) An economical and efficient method of producing disease and virus free banana plants using tissue culture technology. 8th international symposium on natural products chemistry Karachi, Pakistan.

Erklärung zur Dissertation

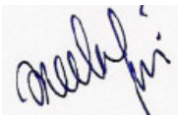
Hierdurch erkläre ich, dass die Dissertation

“Identification and Molecular Characterization of *Rdr1* Resistance Genefrom roses”

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 12. Mai 2010



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(Unterschrift)
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Curriculum vitae

Personal Profile

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Educational Profile

1980-1991	Basic schooling at Pakistan Steel Mills Secondary School, Karachi
1991-1993	College education at Pakistan Steel Mills College, Karachi
1994-1995	B.Sc. (Microbiology) at St. Joseph's College for Women, Karachi
1996-1998	M.Sc. (Biotechnology) at University of Karachi
10/ 2006-present	PhD (Promotion) at University of Hannover, Germany

Professional Profile

1999-2000	Research Officer at HEJ Research Institute of Chemistry, Karachi
2000-2002	Full time Co-operative Teacher at Department of Biotechnology, University of Karachi
2002-2003	Research Officer at PIMA/ RAJA fruits (Pvt) Ltd., Karachi
2003-2010	Lectuer at Department of Biotechnology, Sind Agriculture University, Tandojam
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PhD Studies

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