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Analysis of the genetic background of resistance in potato with special attention to late blight (*P. infestans*) resistance

DOCTOR OF PHILOSOPHY (PhD) THESIS

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ABSTRACT

Potato is the third most important food crop worldwide. However, cultivated potato has many diseases there is a large reservoir of different resistance genes. Sources of resistances are mainly in wild *Solanum* species, which have been partially utilized in breeding. Potato is affected among others by different viruses and possibly the most dangerous disease of it is late blight caused by the oomycota *Phytophthora infestans*. The present study is part of a larger program that aims the detection and characterization of biotic stress response genes of potato for future utilization in breeding. To this end by RNA-sequencing a whole genomic transcriptome (TC) dataset of the cultivar White Lady was generated and different analyses of biotic stress response genes were performed. This cultivar was chosen, since it possesses important resistance genes among others to the PVX and PVY viruses and to *P. infestans*. In the present study we focused on the examination of the genetic background of late blight resistance in this cultivar. By infection tests with different isolates and comparison to the Mastenbroek differential R-lines it was found that White Lady possesses the *R1, R2,* and *R3* race-specific *P. infestans* resistance genes from among the cloned *R*-genes that derive from *S. demissum*. With published sequence specific primers the presence of the *R2, R3a* and *R3b* genes could be revealed, while for the *R1* gene a set of primers based on transcript sequences of the TC analysis were designed here, from among with the R1L333 intron-targeting primer pair a highly similar gene could be detected. It is suggested that this gene is either an allelic version or a homolog of *R1*. For the identification of further late blight resistance genes and homologs potentially present in White Lady the transcriptome dataset was analysed. In total 142 *P. infestans* resistance gene homologs could be identified from among 82 could be used in a phylogenetic analysis. The remaining genes were excluded from the analysis, since their sequence similarity to cloned late blight resistance genes was either too low, or their sequence was too short. In the phylogenetic analysis 21 cloned genes were also used. Results of the analysis revealed that not only *S. demissum* derived *R*-gene homologs are present in White Lady, but also homologs of broad spectrum resistance (*Rpi*) genes of such species which are not present in the genetic background of this cultivar. This indicates the common ancestral origin of *P. infestans* resistance genes in potato and sheds light on their evolution. Further, from the TC dataset 16 genes of four gene families have been chosen which are known to be active in biotic stress response in plants. By quantitation analysis with qPCR for eleven of these genes it was found that they are up-regulated by the *P. infestans* inoculation. The expression of these genes was characterized in seven different time points during the early period of the successful resistance response. Besides all the results of this study contribute to our understanding of the genomic background of biotic stress response in potato, it is believed that these results can be utilized in future development of molecular tools to enhance the effectivity of potato breeding.

KIVONAT

A burgonya a harmadik legfontosabb élelmiszernövény a világban. Noha a burgonyának számos betegsége van, a különféle rezisztenciagének széles repertoár-ja áll rendelkezésre. A rezisztenciaforrások leginkább a vad *Solanum* fajokban fordulnak elő, melyek részben már hasznosításra kerültek a nemesítés során. A burgonyát többek között különféle vírusok támadják meg, és a valószínűleg legveszélyesebb kórokozója a burgonyavészt előidéző petespórás gomba (oomycota), a *Phytophthora infestans*. A jelen tanulmány egy átfogó program része, melynek célja a burgonya biotikus stressz-válasz génjeinek jellemzése és hasznosítása a nemesítésben. E célból, ún. RNS-szekvenálással egy teljes genomi transzkriptom (TC) adatbázist hoztunk létre a White Lady fajtából, és a biotikus stressz-válasz gének különböző vizsgálatát végeztük el. E fajta számos értékes rezisztenciagénnel bír, többek között rezisztens a PVX és PVY vírusokkal, illetve a *P. infestans* hazánkban elterjedt rasszaival szemben. A jelen tanulmányban e fajta burgonyavész rezisztenciájának genetikai hátterét vizsgáltuk. Különböző izolátumokkal történő fertőzésekkel és a Mastenbroek differenciáló R-vonalakhoz történő hasonlítással megállapítottuk, hogy a White Lady az *R1, R2* és *R3* rassz-specifikus *P. infestans* rezisztenciagéneket tartalmazza a *S. demissum* származású klónozott gének közül. Publikált, szekvencia-specifikus primerekkel igazolni tudtuk a *R2, R3a* és *R3b* gének jelenléte, míg az *R1* génre a TC adatbázis alapján különböző primereket terveztünk, melyek segítségével a R1L333 intron-targeting primer pár az *R1* génnel nagyfokú hasonlóságot mutató szekvenciát detektált. Feltételezzük, hogy az a gén egy allélikus verziója vagy egy homológja az *R1* génnek. További lehetséges burgonyavész rezisztenciagének és homológok azonosítása céljából a TC adatbázist elemzésével 142 *P. infestans* rezisztenciagén homológot találtunk, melyek közül 82-t filogenetikai vizsgálatát végeztük el. A többi gént kizártuk a vizsgálatból, mivel vagy a szekvencia-hasonlóságuk volt túl alacsony ismert rezisztenciagénekkel, vagy túl rövidek voltak. A vizsgálatba bevontunk még 21 klónozott *P. infestans* rezisztenciagént. Eredményeink azt mutatják, hogy nemcsak *S. demissum* eredetű *R*-gén homológok vannak jelen a White Lady fajtában, hanem horizontális rezisztenciát biztosító (*Rpi*) gének is olyan fajokból, melyek egyébként nincsenek jelenek e fajta genetikai hátterében. Ez a *P. infestans* rezisztenciagének közös őstől való eredtére utal a burgonyában és fényt vet azok evolúciójára. A továbbiakban a biotikus stressz-válaszban szerepet játszó 4 géncsalád 16 génjét választottuk ki a TC adatbázisból. E gének kifejeződését a fertőzés korai szakaszában hét különböző időpontban vizsgáltuk. E gének közül qPCR vizsgálattal 11 esetében mutattunk ki a *P. infestans* fertőzés hatására bekövetkező aktivációt. A jelen munka eredményei bővítik ismereteinket a burgonya biotikus stressz-válasz genetikai hátteréről, és úgy gondoljuk, hogy eredményeink a továbbiakban hasznosíthatóak a burgonyanemesítés hatékonyságát növelő molekuláris eszközök fejlesztésében.

ABSTRAKT

Kartoffel ist die drittwichtigste Nahrungspflanze weltweit. Jedoch hat Kartoffel viele Krankheiten, der besteht ein großes Reservoir an verschiedenen Resistenzgene. Quellen der Widerstände sind vor allem in der wilden Solanum-Arten, die teilweise in der Zucht verwendet worden sind. Kartoffel wird unter anderem von verschiedenen Viren und möglicherweise von der gefährlichste Krankheit, Krautfäule der durch die Oomycota *Phytophthora infestans* verursacht ist, angegriffen. Die vorliegende Studie ist Teil eines größeren Programms, das die Detektion und Charakterisierung von biotischen Stressantwort Gene der Kartoffel für die zukünftige Nutzung in Zucht anzielt. Zu diesem Zweck wurde durch RNA-Sequenzierung eine ganze genomische Transkriptom (TC) Datensatz von der Sorte White Lady generiert und unterschiedliche Analysen der biotischen Stressantwort-Gene wurde durchgeführt. Diese Sorte wurde gewählt, da es besitzt wichtige Resistenzgene unter anderem zu den PVX und PVY Viren und gegen *P. infestans*. In der vorliegenden Studie haben wir des genetischen Hintergrund der Kraut-und Knollenfäule Widerstand in diesem Sorte untergesucht. Von Infektionstests mit verschiedenen Isolaten und Vergleich mit den Differenz Mastenbroek R Linien wurde festgestellt, daß White Lady besitzt die *R1, R2, R3* und rassenspezifische *P. infestans* Resistenzgene. Mit veröffentlichten Sequenz-spezifischen Primern das Vorliegen der *R2, R3a* und *R3b* Gene wurde erschlossen, während für die *R1*-Gen ein Primer-Set von die Transkript Sequenzen entworfen wurde, und hier aus mit der R1L333 Intron-targeting marker ein hoch ähnliche Gen nachgewiesen werden konnte. Es wird vorgeschlagen, dass dieses Gen entweder eine allele Variante oder ein Homolog von *R1* sei. Für die Identifizierung von weiteren Krautfäule-Resistenz-Gene und Homologen die potentiell in White Lady vorhanden sein sollen, wurde das Transkriptom-Datensatz analysiert. Insgesamt aus 142 *P. infestans* Gen-Homologe 82 konnten in einem phylogenetischen Analyse verwendet werden. Die restlichen Gene wurden aus der Analyse ausgeschlossen, da ihre Sequenzähnlichkeit zu kloniert Krautfäule Resistenzgene entweder zu niedrig oder in ihrer Reihenfolge zu kurz war. In der phylogenetischen Analyse wurden 21 geklonten Gene verwendet. Die Ergebnisse der Analyse zeigten, dass nicht nur *S. demissum* abgeleitet R-Gen-Homologen liegen in White Lady, aber auch Homologen von Breitspektrum-Widerstand (RPI) Gene für solche Arten, die nicht in den genetischen Hintergrund dieser Sorte vorhanden sind. Dies zeigt die gemeinsame Herkunft der Vorfahren der *P. infestans* Resistenzgene in Kartoffeln und wirft Licht auf ihre Entwicklung. Ferner, es wurden aus der TC-Datensatz 16 Gene aus vier Genfamilien ausgewählt, die als in biotische Stressantwort Gene in Pflanzen bekannt sind. Durch quantitative Analyse mit qPCR für elf von dieser Gene es wurde festgestellt, dass sie durch der *P. infestans* Impfung hochreguliert geworden sind. Die Expression dieser Gene wurde in sieben verschiedenen Zeitpunkten während der frühen Periode des erfolgreichen Resistenzreaktion gekennzeichnet. Neben all die Ergebnisse dieser Studie tragen zum Verständnis der genomischen Hintergrund der biotischen Stressantwort in Kartoffel, und es wird angenommen, dass diese Ergebnisse in zukünftigen Entwicklung von molekularen Werkzeugen verwendet werden können, um die Effektivität der Kartoffelzucht zu verbessern.

ABBREVIATIONS

AFLP - Amplified Fragment Length Polymorphism
AHP- Apoplastic hydrophobic protein
Avr - Avirulence gene
BLAST - Basic local alignment search tool
BPB - Brome phenol blue
CAPS - Cleaved amplified polymorphic sequence
CC - Coiled coil domain
4CL-4 coumarate ligase
CDPK- Ca^{2+}-dependent protein kinase
cDNA - Complementary deoxyribonucleic acid
ChIP - chromatin immunoprecipitation
CNL - CC-NB–LRR
CRN- crinkling and necrosis
DGE- Digital gene expression
DNA - Deoxyribonucleic acid
EU- European union
EBN - endosperm balance number
ER - Extreme Resistance
EST- Expressed sequence tag
ETI - Effector triggered immunity
GMO - Genetically modified organism
GSPs - Gene specific primers
GWAS-Genome wide association studies
H0- Null hypothesis
HA- Alternative hypothesis
HR - Hypersensitive reaction
IPTG - Isopropyl β-D-1-thiogalactopyranoside
IT - Intron targeting
JA - Jasmonic acid
LRR - Leucine-rich repeat
MAPK - Mitogen-activated protein kinase
MAS - Marker assisted selection
MPSS- Massively parallel signature sequencing
ML- Maximum likelihood
mRNA - Messenger ribonucleic acid
NADPH-Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NBS - Nucleotide-binding site
NBS-LRR -Nucleotide-binding site leucine reach repeat
NCBI - National Center for Biotechnology Information
NGS - Next generation sequencing
NO^- - Nitric oxide
ORFs- Open reading frames
PAL- phenylalanine ammonia-lyase
PAMPs - Pathogen-associated molecular patterns
PIs- Proteinase inhibitors
PCR - Polymerase chain reaction
Potato-DM - *Solanum tuberosum* group *Phureja* DM1-3 5116R44
PR - Pathogenesis-related genes
PTI - PAMP triggered immunity
Pto - tomato serine-threonine protein kinase
PVX- *Potato virus X*
PVY - *Potato virus Y*
QTL - Qualitative trait loci
qPCR - quantitative- PCR

RBOHs - Respiratory burst oxidase inhibitors
RFLP - Restriction fragment length polymorphism
RNA - Ribonucleic acid
RNA-seq - RNA-sequencing
ROS- Reactive oxygen species
Rpi- *Phytophthora infestans* - Resistance genes
RPKM- Reads per kilobase of exon per million mapped reads
RT-PCR- Real-time polymerase chain reaction
SA - Salicylic acid
SAGE - Serial analysis of gene expression
SAR - Systemic acquired resistance
SCAR - Sequence characterized amplified region
SDS-PAGE- Sodium dodecil sulphate-polyacrylamide gel
SGN- SOL genomics network
StCDPK- *S. tuberosum* calcium-dependent protein kinase
SNP - Single nucleotide polymorphism
SOLiD - Sequencing by oligonucleotide ligation and detection
SSR - Simple sequence repeat
TC- Transcriptome
TDF- Transcripts derived fragment
TE - Tris-HCL, EDTA buffer
TEMED -Tetramethylethylenediamine
TIR - Toll interleukin-1 receptor domain
UK - United Kingdom
USA - United States of America
UHTS- Ultra High-Throughput Sequencing

1. INTRODUCTION

Worldwide, cultivated potato belongs overwhelmingly to *Solanum tuberosum* L. Wild potato species can be found throughout the Americas, but the primary center is the Andean mountain of Peru and Bolivia where about 7000-10,000 years ago potato was domesticated (Spooner et al., 2005). During the domestication process on the Titicaca plateau the Aymara Indians developed more than 200 potato varieties at 3000 to 4600 meters above the sea level (Sleper and Poehlman, 2006). The importance of potato in the societies of the of origin were documented by many representations of potato on ceramic artworks collected from these area (Bamberg and Del Rio, 2005). This crop was unknown to the rest of the world until the 1500's, but afterward its spread was accelerated all over the world so that nowadays it is accounted as one of the most important food crops in the world along with rice and wheat (Haverkort et al., 2009). The tubers of this plant are carbohydrate rich, are a good source of microelements and vitamins, and are highly popular worldwide, prepared and served in very different kinds and methods. Potato is an unrivalled crop among economically important plants, because a diverse pool of wild species with various ploidy levels is at hand which can be utilized in breeding (Carputo and Barone, 2005). Two hundred ninety wild tuber-bearing *Solanum* species were recognized which distributed at wide geographic zones from the southwestern United States to central Argentina and southern Chile (Hawkes, 1990). They have different polyploidity from diploid (2n = 2x = 24) to hexaploid (2n = 6x = 72). Cultivated potato, *S. tuberosum* is a tetraploid (2n = 4x = 48) non-inbred crop species displaying tetrasomic inheritance. To avoid inbreeding depression bred potato should be highly heterozygous, although that complicates the process of improving and makes conventional breeding time consuming.

Potato is vegetatively propagated by tubers. Compared to seeds, with tubers much more diseases and even pests can be transmitted, which then may affect the leaves, stems, roots and the tuber yield. The pathogens which could attack potato belong to different groups

of fungi, oomycota, bacteria, viruses, viroids, phytoplasmas. Besides them also nematodes can be transmitted by tubers and decrease the quantity and quality of yield. Among the pathogens *Phytophthora infestans* that can cause late blight and some viruses like PVY and PVX pose a considerable threat to the crop in potato production areas all around the world. In the twentieth century, shortly after discovery of Mendel's laws of inheritance, a source of genetic resistance to *P. infestans* was discovered in a tuber bearing wild *Solanum* species (Gebhardt and Valkonen, 2001). Afterwards many wild *Solanum* species and accessions of cultivated potato were found to have late blight resistance genes which could be used in classical breeding and in cis-genetic molecular breeding for resistance (Park et al., 2009). In this aspect, localization of traits on the chromosomes, functional characterization of genes and analysis of gene variations have special importance. Nowadays, molecular markers are used as valuable and reliable tools for crop improvement, due to their usefulness in characterizing and mapping genetic loci responsible for monogenic and polygenic resistance traits. The molecular markers can effectively be employed in marker assisted selection (MAS) when they co-segregate with the target gene, they have a high polymorphic resolution, when their use is cost effective, simple and are applicable in high-throughput genotyping systems (Xu et al., 2003; Mohler and Singrün, 2005).

The mechanism of resistance in plants to biotic stresses is complicated and is not completely understood. Several physiological procedures in cells are involved to prevent progression of pathogen invasion locally and systematically through hypersensitive responses which is mediated mostly by major R genes. These R genes encode intracellular nucleotide binding – leucine rich repeat (NB-LRR) molecules which are assumed to regulate the production of biomolecules in signal transduction pathways (Leipe et al., 2004). In order to understand in details the resistance response, it is essential to figure out the role of defensive mechanisms. The quantitative (real-time) PCR technology allows to measure the relative expression level of a particular transcript in a given tissue or cell type and determine the fold change expression of it after being exposed to a specific alteration (Bookout and Mangelsdorf, 2003). More recently transcriptome based analysis of genes and signaling pathways help to better understand biological processes like organogenesis, fertilization or responses to biotic and abiotic

stresses (Yoo and Wendel, 2014). For many years, microarray and serial analysis of gene expression (SAGE) were the primary tools for transcriptome analysis, but recently a promising new ultra high-throughput sequencing (UHTS) technology called next generation sequencing (NGS) with multifunctional purposes was developed. NGS is used for RNA-sequencing (RNA-seq) for assessing the copy number of transcripts and to elucidate more details about any kinds of a transcriptome (Wang et al., 2012). This technique make millions number of reads of genes thereby provide rapid genome-wide expression profiling (Marguerat et al., 2008). In order for screening and selection of the gene homologs which are involved in resistance against *P. infestans,* and for the detection of R-genes with transcript derived markers, a bulked transcriptome analysis of the highly late blight resistant potato cultivar White Lady was performed in the current research. The cumulated dataset obtained by RNA-sequencing was analyzed by different bioinformatics software and stress induced expressional changes of some genes in probable role in stress response to *P. infestans* were examined by qPCR.

Research objectives

The research objectives of the present study are the followings:

1) Exploring race-specific resistance genes to *Phytophthora infestans* in White Lady, a Hungarian potato variety with high late blight resistance.

2) Evaluation of biotic stress induced expressional changes in White Lady by analysis of RNA-sequencing generated transcriptome dataset.

3) Phylogenetic analysis of the *P. infestans* resistance gene homologs of White Lady.

4) Based on the transcriptome data of White Lady, development of intron-targeting (IT) primers for the detection of R-gene homologs.

5) QPCR analysis of the expressional profile of some selected genes known to be involved in biotic stress response.

2. LITERATURE REVIEW

Potato (*Solanum tuberosum* L.) is the third most important food crop in the world after rice and wheat (Haverkort et al., 2009). This crop is rich in carbohydrates, microelements and vitamins, and is highly popular worldwide. Nevertheless, potato is the host of many pathogens, including fungi, bacteria, phytoplasmas, viruses, viroids and nematodes, which cause reductions in yield quantity and quality.

Among the fungal diseases, *Phytophthora infestans* (Mont.) de Bary causing late blight is one of the most important and destructive diseases of potato. In the 1840s it caused the Irish potato famine. Recently, new strains with capability to reproduce sexually are spreading that is associated with increased genetic diversity and survival in many parts of the world (Fry, 2008).

2.1. Potato, an overview on origin, variation and production

The Inca Indians in Peru were the first people who domesticated the potato around 8,000 B.C to 5,000 B.C. After the Spanish conquered the Inca empire, they introduced the potato to Europe in the second half of the 16th century. Since then, it was spread around the world and became as a staple crop in many countries (Hawkes and Francisco-Ortega, 1993).

The genus *Solanum* includes more than 2000 species which is distributed throughout the Americas from the United States to central Argentina and southern Chile (Hijmans and Spooner, 2001). The tuber bearing potatoes are in a range of polyploidy from diploid (2n = 2x = 24) to hexaploid (2n = 6x = 72). The cultivated potato *Solanum tuberosum* L. is a tetraploid (2n = 4x = 48) that displays tetrasomic inheritance and is placed in the series of *tuberosa*. The tetraploid potato (*S. tuberosum*) arose from hybridization of *S. stenotomum* which is domesticated from wild prototype *S. leptophyes,* and a wild diploid species, *S. sparsipilum* (Hawkes, 1988). There are two genetically distinct population groups of *S.*

tuberosum, one is a short-day adapted landrace population of the Andes and the other is long-day adapted of coastal Chile. They have been classified as separate subspecies *S. tuberosum* subsp. *andigena* and *S. tuberosum* subsp. *tuberosum* which are referred to as Andigena and Chilean Tuberosum potatoes respectively (Raker and Spooner, 2002). Although, most of the current potato varieties are derived from Chilean lowland races potato, but root testing of potato varieties and wild species showed that they all from a single origin located in southern Peru and northwest of Bolivia (Innovation, 1989; Spooner et al., 2005).

Nowadays potato germplasm preservation in the world is confined to the countries which have one of the followings specificities: i) enriched sources of genetic variation of potato, ii) technologies of preservation, production of pathogen-free seedlings and seed tubers and improving potato by breeding programs (Kaczmarczyk et al., 2011).

According to the FAO statistics in 2012, the total amount of potato production was 364,808,768 tons from 19,202,082 hectares under cultivation (Fao, 2012). Considering 1990 as a base, it can be concluded that potato production dramatically increased until 2012 with about 98,000,000 tons, while the land used for production increased just with 1,546,000 hectares during this 22 years period. This can be due to progress in knowledge and using of new technologies in the fields of crop management and breeding. In the first decade of the 21st century, an average annual diet of a person was about 33 kg of potato. However, the local importance of potato is extremely variable and rapidly changing. It remains an essential crop in Europe (especially eastern and central Europe), where per capita production is still the highest in the world, but the most rapid expansion over the past few decades has occurred in southern and eastern Asia (Hijmans and Spooner, 2001). China is now the largest potato-producing country in the world with nearly 24 percent of total production (Fao, 2012).

2.1.1. Potato production in Europe

The first report about cultivation of potato outside South America was in the Canary Island in 1567 and soon thereafter it was brought to Spain in 1573 (Hawkes, 1990; Hawkes and Francisco-Ortega, 1993). Afterwards potato was distributed to whole Europe

and subsequently was exported and cultivated in many other parts of the world and therefore potato is referred as a "European" crop (Hawkes and Francisco-Ortega, 1993).

There is a long controversy about the origin of potato in Europe. Juzepchuk and Bukasov (1929) propose that the European potato originally derived from landraces of Chile (Juzepchuk and Bukasov, 1929), while British investigators believed that it came originally from the Andes and persisted until the occurrence of the big European potato late blight epidemic in 1845 (Salaman, 1937; Salaman and Hawkes, 1949), after which it was replaced with Chilean germplasm through introductions and breeding efforts. Chronological studies with a plastid DNA deletion marker on 49 European herbarium specimens of *S. tuberosum* distinguished germplasms originating from the high Andes and from lowland Chile. Results of this study indicated that Andean potato was predominant in Europe in the 1700s, and the Chilean potato was introduced into Europe as early as 1811 and became predominant long before the late blight epidemics in the UK (Ames and Spooner, 2008).

2.1.2 Potato production in Hungary

Potato is the most consumed vegetable in Hungary. Production area in the Hungary dramatically decreased during the last 15 years from 50.000 to 22.000 ha. However, the average yield increased from 16 ton/ha to over 23 ton/ha during this period. After Hungary joined the EU, the seed potato production area also significantly decreased from 1500 ha to 350 ha. The total production reached 511,100 tons while 54,800 tons were only seed potatoes (Fao, 2012) which is less than 1% of EU's total potato production and could just cover the needs of the local market. Out of the total consumption less than 10% is consumed as processed food. The average consumption of potato is approximately 65 kg/year/capita in Hungary. According to FAO's report, in terms of production Hungary is in the 21st position in potato production and has the 23rd position in terms of production area in Europe. Hungarian varieties are produced on twenty percent of the total production area. All of these varieties were developed by the Potato Research Centre (PRC) of the University of Pannonia located in Keszthely which is the only institute dedicated to potato research and breeding in Hungary.

The Potato Research Centre has a more than 50 years long tradition on potato breeding and R&D on production technologies. The Centre due to its consistent resistance-breeding efforts has utilised germplasm partially originating from wild species and developed 12 varieties which are registered also on the EU list (Arany Chipke, Démon, Balatoni rózsa, Katica, Lorett, Góliát, Rioja, Hópehely, White Lady, Vénusz Gold, Luca XL and Kánkán). These varieties due to their complex resistance against major pests and pathogens, high yielding potential and outstanding consumption quality are unique in their kind. Some of them are especially advised for organic production.

2.2. Impact of late blight disease on potato and strategy of control

Undoubtedly late blight, caused by *Phytophthora infestans* is the most destructive disease of potato. The pathogen *P. infestans* belongs taxonomically to the oomycetes. This pathogen first made its impact outside of Mexico in the mid-1840s when severe epidemics swept through North America and Europe and resulted in the Irish potato famine (Large, 1940). Over 160 years later, still it remains a major and complicated threat for potato cultivation despite different strategies for controlling and holding its aggressiveness down in potato cultivation zones. Annual potato crop losses due to late blight are conservatively estimated about $ 6.7 billion worldwide (Haverkort et al., 2008). This pathogen is equipped with genetic changes that can overcome the resistance in potato even though in potato cultivars with high level of partial resistance (Inglis et al., 1996; Tai, 1998).

2.2.1. Genetic diversity of *P. infestans*

P. infestans is heterothallic, requiring two mating types (designated as A1 and A2) for sexual reproduction and the production of oospores. The presence of both mating types allows sexual reproduction that contributes to the formation of resistant oospores in early infections and the adaptation of the pathogen to certain fungicides and also to host resistance. Generally sexual recombination leads to the generation of particularly fit lineages that have new combinations of troublesome traits (Smart and Fry, 2001;

Turkensteen et al., 2008). Another mechanism involved in genetic diversity in the agricultural zone is pathogen migration. This phenomenon appears to define population dynamics of *P. infestans*. Population displacement by genotypes with increased fitness is a recurrent event (Vleeshouwers et al., 2011b).

Before 1980, the worldwide population of *P. infestans* outside Mexico appeared to be asexual and consisted of a single clonal lineage (US-1) of A1 mating type characterized by this single genotype. In contrast, the population in the highlands of Mexico was sexual and consisted of both A1 and A2 mating types which were genotypically highly diverse (Grünwald and Flier, 2005). The global situation was disrupted by at least two different migrations from Mexico in the twentieth century. Due to these events the population genetics of *P. infestans* was dramatically altered and is now recognized as a second wave of introductions (Fry et al., 2009). The first migration of the A2 compatibility type was possibly to Europe and was detected in the early 1980's in Switzerland (Hohl and Iselin, 1984). It is widely believed that new strains migrated within consignments of ware potatoes imported into Europe in the dry summer of 1976 (Niederhauser, 1991). Since European producers sent tubers to many locations throughout the world, the fungal population was widely distributed to South America, North Africa and Asia. The second migration event of the A2 type was from Mexico to the United States and Canada (Lamour and Kamoun, 2009).

2.2.2. Strategies for disease management

Rapid changes in the population of *P. infestans* could be managed by two alternative strategies including application of more fungicides or use of potato cultivars with durable resistance to the pathogen. The second strategy could reduce fungicide applications also and bring less costs of crop production for farmers and less environmental pollutions. Therefore developing resistant cultivars is in the focus of modern breeding programs (Inglis et al., 1996; Peters et al., 1999).

The need for resistant cultivars was clear and an apparent breakthrough came in 1909 when Salaman recognized the Mexican wild species *S. demissum* as a source of extreme resistance that could be backcrossed into *S. tuberosum* (Müller and Black, 1952). Breeding for late blight resistance therefore concentrated on using *S. demissum*'s major

dominant R-genes, of which 11 were identified (Müller and Black, 1952; Malcolmson and Black, 1966; Malcolmson, 1969). Afterwards, some other wild potato species were identified to have one or more genes or allelic variants responsible for late blight resistance.

2.3. Physiological aspects of resistance

There are two important lines at the plant cell level which act as defense barrier against pathogenic organisms. The first is a line of surface-exposed pattern recognition receptors which mediate the recognition of highly conserved microbial molecules called PAMP-triggered immunity (PTI). PAMP stands for pathogen-associated molecular pattern that recognizes different components of the pathogens, like peptides derived from bacterial flagella, elongation factors, conserved secreted proteins from bacteria, fungi or oomycetes, polysaccharides like chitin and beta-glucans (Postel and Kemmerling, 2009). PTI is activated through receptor-like proteins or receptor-like kinases and the recognition is peripherally located on the plant cell surface. This line in plants is thought to be the main mediator of basal immunity against pathogen attack (Jones and Dangl, 2006). To cope with this, pathogens use effectors to block PTI and convert to virulence.

The second line of the defense barrier evolved to recognize effectors of the pathogen thus is called effector-triggered immunity (ETI). If pathogens block the first line and pass through the cell, they encounter ETI. This line is stronger and is more effective against the pathogen and evolved to produce resistance (R) proteins. The majority of *R*-genes contain nucleotide binding site - leucine rich repeat (NBS-LRR) receptors and are able to specifically recognize cytoplasmic effectors of the pathogen.

Although it is generally known that PTI and ETI share many signaling components, it has been proposed that immune responses in ETI occur more quickly, are more prolonged, and are more robust than those in PTI, suggesting that PTI is a weak variant of ETI (Tao et al., 2003; Jones and Dangl, 2006; Tsuda et al., 2009; Tsuda and Katagiri, 2010; Thomma et al., 2011). Typically, the propensity to trigger ETI is pathogen strain or race specific and is associated with a hypersensitive reaction (HR) and systemic acquired resistance (SAR), while PTI is not. Although, it is demonstrated that HR is not

exclusively restricted to ETI but can also occur in PTI responses (Wei et al., 1992; Khatib et al., 2004; Ron and Avni, 2004; Thomma et al., 2011) and PAMP perception may also result in SAR (Mishina and Zeier, 2007). Finally, it should be noticed that accumulating evidence indicates that the separation between PAMPs and effectors, and between pattern recognition receptors and R proteins, and thus also between PTI and ETI, cannot strictly be maintained. Rather, there is a continuum between PTI and ETI (Thomma et al., 2011). The molecular interaction between plant cell and *P. infestans* during their encounter is schematized in Figure 1.

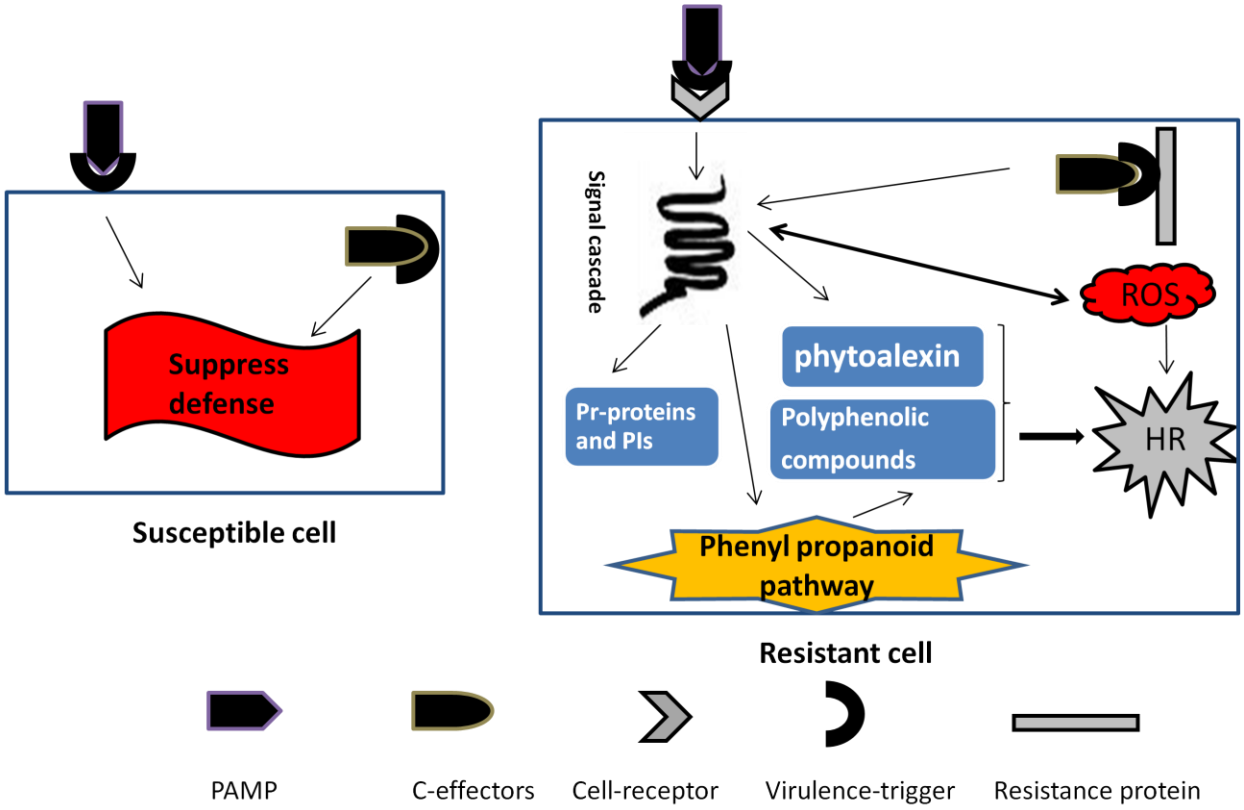

Fig.1. A schematic view of host interaction with *P. infestans.* In a susceptible cell pathogen effectors are not recognized by plant cell receptors and the disease may progress. In a resistant cell different receptors on the cell surface and in cytoplasm (resistance R protein) recognize the avirulence compound of the pathogen that triggers defense response in hypersensitive reaction (HR).

Abbreviations: ROS: Reactive Oxygene Species; HR: Hypersensitive Reaction; Pr-proteins: Pathogenesis related proteins; PIs: Protein Inhibitors; PAMP: Pathogen Associated Molecular Patterns; C-effector: Cytoplasmic effectors.

2.3.1. Recognition of pathogen effectors by R-genes

Research has shown that oomycete plant pathogens, such as *P. infestans*, secrete an arsenal of effector proteins that modulate innate immunity of host and enable parasitic infection (Kamoun, 2007). Although these effectors primarily function as virulence factors, but it is possible that they are recognized by plant R proteins in particular host genotypes resulting in activation of effector-triggered immunity. In such cases, the effectors are said to have an avirulence (*Avr*) activity. In ETI the Avr proteins induced plant response in most cases is a HR, a form of programmed cell death, followed by restriction of the invading pathogen (Jones and Dangl, 2006; Torres, 2010). In the gene-for-gene model (Flor, 1971), the presence of both the *R* gene in plant and the corresponding avirulence (*Avr*) gene from the pathogen results in resistance (incompatible interaction), whereas absence of either the *R* gene or the *Avr* gene results in disease (compatible interaction). In fact, HR is a part of plant innate immunity and its aim is to limit the invading pathogens to the infected area by depriving them from the source of nutrients. Combination between race of pathogen and *R* gene may be an important trigger to switch on some signal transduction pathways for production of defense-associated compounds.

In nature, numerous races of *P. infestans* have evolved which are able to infect plants containing some *R* genes. On the other hand many different resistance genes evolved in potato, thus, late blight resistance proteins account as one of the largest group among devastating pathogens in this crop.

In potato many different types of signal molecules were found which trigger defense responses. In systemic acquired resistance (SAR) signals are transported from the infection site to other parts of the plant to produce molecules designated as components of the defense response pathways. Components which are pronounced in pathogen induced hypersensitive reaction belong to many different groups including reactive oxygen species (ROS), pathogenesis related proteins (PR), proteinase inhibitors and antimicrobial compounds among others (Pieterse et al., 1992; Vleeshouwers et al., 2000a; Yoshioka et al., 2003; Tian et al., 2004; Doke, 2005; Guevara et al., 2005; Fernández et al., 2012).

2.4. Hypersensitive Reaction (HR) mediated defense in *Phytophthora infestans* challenged potato

When potato and the pathogen of the late blight disease come into contact many changes in the metabolisms of the host occur. Molecular crosstalk between *Phytophthora* and plants involves a multitude of signal exchanges. The pathogen produces effectors which are molecules that manipulate host cell structure and function by facilitating infection (virulence factors) or triggering defense responses which is induced by avirulence (Avr) factors or specific elicitors. The Avr molecules induce expression of defense response genes and the production of antimicrobial compounds in host cells. During the initial stages of infection when the pathogen penetrate into the host, Avr factors of incompatible race of the pathogen activate corresponding R genes in the host plant (Flor, 1971; Dangl and Jones, 2001; Collier and Moffett, 2009) and consequently signals transfer from stressed exposed tissue to distal parts. So combination between race of pathogen and R gene may be an important trigger to switch on signal transduction pathways for the production of defense-associated compounds. Production of these compounds leads to the induction of hypersensitive reaction (HR) in which the pathogen is localized around the site of infection and cannot progress anymore. Recently, several candidate signaling molecules have been studied including SA (salicylic acid), JA (jasmonic acid), methyl salicylate, an as yet undefined glycerolipid-derived factor, and a group of peptides that are involved in cell-cell basal defense signaling and systematically acquired resistance (Vlot et al., 2008). Some of these signals induce defense responses in both susceptible and resistant cultivars but others can do it only in resistant ones (Huitema et al., 2004).

These signals are transported in the plant and stimulate the meristems or stems to produce molecules designated as components of the defense response pathways including resistance proteins like pathogenesis related (PR) proteins, proteinase inhibitors, reactive oxygen species, antimicrobial compounds or various other plant molecules involved in the hypersensitive reaction (HR).

Evidence on the process of hypersensitive reaction in plants suggest that in many aspects this is a genetically programmed and active process likewise to apoptosis in animals

(Torres, 2010). This localized response at the site of pathogen attack displays as a programmed cell death and could contribute to limit the spread of the pathogens or be a source of signals for establishment of further defenses (Mur et al., 2008a).

As in animal cells, this process is regulated by proteolytic cleavage with a number of cellular proteins and different protease enzymes are involved. Plants used to apply many similar enzymes and proteins for developing HR after being attacked by pathogens (Fig. 2).

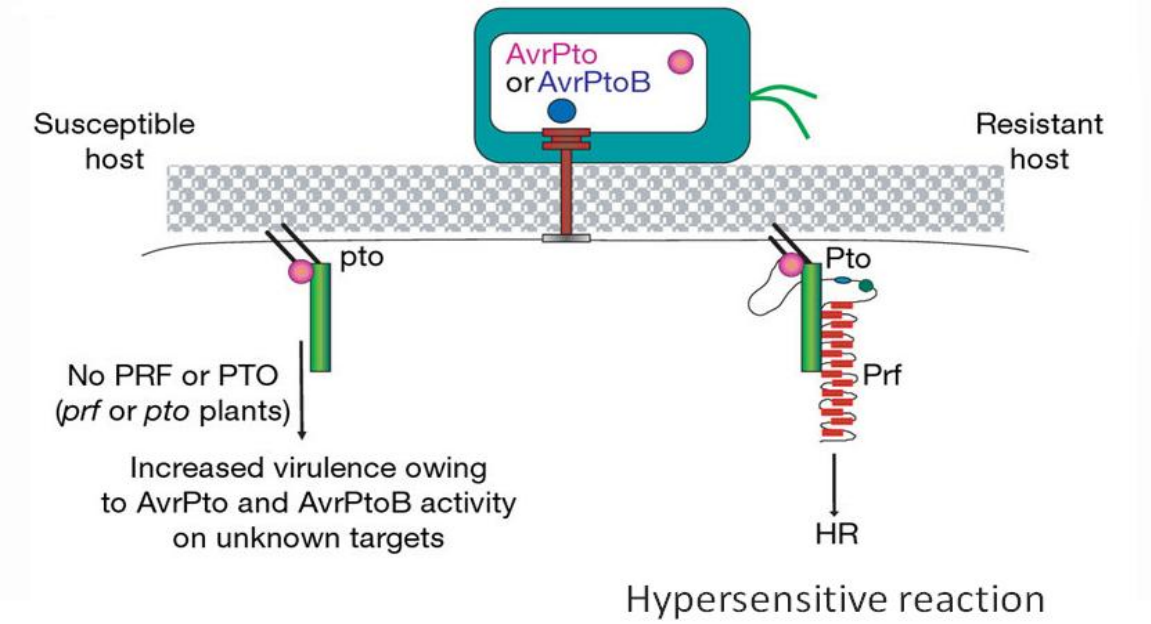

Fig.2. Proteins involved in resistance response in tomato against *P. syringae*. Pto is a tomato serine-threonine protein kinase. Pto is polymorphic and hence satisfies the genetic criteria for the definition of a disease resistance protein. Pto activity requires the NB-LRR protein Prf, and the proteins form a molecular complex. Prf is monomorphic, at least in the tomato species analysed to date. Pto is the direct target of two unrelated *P. syringae* effectors, AvrPto and AvrPtoB, each of which contributes to pathogen virulence in *pto* mutants (Jones and Dangl, 2006).

2.4.1. Role of reactive oxygen species in hypersensitive response to *P. infestans*

To control a large array of biological processes ranging from regulation of development, growth and response to biotic/abiotic stresses plants deploy reactive oxygen species (ROS) like superoxide or hydrogen peroxide (Mittler et al., 2011). In plant-pathogen interactions, ROS molecules are involved in hypersensitive response (Fig. 3) that is a common short term response in which cells surrounding the site of infection either are killed or signaled to undergo programmed cell death, in order to prevent the spread of the pathogen to other parts of the plant (Kiraly et al., 1972; Mur et al., 2008b). ROS generate lipid derivatives by non-enzymatic oxygenation that can produce membrane damage or they are functioning as signaling molecules (Montillet et al., 2005). By acting as signal molecules, ROS can mediate the generation of phytoalexins and secondary metabolites that inhibit further pathogen growth (Thoma et al., 2003). Different types of ROS as derivatives of superoxide including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO) were known to be highly reactive and toxic, and can lead to the oxidative destruction of cells (Mittler et al., 2004).

Several studies have shown that biotic and abiotic stresses are accompanied by an oxidative burst mediated by NADPH oxidases called respiratory burst oxidase homologs (Rboh) (Cazalé et al., 1998; Miura et al., 1998; Fodor et al., 2001; Torres and Dangl, 2005; Suzuki et al., 2011). These molecules are assigned to produce the main source of ROS and are an essential intermediate step in plants to recognize effectors of the pathogen, both in PTI and ETI and other abiotic stimuli, as well as in the activation or amplification of defense responses.

In potato the *S. tuberosum* calcium-dependent protein kinase (*StCDPK5*) has been shown to phosphorylate the N-terminal region of plasma membrane Rboh proteins, and participate in *StrbohB*-mediated reactive oxygen species burst. By transgenic approaches it was proven that the constitutively active form, *StCDPK5VK*, provides resistance to *P. infestans* by ROS production at the infection sites (Kobayashi et al., 2012). Yamamizo et al. (2006) found that in potato the attack of *P. infestans* activates the mitogen-activated protein kinase (MAPK) cascade that induces a large array of defense genes, including the *StrbohC* and *StrbohD* NADPH oxidases. The strong induction of both genes indicates

that *StrbohC* and *StrbohD* may be responsible for the oxidative burst in response to the pathogen attack in the potato leaves and for the resulted hypersensitive response-like phenotype. These results indicate that Rboh-dependent ROS contribute in potato to basal defense against *P. infestans* (Yamamizo et al., 2006).

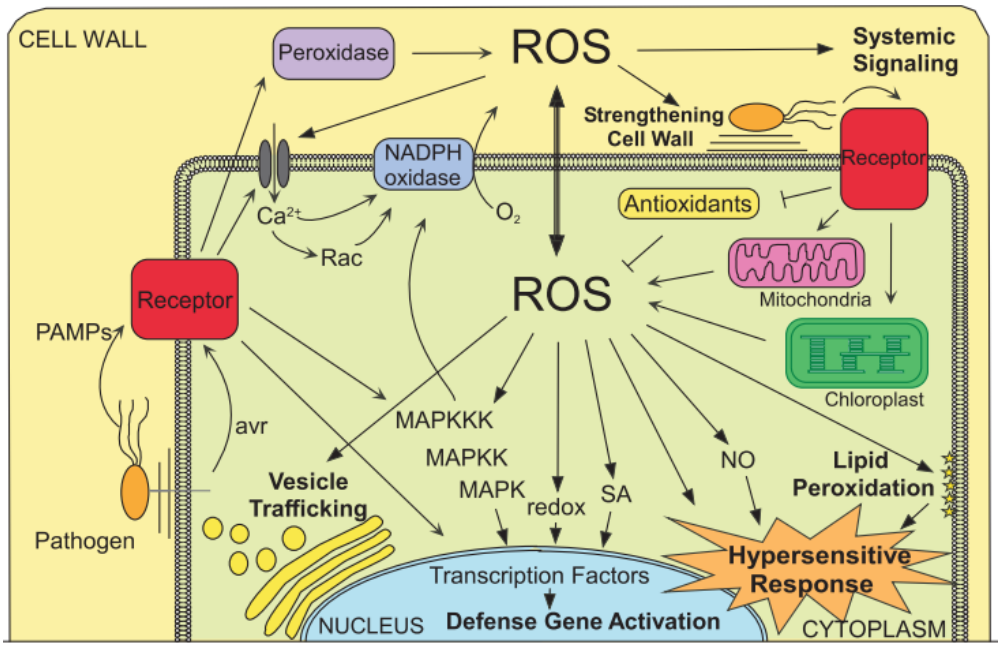

Fig. 3. Pathogen recognition leads to ROS production that has different functions associated to activation of plant defenses. Thin arrows depict signaling events that point to ROS production both in the apoplast and inside the plant cell.

Double-head arrow: indicates the cross talk between ROS in these compartments.

Thick arrows: point to the functions of these ROS in relation to activation of plant defenses.

Blocked end line: indicates inhibitory effect (Torres, 2009).

2.4.2. Proteinaceous compounds as inhibitors to *P. infestans*

Among proteinaceous compounds the pathogenesis related (*PR*) proteins have an important role in plant defense elicited by environmental stress or by developmental stimuli (Edreva, 2005). The PRs which are able to inhibit the growth of pathogens, are divided into 17 subgroups (*PR1-PR17*) based on similarity in amino acid sequence data

and molecular masses (Van Loon and Van Strien, 1999; Okushima et al., 2000; Park et al., 2004). Moreover, some subgroups of PR-proteins are members of multi-gene families, or example up to six members has been detected in *PR-1* gene. The role of many different PR subgroups in resistance to late blight disease was determined and probably more genes of these groups will be identified and functionally characterized in future. (Niderman et al., 1995). They are accumulated locally at the site of infection, and are systematically transferred to the whole plant as a part of systemic-acquired resistance to control further infection. Many research works also indicate that these components are produced constitutively in different plant organs and in seeds, regardless to the stress conditions. These findings suggest a possible role of preformed defense barriers (Vigers et al., 1991; Buchel and Linthorst, 1999). An important common feature of most PRs is their antifungal effect but some PRs exhibited also antibacterial, insecticidal, nematicidal, and antiviral action. PRs target different cell organelles for instance *PR-2, PR-3, PR-4, PR-8* and *PR-11* target the cell wall of the pathogen, *PR-1* and *PR-5* attack the cell membrane, *PR-10, PR-6* and *PR-9* threaten RNA of the pathogen and further up-to-now undefined proteins of it (Gurr and Rushton, 2005). Toxicity of PR proteins could be due to their role in hydrolytic, proteinase-inhibitory, peroxidase activator and permeabilization reactions of membrane metabolisms (Woloshuk et al., 1991; Beerhues and Kombrink, 1994; Niderman et al., 1995; Edreva, 2005). Several studies have shed light on the role of pathogenesis related proteins in the major *R*-gene mediated resistance of potato to *P. infestans.*

Vleeshouwers et al. (2000a) studied if basal *PR* gene expression contributes to non-specific resistance to *P. infestans*. Analyzing the *PR*-1, *PR*-2 and *PR*-5 mRNA levels in 13 wild *Solanum* clones (*Solanum berthaultii*, *S. arnezii x hondelmannii*, *S. circaeifolium* ssp. *circaeifolium*, *S. microdontum*, *S. sucrense*, *S. vernei*, ABPT hybrid, *S. nigrum*) and in five cultivars (Bintje (susceptible), Ehud (*R1* gene), Estima (*R10* gene), Premiere (*R10* gene) and Robijn (multiple *R* genes) they concluded that constitutive expression of *PR* genes may contribute to non-specific resistance to *P. infestans* in *Solanum*, and therefore, *PR* mRNAs could serve as molecular markers in potato breeding programs. (Vleeshouwers et al., 2000a).

In potato members of the *PR-1* family, *PR-1b1* and *PR-1b2* were identified to be involved in *P. infestans* resistance and it is suggested that *PR-1b2* is a homologue of the *PR-1* genes of tomato and tobacco (Evers et al., 2006). Strong accumulation of *PR-1b* mRNA and protein occurs in leaves in response to *P. infestans* infection. *PR-1b* mRNA and protein accumulation is initiated at the infection site, but a delayed and sustained accumulation can also be observed in neighbouring, uninfected leaves of potato plants (Hoegen et al., 2002). Homologs of osmotin as a *PR-5* protein which is inducible by pathogens and osmotic stress in tomato and potato have been suggested to have anti oomycete activity against *P. infestans*, since *in vitro* and transgenic tobacco and potato plants have enhanced resistance against this pathogen (Singh et al., 2013)

Proteinaceous compounds also have a noticeable role in protection of plants against metabolites of microorganisms during infection. Biosynthesis of proteinase inhibitors in response to *P. infestans* was reported initially in tomato and a correlation between increased content of trypsin and chymotrypsin inhibitors and plant resistance to the pathogen was described (Peng and Black, 1976). Different kinds of protease inhibitors including the Kunitz-type protease inhibitor, aspartic protease inhibitor, Kazal-like serine and cysteine protease inhibitors as apoplastic hydrophobic proteins are known to be effective compounds in resistance against *P. infestans* in potato. These inhibitors may play a significant role in the natural defense mechanisms of the potato plant against insect and phytopathogen attack and have a high toxicity toward the pathogen by inhibiting the germination of hyphae and accelerating the destruction of fungal spores (Tian et al., 2004; Guevara et al., 2005; Fernández et al., 2012).

2.4.3. Phytoalexins as anti-fungal compounds produced in potato against *P. infestans*

A heterogeneous group of low molecular mass secondary metabolites with antimicrobial activity that are induced by stress are collectively named phytoalexins (Hammerschmidt, 1999). Phytoalexins are an important part of the plant defense repertoire and are considered as molecular markers of disease resistance (Shinbo et al., 2006; Schmelz et al., 2011). These were first described by Müller and Börger (1939) during studies on *P. infestans* - *S. tuberosum* interactions(Mueller and Börger, 1939). Although, since then, the field has evolved extensively, the biosynthesis of most phytoalexins, the regulatory

networks involved in their induction by biotic and abiotic stress, and the molecular mechanisms behind their cytotoxicity are largely unknown. For most species and cultivars the phytoalexins have yet to be characterized (Ahuja et al., 2012).

The production of phytoalexins is either induced by elicitors of the pathogens or by wounding when plant signal compounds like jasmonates, NO^- and ROS are released. (Pieterse et al., 1992). Hence, phytoalexins are involved not only in the short-term hypersensitive response, but also in the long-term response i.e.: the systemic acquired resistance.

Potatoes produce a number of antinutritional phytoalexins such as sesquiterpenoid compounds including rishitin, phytuberin, lubimin and solavetivone (Metlitskii et al., 1970; Kuc, 1982) or the steroid glycoalkaloids α-solanine and α-chaconine. Nevertheless, besides their beneficial role in plant defense these latter phytoalexins display a certain level of toxicity for humans. (Matthews et al., 2005) Therefore, their production in tubers should be avoided or kept at minimal level.

Here the phenylpropanoid pathway has a central role in rapid browning and hypersensitive cell death during incompatible interaction of potato leaves and tubers with *P. infestans*. Rapid increments of transcription rate of two genes encoding phenylalanine ammonia-lyase (*PAL*) and 4-coumarate: *CoA* ligase (4CL) were detected within a few hours post inoculation with the pathogen (Fritzemeier et al., 1987).

Further anti-fungal compounds which play major role against *P. infestans* infection in potato are phenolic compounds. Scanning-electron microscopy and staining light-microscopy revealed depositions of phenolic compounds as extracellular globules in hypersensitive cells of the epidermis and mesophyll layer in response to infection by the late blight pathogen (Vleeshouwers et al., 2000b).

2.5. Role of NBS-LRR molecules in host defense aginst late blight disease

The broad spectrum *R*-genes against *P. infestans* (*Rpi*) which provide non-race-specific resistance, typically encode immune receptor intracellular plant proteins (Ballvora et al., 2002; Huang et al., 2005; Lokossou et al., 2009; Pel et al., 2009). These proteins belong to the nucleotide binding site - leucine-rich repeat (NBS-LRR) class, and contain two important parts including nucleotide binding (nb) site which is central NB

domain and leucine reach repeat (Lrr-domain) which have a C-terminal (Sacco and Moffett, 2009). More than 50 functional NB-LRR genes have been cloned from potato and related members of the *Solanaceae* (Hein et al., 2009). Recently, based on an amino acid motif based search of the annotated potato genome 438 NB-LRR type genes were identified among about 39,000 potato gene models. Of the predicted genes, 77 contain an N-terminal toll/interleukin 1 receptor (TIR)-like domain, and 107 contain an N-terminal coiled-coil (CC) domain (Jupe et al., 2012).

All homologs of the functionally characterized late blight R resistance genes including R1, R2, Rpi-bt1, Rpi-blb2, Rpi-blb3 and Rpi-vnt1 were CNL (CC-NB LRR) type (Jupe et al., 2012).

2.5.1. Co-evolution of host-pathogen genes in late blight resistance

The genome of several oomycota pathogen including *P. infestans* has been sequenced. The 240 Mbp genome of *P. infestans* is remarkable large in the genus. It is three to fourfold larger than the genome of two other analyzed species, *P. soja* and *P. ramorum*. This increase in the amount of DNA is mainly due to transposons and other repetitive sequences which account for 74% of the *P. infestans* genome. While most gene families are not expanded the RXLR (effectors carrying an N-terminal type signal peptide) and CRN (crinkling and necrosis) effector families which occupy repeat rich regions in the genome that accelerate effector evolution expanded twofold or more in *P. infestans* compared to that other two species (Thines and Kamoun, 2010). The RXLR effectors are secreted by *Phytophthora* species across the haustorial host-pathogen interface and target host proteins as well as cellular processes to enhance susceptibility. CRN proteins are another class of host translocated effectors of *Phytophthora* species and can be found also in other pathogenic oomycetes. CRNs target cytoplasmic host factors and induce death of the host cell. Dynamic evolution by non-allelic homologous recombination and tandem gene duplication characterizes these effector gene families (Lamour and Kamoun, 2009; Hardham and Cahill, 2010; Oliva et al., 2010). Subsequently, the pathogen may evolve to escape of being recognized by *Rpi* genes. This may occur in the host plant with different mechanisms including: i) alteration of binding site of the effectors; ii) by evolution to

overcome host defense; iii) or simply by entire gene deletion (Kamoun, 2006; Whisson et al., 2007; Lamour and Kamoun, 2009; Nowicki et al., 2012). The plant genome may in turn evolve fortuitous compensations that restore recognition of altered effectors (Friedman and Baker, 2007). The *Rpi* genes against *P. infestans* typically encode immune receptor proteins of the coiled coil - nucleotide binding - leucine rich repeat (CC-NB-LRR) class of intracellular plant proteins (Ballvora et al., 2002; Huang et al., 2005; Lokossou et al., 2009; Pel et al., 2009). Recognition of the pathogen effectors occurs in the LRR domain of R proteins. The LRR domain undergoes a higher rate of changes than the other parts of the gene to get the ability of recognition of effectors which may have been lost due to the evolution of the pathogen.

2.5.2. Evolution of different *Rpi* genes in potato

Rpi genes have been proposed to follow either of two distinct evolutionary patterns. Some of them are fast-evolving and others are slow-evolving and are designated as type I or type II *R* genes, respectively (Friedman and Baker, 2007). For both types, sequence exchanges mostly occur between clade members. However, the rate of sequence exchange between paralogs in clades of type I is higher, so they may have higher haplotypic diversity, whereas paralogs in clades of type II show infrequent sequence exchanges and keep orthologous relationships (Friedman and Baker, 2007). One of the most noticeable mechanism involved in rapid evolution of *R* genes is the unequal crossing over which results in local duplications (Kuang et al., 2004; Leister, 2004; McDowell and Simon, 2006).

Frequent sequence exchanges and conserved intron region characterize the *R1* gene, a race specific resistance gene to *P. infestans* originating from the hexaploid *S. demissum* Lindl., that represents a type I gene with fast-evolution and has divergent homologs with typical chimeric structures (Kuang et al., 2005). An interesting finding in *R1* gene clusters is the high rate of sequence exchanges confined to specific regions of this gene, while other regions show a normal pattern of evolution with slower rate of sequence exchanges (Friedman and Baker, 2007).

The *R3 P. infestans* resistance gene also from *S. demissum,* is representing the dynamic evolution of the potato genome regarding co-evolution with *P. infestans*. The genomic region of *R3* is functionally diverse for *P. infestans* resistance. The *R3* locus consists of two distinctly functional *R* genes, the *R3a* and *R3b* (Huang et al., 2004). Even it was shown that there is a large expansion in the *R3a* subfamily, with the capacity to recognize additional elicitors from *P. infestans* (Bos et al., 2006). It is suggested that the *R3* locus might have passed through multiple rounds of gene duplication and diversifying selection to produce new specificities for *P. infestans* resistance (Huang et al., 2004).

Unlike the *R1* and *R3* resistance genes which were classified as type I resistance genes, no obvious sequence exchanges were found among paralogs of the RB gene, a *P. infestans* resistance gene that derives from the diploid species *S. bulbocastanum* (Song et al., 2003). Despite recognition of a large spectrum of *P. infestans* races, this gene shows a clear orthologous relationship in resistant and susceptible haplotypes and its evolutionary pattern is attributed to type II, i.e.: it is a slow-evolving gene. Current models for NB-LRR proteins suggest a dual role for the LRR domain, not only as recognition specificity determinants, but also as repressors of inappropriate nucleotide binding activation (Belkhadir et al., 2004). Furthermore, evolutionary analyses of R proteins have shown selection pressure on several domains within them especially in the LRR region and in the b-strand/b-turn motif of it. It is suggested that this region may undergo co-evolution with the pathogen to establish and maintain recognition of the effectors (Meyers et al., 1998; Ellis et al., 1999; McDowell and Simon, 2006). On the other hand new findings proposed a different theory which implies rather conservation in the LRR domain and more variation in the NBS domain as it was found in some *Rpi* genes. This may bring to mind the existence of a different signaling pathway or additional effectors being recognized by these later type of *Rpi* proteins (Lokossou et al., 2009).

2.6. Characteristics and advantages of major *P. infestans* resistance genes in breeding

Since the organization of genes controlling disease resistance in the potato genome including late blight resistance (R) genes and quantitative trait loci (QTLs) were reviewed (Gebhardt and Valkonen, 2001), significant progress has been achieved at molecular level in mapping, cloning and marker assisted selection (MAS). Wild and primitive cultivated *Solanum* species are often used in potato breeding programs, especially for resistance breeding, because their extensive phenotypic diversity makes them valuable resources for high value traits (McCann et al., 2010). Two types of major R genes were found from wild *Solanum* species. R genes derived from *Solanum demissum* confer race-specific resistance whereas the identical Rpi genes from the wild potato species *Solanum bulbocastanum* confer high levels of resistance to a range of *P. infestans* isolates with complex race structures (Helgeson et al., 1998; Song et al., 2003; van der Vossenet al., 2003).

Currently grown potato cultivars lack adequate late blight tolerance. In earlier bred cultivars disease resistance genes were used which confer immunity only to some strains of the pathogen harboring the corresponding avirulence genes. Specific resistance gene-mediated immunity and also control chemicals are rapidly overcome in the field when new pathogen races arise through mutation, recombination, or migration from elsewhere. (Yamamizo et al., 2006) The most sustainable strategy to protect potato plants from late blight would be the introgression of multiple *R* genes especially broad-spectrum *P. infestans* resistance (*Rpi*) genes into cultivars (Pink and Puddephat, 1999; Jones, 2001; Park et al., 2009; Rietman et al., 2012). In addition to durability, pyramiding of resistance genes has the advantage that multiple *R* genes strongly delay the onset of late blight symptoms in the field (Tan et al., 2010). Allele stacking i.e.: the introgression of different alleles or same alleles of one gene (allele-dosage) and even defeated *R* genes may enhance resistance in multiple condition (Tan et al., 2010).

Sixty-three resistance genes to *P. infestans* have been identified from various *Solanum* species and out of them twenty-seven have already been cloned (Rodewald and Trognitz, 2013). Eleven major resistance genes (*R1-R11*) which derive from the hexaploid wild potato species *S. demissum* Lindl. (Black et al., 1953; Bonde et al., 1959; Malcolmson

and Black, 1966; Malcolmson, 1969) were identified and have been introgressed into cultivated potato (Gebhardt and Valkonen, 2001). These resistance genes are race-specific, thus they provide non-durable resistance and are rapidly overcome by virulent strains of the pathogen (Malcolmson and Black, 1966). From among the *S. demissum* derived *R* genes four have been cloned and analyzed. These are *R1* (Ballvora et al., 2002), *R2* (Li et al., 1998; Park et al., 2005a; Lokossou et al., 2009) *R3a* (El-Kharbotly et al., 1996; Huang et al., 2004; Huang et al., 2005) and *R3b* (Huang et al., 2004; Huang et al., 2005; Li et al., 2011).

Recently, *Rpi* genes were identified in the diploid wild potato species *S. bulbocastanum* Dunal, and some of them were cloned. These cloned genes are the *Rpi-blb1* (Van Der Vossen et al., 2003) also known as *RB* (Song et al., 2003), the *Rpi-blb2* (Vossen et al., 2005), the *Rpi-blb3* (Lokossou et al., 2009) and the *Rpi-bt1* (Oosumi et al., 2009). Another *Rpi* gene which possibly derives from *S. bulbocastanum* is the *Rpi-abpt* (Lokossou et al. 2009), that was isolated from a complex quadruple hybrid of *S. acaule* Bitter. *S. bulbocastanum, S. tuberosum* group Phureja and *S. tuberosum* L. (Park et al. 2005b). Several late blight resistance genes were cloned from *S. stoloniferum* Schltdl (Vleeshouwers et al., 2008), from *S. edinense* Berthault, *S. hjertingii* Hawkes, *S. schenckii* Bitter., (Champouret, 2010) and from *S. venturii* Hawkes & Hjert (Foster et al., 2009). Late blight resistance genes from *S. okadae* Hawkes & Hjert., and *S. mochiquense* Ochoa were isolated and patented. While the *R1-R11* genes which derive from *S. demissum* are race specific *P. infestans* resistance genes, the *Rpi* genes from *S. bulbocastanum* confer horizontal resistance against all known races of the pathogen.

2.6.1. *R* gene homologs

Whole-genome sequencing of the potato (Potato Genome Sequencing Consortium, 2011 –Xu et al., 2011), the Sol Genomics Network database (http://solgenomics.net), transcriptome analyses and data base mining (Vleeshouwers et al., 2011a) indicate that *R* gene homologs are abundant in potato. However, these homologs show high level of sequence similarity to late blight resistance genes, the structure based prediction of the function of these genes is challenging, since homologs proteins often have distinct and

sometimes multiple functions (Alberts, 2002). These homologs may reflect to the fast evolution of *R genes*, hence it would be important to determine the real function of them for potential utilization in marker assisted breeding.

High variation in length and homologue numbers of the *R1* gene was found in three different haplotypes. Structural comparison of these *R1* homologs showed three distinct groups with frequent sequence exchange among them indicating that this gene is under rapid evolution. (Kuang et al., 2005).

For the *R2* gene eleven orthologues from different *Solanum* species have been identified which confer resistance to *P. infestans* (Vleeshouwers et al., 2011b). These are: *R2* from *S. demissum, R2-like* from *S. edinense, Rpi-blb3* from *S. bulbocastanum, Rpi-abpt* from a quadruple hybrid (*S. acaule, S. bulbocastanum, S. tuberosum* group Phureja and *S. tuberosum*), *Rpi-mcd1.1* from *S. microdontum, Rpi-snk1.1* and *Rpi-snk1.2* from *S. schenckii, Rpi-edn1.1* from *S. edinense, Rpi-hjt1.1, Rpi-hjt1.2,* and *Rpi-hjt1.3* from *S. hjertingii*. These *R2* gene homologs derive not only from different potato species but also from different regions of South- and Central-America and show adaptation to their regional *P. infestans* populations, which may have led to distinct recognition spectra and degree of resistance (Lokossou, 2010; Vleeshouwers et al., 2011b). In spite of these differences of the *R2* gene homologs, they are all located in the same region on potato chromosome IV. Structural comparison of the homologs revealed high frequency of sequence exchanges among them (Lokossou et al., 2009). All these findings regarding the *R2* homologs suggest common ancestral origin and evolutionary changes during speciation.

For the *R3* it was found that it is a complex late blight R locus where besides the two resistance genes, *R3a* and *R3b* paralogs are also known (Huang et al., 2004; Bos et al., 2006).

2.6.2. Distribution of *R* gene hot spots in the potato genome

In some cases, loci of *R* genes are conserved across genera and some alleles at these loci maintain similar function and specificity for the same or related pathogen taxa. Several *Rpi* genes are found in clusters of various *R* genes which sometimes co-localize with homologs of unknown function. This clustering is more pronounced when the *R* genes

and homologs of different *Solanaceous* crops like tomato, red pepper and potato are combined on a comprehensive map (Grube et al., 2000).

Several late blight resistance genes and also QTLs are located in resistance gene hotspots, that enables the effective transfer of different resistance characteristics and genes from the donor by marker assisted breeding.

As shown in figure 4, the short arm of potato chromosome IV is a hotspot for resistance, harboring several distinct *R* gene clusters with resistance specificities to different pathogens. Here, the cloned root knot nematode *R* gene Hero (Ganal et al., 1995) is part of an extensive *R* gene cluster that is located distal to *Rpi-blb3*. Also the cyst nematode R locus *Gpa4* (Bradshaw et al., 1998), the virus R locus Ny_{tbr} (Celebi-Toprak et al., 2002), and quantitative trait loci for late blight resistance (Leonards-Schippers et al., 1994b; Oberhagemann et al., 1999; Sandbrink et al., 2000) are located here.

The *R1* gene is located within a hot spot for pathogen resistance on potato chromosome V (Birch et al., 2006). The *Rx2* resistance gene to the potato virus X (PVX) (Ritter et al., 1991; De Jong et al., 1997) and QTLs for root cyst nematodes (Kreike et al., 1994; Rouppe van der Voort et al., 2000), as well as major QTLs for late blight resistance (Leonards-Schippers et al., 1994a; Collins et al., 1999) are localized in this region also.

On potato chromosome VI besides *Rpi-blb2* which is located on distal part of the chromosome QTLs for resistance to *P. infestans* and *Erwinia carotovora* have also been described in the same region (Gebhardt and Valkonen, 2001).

A gene providing high level of late blight resistance was localized on chromosome VII. This gene is the *Rpi-mch1*, originating from *S. michoacanum* (Bitter.) Rydb., which is a natural hybrid of *S. pinnatisectum* Dunal and *S. bulbocastanum*. (Śliwka et al., 2012a).

The potato chromosome IX at the distal end of the long arm harbors two resistant genes against late blight including *Rpi-mochi* and *Rpi-dlc1*. The *Rpi-mochi* locus is close to a QTL *Ph-3* near TG591A marker in *Lycopersicon pimpinellifolium*.

On potato chromosome XI at the *R3* gene cluster most likely also the newly identified *Rpi-Smira1* late blight resistance gene is located on a sister chromatid (Rietman et al. 2012). In the same genomic region with the *R3* cluster also further R genes, the R6 and R7 were mapped (El-Kharbotly et al. 1996).

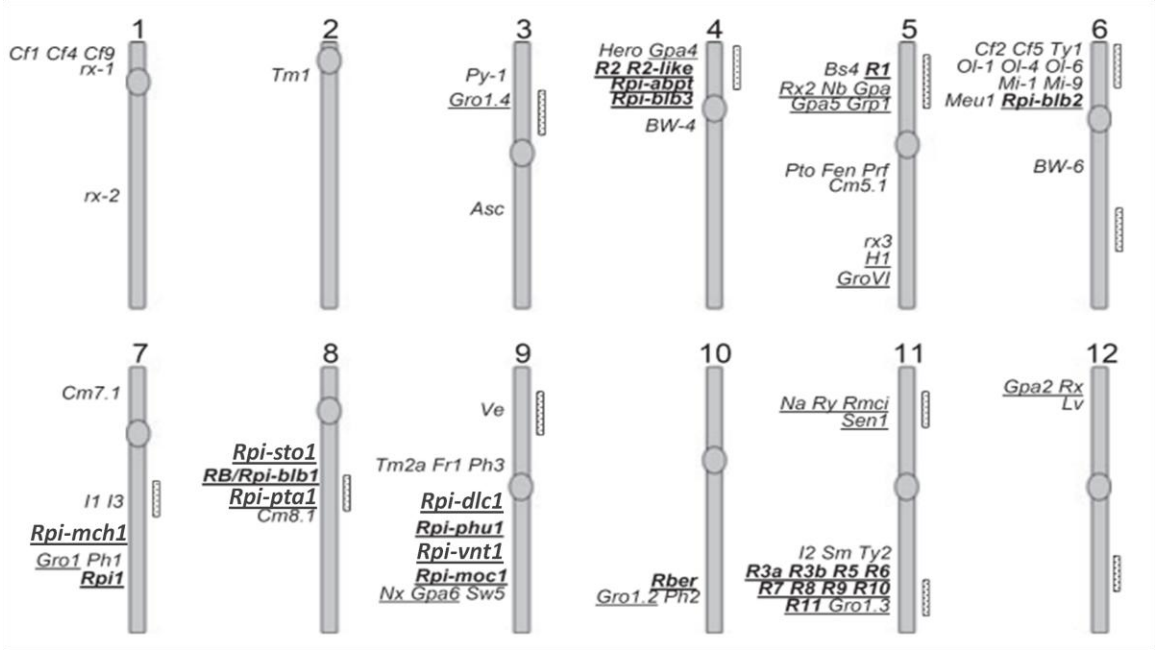

Fig. 4. Potato and tomato map for disease resistance. Position of late blight R genes underlined with solid letters and for QTL with dotted bars at the right of each chromosome (Park et al.,2009).

2.6.3. Utilization of *R* gene resources in breeding programs

Different homologs of some *R*-genes were found in different *Solanum* species and considered as an enriched source of resistance which would be applicable in breeding program to *P. infestans*. However, limited progresses have been achieved in the utilization of *R* genes of wild *Solanum* germplasm in molecular breeding (Park et al., 2009). But newly it got accelerated and many research works on potato breeding goes to exploit this approach to introduce new late blight resistant cultivars armed with R genes derived from wild *Solanum* species (Vleeshouwers et al., 2011b).

By an effectoromics approach Rietman et al., (2012) unraveled the genetic basis of late blights resistance of 'Sarpo Mira'. This Hungarian bred cultivar is one of the few that have been reported to retain resistance in the field for several years and is a candidate for delivering durable late blight resistance (Kim et al., 2012). They found that the resistance is based on the combination of four pyramided qualitative *R* genes and a quantitative *R* gene that was associated with field resistance. The qualitative *R* genes include *R3a*, *R3b*, *R4*, and a newly identified *R-gene*, *Rpi-Smira1*. The quantitative resistance was

determined to be conferred by a novel gene, *Rpi-Smira2*. This later was only detected under field conditions and was associated with responses to the RXLR effector AvrSmira2.

In order to bring the effective potentials of *R* genes, QTLs and other genes involved in resistance response into commercial cultivars through crossing, some manipulations on ploidy level of wild *Solanum* species with due regard to endosperm balance number (EBN) must be done (Bradshaw, 2008). Ploidy level in potato clones of wild species can be enhanced by polyploidization sexually or somatically (Bradeen and Kole, 2011). Unilateral or bilateral sexual polyploidization of wild *Solanum* species and production of haploid genotypes (2n=2x=24) derived from common potato (2n=2x=48) have been successfully used for hybridization of cultivated and wild tuber-bearing *Solanum* species (Muthoni et al., 2014).

Pyramiding individual *Rpi* genes in new cultivars seems to be a promising way to develop late blight resistant cultivars. This could be most effectively achieved by marker assisted selection where inheritance of the individual *R* genes is simply followed by DNA markers. Especially the late blight resistance genes from *S. bulbocastanum* are promising, since during the more than thirty years of their application in breeding programs it was noticed that potato clones harboring the *Rpi-blb2* gene remained resistant to all known races of *P. infestans* and no or hardly any spore-bearing lesion could be observed (Flier et al., 2003).

2.7. Field resistance

Quantitative resistance, the so-called field resistance is assumed to be controlled by more than one single genetic locus and is influenced by environmental conditions (Collins et al., 1999). Quantitative resistance genes often segregate as quantitative trait loci (QTL) in mapping populations of potato (Vleeshouwers et al., 2011b). In this type of resistance the progress of lesion development is slow that substantially decelerates late blight development on the plants. It is characterized with general suppression but not elimination of symptom development, thus it could be considered as rate-reducing or, in some terms, partial resistance, and was effectively used in resistance breeding programs (Song et al., 2003). Unlike to potato varieties containing *S. demissum* derived R genes no

obvious necrotic lesions which are characteristic of the classical hypersensitive response can be found in this type of resistance.

By the end of the 1950s, most potato breeders had switched to use germplasm with partial/quantitative resistance (Hawkes, 1979). However, they were not successful in combining durable resistance against *P. infestans* with early maturing foliage. Resistance was lost when short days induced early maturity (Colon, 1994). Thus, it was suggested that late blight resistance and foliage maturity type are either controlled by closely linked genes or the loss of resistance during foliage maturation is due to the physiological processes of ageing (Colon et al., 1995). The hypothesis of the role of physiological changes during foliage maturation is supported by the influence of photoperiod on resistance to late blight. In most studies it was found that in quantitative late blight resistance there is an epistatic interaction among the QTLs located on different chromosomes (Ewing et al., 2000). Two epistatic QTLs were detected by Visker et al. (2003) for foliage resistance against *P. infestans* on chromosomes III and V and another QTL for foliage maturity also on chromosome V. The foliage resistance and the foliage maturity QTLs on chromosome V were located in indistinguishable position and it was suggested that this is a single gene with pleiotropic effect on both traits. (Visker et al., 2003). Two linkage groups also on potato chromosome V associated with late blight resistance were identified in a tetraploid population, and interestingly one of them was not associated with late maturity. (Bradshaw et al., 2004). By QTL mapping of a full-sib backcross population of potato a region on chromosome IV was also identified that conferred both foliage and tuber resistance (Bradshaw et al., 2006). Park et al. (2005a) identified in a *S. microdontum* derived clone a QTL also on chromosome IV that was associated both with tuber and foliage late blight resistance.

Three QTLs were identified on chromosomes III, V, and XI in a population derived from a cross between two hybrid *S. phureja* x *S. stenotomum* clones, and accounted for 23, 17 and 10% of the total phenotypic variation to late blight resistance (Costanzo et al., 2005). Late blight resistance associated QTLs were identified in the tuber bearing wild potato species *S. paucissectum* Ochoa. on chromosomes X, XI and XII. However, partial reproductive barriers may exist between this wild species and cultivated potato, the few

seeds obtained after crossing indicate it is a potential donor for late blight resistance in potato (Villamon et al., 2005).

Danan et al. (2011) constructed a meta-QTL map of potato by analyzing data of twenty-one QTL maps and eight reference maps. By this approach they succeeded to reduce by six-fold the number of late blight resistance QTLs to 24 meta-QTLs, and by five-fold the maturity QTLs to eight meta-QTLs. Late blight resistance meta-QTLs were localized on each chromosome, while maturity meta-QTLs only on six chromosomes (Danan et al., 2011).

It can be concluded that late blight resistance QTLs are spread throughout the potato genome and only a part of them indicate pleiotropic control with maturity. Late blight resistance QTLs are independent from major *R*-genes, and contain genes involved in physiological pathways.

QTLs contribute to the general resistance against late blight and their introgression into cultivated tetraploid potato cultivars could be realized through marker-assisted selection using the same genotypes for donors in which those QTLs were detected.

2.8. Marker assisted selection in potato resistance breeding

There are two reasons for genetic variation among cultivars of a polyploid plant species like cultivated potato. The first factor is heterozygosity which refers to the presence of more than one allele at a locus, and the other one is allele dosage that means combination of alleles over the four homologous chromosomes.

To achieve high precision in selection of genotypes which harbor the desired *R* genes and QTLs, use of allele-specific primers and closely linked molecular markers is inevitable. Gebhardt et al. (2006) indicated that MAS could be efficiently used in resistance breeding programs. In addition to accuracy, efficiency of molecular markers could increased when multiplex PCR reactions are elaborated which allow the detection of more than one specific sequence/marker in a single reaction (Gebhardt et al., 2006).

Some of the molecular markers like restriction fragment length polymorphism (RFLP) or amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) are usually used for gene-mapping in potato. RFLP and AFLP markers are a reliable molecular technique but they are expensive, the technique is time consuming and laborious. So they are not convenient for marker assisted selection. However, among multilocus markering technologies simple sequence repeat (SSR) technique is an exception. This technique although is long and expensive, but it is frequently used, because of the co-dominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing marker size variations (Maroof et al., 1994). The SSR markers are highly reproducible and can be used in different laboratories with consistent results.

Other molecular techniques including sequence-characterized amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) are simple to use. Sequence-characterized amplified region markers make use of polymorphisms in the primer sites resulting in an absence or presence of an amplified band, whereas CAPS markers make use of a restriction site polymorphism after PCR amplification. Moreover, with the advancement in molecular biology of potato, applying of molecular markers linked to resistance genes in genotype selection has been demonstrated(Gebhardt et al., 2006).

Recently mapping analysis of a *S. demissum* derived *R* gene, the so called *R8* in a tetraploid potato clone was done by using of SSR, CAPS and SCAR markers and showed that it is located on the distal end of the long arm of chromosome IX. Also, a co-segregating CAPS marker was developed, which will be useful for marker assisted selection (Jo et al., 2011).

Many different molecular marker types like RFLP, SSR, AFLP and CAPS also have been used for mapping quantitative trait loci in potato. The most valuable diagnostic DNA-based markers are those derived from polymorphisms in the genes causal for a trait of interest, as such markers are in complete linkage disequilibrium with the quantitative trait alleles (Pajerowska-Mukhtar et al., 2009).

Recently, loci of many *R* genes and QTLs were identified, but most of these genes still remain to be cloned and functionally characterized.

2.8.1. Intron Targeting Markers

The relatively conserved nature of the gene structures in *Solanaceous* plants makes it possible to use intron sequences as molecular markers. This high degree of conservation may be due to the fact that *Solanaceae* genomes have undergone relatively few genomic rearrangements and duplications and therefore have similar gene content and order (Mueller et al., 2005). One effective strategy for exploiting this information and to generating gene-specific co-dominant markers is a method called Intron Targeting (IT). This method was first applied by Choi et al. (2004) to construct a linkage map of the legume *Medicago truncatula* Gaertn. (Choi et al., 2004).

The basic principle of IT relies in the fact that intron sequences are generally less conserved than exons, and they display polymorphism due to length and/or nucleotide variation in their alleles. Expressed sequenced tag (EST)-specific primers and NGS-derived flaking exon primers allow the amplification of genomic DNA across intron regions producing the PCR products that exhibit size or presence/absence polymorphisms (Poczai et al., 2010; Ahmadvand et al., 2014). The basic assumption for this strategy is that introns contain more DNA polymorphisms than exons. Introns, as non-coding regions evolve much faster, than the coding regions (exons). Therefore, intron-targeting strategy of primer design is expected to yield higher polymorphism frequency and therefore has more efficiency than other EST–PCR-based conventional strategies. It is simple to use due to being agarose-based and produces co-dominant markers for potato research and breeding, as well as for genetic diversity analysis in the genus *Solanum* (Poczai et al., 2010).

2.9. Sequencing of transcriptome

Transcriptomic studies are often limited by the number of genes that can be surveyed simultaneously. From the 1990s to early 2000s, many analytical methods were developed for high-throughput profiling of the gene space including differential display, serial

analysis of gene expression (SAGE) microarray, cDNA- amplified fragment length polymorphism (AFLP) and massively parallel signature sequencing (MPSS). Among these tools, hybridization-based microarrays became the dominate platform and has been routinely used to analyze transcriptional changes in many species (Wang et al., 2012). Although serial analysis of gene expression (SAGE) has good capability, but the level of expression was lower than for microarrays.

Another method of transcript profiling is Digital gene expression (DGE) or Digital tag profiling which is qualitatively similar to SAGE analysis however, single transcripts induced identified a 3' end tag by this method. Although DGE method is a vast increasing in throughput by single read of cDNA which accounted as an advantage but it suffers a high difference in generating transcripts library of a population if multiple restriction enzymes are used to generate 3' end tags (Wang et al., 2012). Another disadvantage of both methods in application is producing a large number of small tags which lead to additional cost of DNA sequencing (Nobuta et al., 2010).

Such experiments will be entirely feasible with next-generation technologies, especially with the benefit of low input amount of each type of biomolecule required for a suitable library and the high sensitivity afforded by the sequencing method (Mardis, 2008b).

2.9.1. Next generation sequencing

Many analytical methods were developed for high-throughput sequencing among them a new molecular genetics approach, called next generation sequencing (NGS) is an effective and multipurpose technique. The high throughput, short read NGS systems have been successfully used in several studies for quantitative and qualitative transcriptome analysis in animal, plant and microbial model systems (Cloonan et al., 2008; Marguerat et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Shendure and Ji, 2008) This technology is an ultra-low cost per base of sequencing and has an overwhelmingly high data output. High speed and throughput analyzing methods enable the real-time profiling of whole genome transcripts which may take several years with Sanger technique. The development and application of NGS technologies have greatly facilitated the ability to provide genome sequences of more plant species. Furthermore re-sequencing of entire

plant genomes or transcriptomes is possible by this technology (Varshney et al., 2009). Rather than sequencing individual genomes, it is possible to sequence hundreds or even thousands of related genomes to sample genetic diversity within and between germplasm pools by NGS. However, a high cost for generating the sequence, short read lengths and generating huge amount of output data which need to develop appropriate analyzing software and more efficient computer algorithms are of its limitation (Ansorge,2009)

The NGS technology can have significant implications for crop genetics, genomics, and crop breeding (Varshney et al., 2009). In the latter feature one more point to put forward is breeding for developing plant against biotic and abiotic stresses. The valuable capability of this technique made a wide range of opportunities for the discovery of stress-related genes and pathways that can serve as the foundation for crop improvement during breeding program (Ma et al., 2012). For example, NGS technology can be used for high-throughput transcriptome profiling to investigate genome-wide changes in transcripts in response to stresses (Molina et al., 2011). The DNA–protein interactions which play a key role under the stress condition that underlie this type of regulation of gene expression are frequently determined by chromatin immunoprecipitation (ChIP). More recently, NGS technologies have replaced configure of former ChIP-chip in microarray with so-called ChIP-sequencing, which entails conventional ChIP followed by direct sequencing. Capability of NGS technique in sequencing of Chip-derived DNA bound by a transcription factor of interest is paving the way for whole genome transcriptomics (Wang et al., 2012). In ChIP-seq method, a data of library of the released immunoprecipitated fragments which is made by ligation with an adaptor could be analyzed by bioinformatics software followed by sequencing. This capability provide identification of genome in protein binding sites with exquisite specificity (Mardis, 2008a).

Although NGS technology is still in the early stage of its application, it has proved to be a robust tool for the genome-wide identification of genetic variation. NGS, combined with GWAS (genome-wide association studies), has been used to identify potential molecular markers, such as single nucleotide polymorphisms, insertions and deletions, and copy

number variations, which are associated with growth and development and/or stress responses (Ma et al., 2012).

There are four commercially available NGS technologies: 454 Life Sciences (acquired by Roche), Solexa (acquired by Illumina), ABI SOLID (acquired from Agencourt Biosciences), and Helicos Biosciences. Although all have their specific features, generally they can be grouped into two classes based on the lengths of the sequence reads produced. Solexa, ABI SOLID, and Helicos all produce very short reads in very large quantities, while the 454 platform can produce a more moderate amount of sequence, but with much longer read lengths (Rounsley et al., 2009; Willenbrock et al., 2009).

2.9.2. Gene quantification by NGS technique

Previous studies on gene expression based on high throughput of the transcriptome relied on microarrays and serial analysis of gene expression (SAGE) (Varshney et al., 2009). Analyzing of transcriptional changes in many species has been routinely used by hybridization-based microarrays as a dominate platform (Wang et al., 2012). Recently, however, a digital gene expression as a promising and new platform for assessing the copy number of transcripts by next-generation sequencing has been introduced, thereby providing a digital record of the numerical frequency of a sequence in a sample (Willenbrock et al., 2009). Unlike microarrays, NGS technologies are not limited to sequences of previously known genes because they generate tags without having any knowledge of gene annotation, however they require extensive sequencing and a reference genome to determine gene identity (Varshney et al., 2009).

A unique feature of high-throughput transcriptome sequencing coming by different techniques is the versatility of the data, which can simultaneously be analyzed to provide insight into the structure of genomic loci, sequence variation, exploring of single nucleotide polymorphisms (SNP) present at loci and level of gene expression (Morozova and Marra, 2008).

Quantification of transcripts is achieved by counting the density of the reads that are mapped to the exon regions of a specific gene. The calculation is usually corrected and normalized for transcript length (Wang et al., 2012). Normalization process on transcript reads over gene length and total number of mapped reads could be reflected as reads per kilobase of exon per million mapped reads (RPKM) which is a common output for mRNA expression values (Mortazavi et al., 2008; Fahlgren et al., 2009).

In order to examine transcripts of RNA during gene expression, the small fragments in exon-exon junction should be incorporated in RPKM estimation (Wang et al., 2012). This ability of NGS in which shorter reads of transcripts could be used in RPKM estimation make it more precise and high resolution in its application (Varshney et al., 2009).

2.10. Quantitative analysis by real-time PCR

Characterization of expression pattern of induced genes under various altered condition due to stresses and artificial treatments would be an important goal in order to determine the gene behavior and their role in these situations. Many different methods including RT-PCR, northern blot, southern blot and western blot were used for expression analysis of genes involved in resistance to *P. infestans*. RT-PCR has been more implemented recently due to its high sensitivity for quantification of rare transcripts and small changes in gene expression. Moreover this technique is easy to perform, provides the necessary accuracy and produces reliable as well as rapid quantification results (Pfaffl, 2001). Concerning to RT-PCR, two types of fluorescence methods by using of different reagents including SYBR green and Tag Man have been used for monitoring copy numbers of target genes (Applied Biosystems, life technologies, USA). There are pros and cons to each of the chemistries which is used for quantitative-PCR (qPCR) (Bookout and Mangelsdorf, 2003). Although Tag Man has been considered to be more sensitive, but SYBR Green may have a slight edge in sensitivity at low abundance of transcripts (more than >10 copies) because the reporter dye binds to any double-stranded DNA present in the sample and it is not necessary for PCR cycles to be beyond the range of detection cycle as it is in the Tag Man (Wittwer et al., 1997).

2.10.1. Expressional changes of resistance genes against late blight of potato

P. infestans is a hemibiotrophic pathogen which could parasite the host plant through biotrophic and necrotrophic phase. The pathogen is in biotrophic phase at around 24th hour post inoculation (hpi) in compatible interaction. It progresses into the host cells and turns to highly destructive in the necrotrophic phase at around 46 hpi (Vleeshouwers et al., 2000c).

Quantitation of isolated proteinase K inhibitor in potato was measured between resistant and susceptible cultivars by using of western blot technique and results showed a 19-fold increase of inhibitory activity in resistant cultivar at 24 hours after inoculation. Moreover the activity of the gene in extracts at 48 hours after inoculation was lower than the activity after 24 hours but still remained at a higher level (9-fold) than in control healthy plants and in the susceptible cultivar (Feldman et al., 2000).

Expression pattern of P69 protease genes which is a kazal-like extracellular serine protease inhibitor was studied during infection of tomato by *P. infestans* by Northern blot and semi RT-PCR analyses. Semi-quantitative RT-PCR amplifications using primers specific for P69A, P69B, and P69D showed that P69B was the only gene which was up-regulated during interaction with *P. infestans* and the highest level of expression occurred 2 and 3 days after inoculation (Tian et al., 2004).

Northern blot analyses of RNA from potato leaves were performed on control and infected plants for one of the aspartic proteinases (StAsp) which has an antimicrobial activity. The assay was done on two cultivars, Bintje (susceptible cultivar) and Pampeana (resistant cultivar) to *P. infestans*. Expression analysis revealed accumulation of StAsp mRNA post inoculation in both cultivars. In cv. Pampeana the StAps mRNA level increases at 8, 12 and 24 hpi, while in cv. Bintje the StAps mRNA increases at 8 and 12 hpi with *P. infestans* but then decreased at 24 hpi. The signal intensity of StAsp mRNA levels estimated by densitometry in different treatments and control leaves at 8, 12 and 24 hpi, showed 3.54, 1.6, 1.4 and 3.27 higher fold change in cv. Pampeana than in cv. Bintje (Guevara et al., 2005).

Cysteine protease (cyp) gene is tightly up-regulated in leaves of both *R*-gene mediated and quantitatively high field resistant potato cultivars at 15 hour post inoculation with *P. infestans* (Avrova et al., 1999).

Analyses of apoplastic hydrophobic protein (AHP) type in potato between two different potato cultivars showing resistance and susceptibility to *P. infestans* reveal constitutive differences of AHPs level in these two cultivars, which is in correlation with potato defense response to the pathogen. Different kinds of protease inhibitors including Kunitz type, aspartic and cysteine protease inhibitors were expressed more in resistant cultivar compared to the susceptible (Fernández et al., 2012).

The activation of different metabolites of defense response including *PR-1, PR-5, PAL-1* and HMG-2 against *P. infestans* was shown to be under influence of potato cultivar. These genes were much strongly up-regulated in Kennebec (a moderately resistant cultivar) which carries the *R1* resistance gene than in the highly susceptible Russet Burbank cultivar (Wang et al., 2008). Two members of *PR-1* including *PR-1b1*and *PR-1b2* were isolated from a potato clone of the species *S. phureja* with horizontal resistance to *P. infestans.* Maximal induction of both *PR-1* members was observed on the 2nd day in the resistant parent, while in the susceptible it was on the 4th day after infection. Although sequence alignments of these two members showed some difference but they had a similar expression pattern in both the resistant and the susceptible clones in Northern blot and RT-PCR analyses (Evers et al., 2006).

Existence of PR-proteins in some non-specific resistances to *P. infestans* suggests that these genes may be expressed constitutively in different *Solanum* species and *S. tuberosum* cultivars. It was found that there is a significant positive correlation between PRs mRNA levels and resistance levels of *Solanum* species and cultivars of *S. tuberosum* to *P. infestans* (Vleeshouwers et al., 2000a). Some transcripts derived fragment (TDF) involved in potato-*P. infestans* interaction were analyzed by using of quantitative RT-PCR in two cultivars Sarpo Mira (resistant) and Bintje (susceptible). It was shown that different TDFs including patatine like protein3, Cytochrome p450, peroxidase, chitinase B, ascorbate oxydase, transcriptional factors including heat shock and WRKY were

significantly induced more higher in resistant cultivar during the early stage of infection (Orłowska et al., 2011).

2.10.2. Quantitative analysis of *Rpi* genes in potato

Many studies were done on expression pattern of R genes in compatible and incompatible interaction between potato plants and *P. infestans*. The results of some researches showed that the expression of some R genes can be influenced by host genetic background and environmental factors (Wang et al., 2001; Cao et al., 2007). In order to evaluate the expression pattern of *R3a* and their homologs and I2 gene analogues (*I2GAs*), semi-quantitative RT-PCR was performed on mRNA isolated from *P. infestans* and mock-inoculated leaves of resistant potato clone SH at different time points including 0, 8, 16, 24, 32, 48, and 72 hours post-inoculation. Results showed that all genes were expressed at constitutive level in all treatments (Huang et al., 2005). Relative quantity of a highly resistant gene "*Rpi-phu1"* to late blight was measured by RT-PCR. Expression profile of this gene showed a constitutive low level in a tetraploid breeding line, however transcription of *Rpi-phu1* was under influence of developmental stage and genotypes of potato. So that expression level of this gene was enhanced in diploid line and young age of a tetraploid line after challenging with pathogen (Śliwka et al., 2012b).

Ros et al. (2004) detected two to four fold changes induction of three resistance genes including *R1*, *Rx1* and the *Cf-9* gene cluster in two different time points including 16 and 72 hpi of potato cultivars Indira and Bettina with *P. infestans*. No significant changes in gene induction were found in both cultivars at the early stage of the infection process. Expression of the three resistance genes occurred only at 72 h post-infection in both 2- and 4-week-old plants (Ros et al., 2004).

Transcript level of the RB gene was increased significantly after infection of the wild species *S. bulbocastanum* and different lines of the tetraploid transgenic potato cultivar "Katahdin" with the late blight pathogen. The level of transcription in the wild species was much higher than in the transgenic lines. Level of resistance in transgenic lines was correlated with amount of RB transcript (Kramer et al., 2009).

3. MATERIALS AND METHODS

3.1. Plant material and pathogen isolates

3.1.1 Plant material

The potato cultivar White Lady that possesses high tolerance to *Phytophthora infestans* and extreme resistance to PVX and to PVY has been used in the whole analysis. The late blight susceptible breeding line S440 was used in crossings with White Lady to generate an F_1 population (White Lady x S440) for assessing the efficiency of NGS derived intron-targeting markers. A differential set of Mastenbroek R lines was used for assessing phenotypical reactions of potato to *P. infestans* (Table1).

Table 1. Specification of the used differential set of Mastenbroek R lines

Differential R-line	Cultivar/clone[1]	R-gene content	Cross combination/line[1]	Country of origin[2]	Potato species Origin of R gene
r	**Craig royal**	-	line	United Kingdom	-
R1	**Craigs snow white**	*R1*	CRAIGS DEFIANCE	United Kingdom	*S.demissum*
R2	**1512(16)**	*R2*	Unknown	-	*S.demissum*
R3	**Pentland Ace**	*R3*	CRAIGS DEFIANCE	United Kingdom	*S.demissum*
R4	**1563c(14)**	*R4*	CRAIGS DEFIANCE	United Kingdom	*S.demissum*
R5	**3053-18[3]**	*R1, R2, R3b*	Line	-	*S.demissum*
R6	**XD2-21[3]**	*R1, R2, R3a*	Line	-	*S.demissum*
R7	**218ef(7)[3]**	*R3, R4*	Line	-	*S.demissum*
R8	**2424a(5)**	*R3a, R4, R8*	Line	-	*S.demissum*
R9	**2573(2)[3]**	*R1, R2, R3, R9*	Line	-	*S.demissum*
R10	**3681ad(1)**	*R3b, R10*	Line	-	*S.demissum*
R11	**5008ab(6)**	*R3b, R10*	Line	-	*S.demissum*
R1, R2, R3, R4	**2070(54)**	*R1, R2, R3, R4*	Line	-	*S.demissum*

1,2: Data retrieved from http://www.plantbreeding.wur.nl/potatopedigree, (Pieterse et al., 1992)

3: Presence of additional R genes in these clones, besides their R genes contents

The applicability of some intron-targeting markers developed from NGS transcriptome dataset for identification of R genes derived *S. demissum* was analyzed. Plant materials for this analysis include F_1 population of White Lady X S440 and 11 potato cultivars with known resistance or susceptibility to *P. infestans* (Table 2). Analyses of the reaction of potato all genotypes were done on healthy plants grown in a vector-free greenhouse.

Table 2. Potato cultivars used for IT marker evaluation

Cultivar	Country of origin[1]	Reaction to late blight	Cross combination[2]	Pedigree
White Lady	Hungary	R	KE.40 x 1-71.17/6 N+B	-
Victoria	England	R	AGRIA x ROP J 861	-
Luca XL	Hungary	R	79.60 x CHIEFTAIN	-
Agria	Germany	S	QUARTA x SEMLO	-
Rosita	Germany	R	Unknown	-
Vénusz Gold Gold	Hungary	R	Unknown	-
Desiree	The Netherlands	S	URGENTA x DEPESCHE	-
Sante	The Netherlands	R	Y 66-13-636 x AM 66-42	CPC 1673-20
Russet Burbank	USA	S	BURBANK mutant	Rough purple chili seedling
Démon	Hungary	S	88.635 x KURODA	-
Cara	Irland	R	ULSTER GLADE x A 25/19	-

1,2: Data retrieved from http://www.plantbreeding.wur.nl/potatopedigree

3.1.2. Pathogen isolates

Aggressive isolates of *P. infestans* (collected by István Wolf) with different avirulence genes (*avr*) were maintained in the Potato Research Centre (Table 3.). Also a pathogenic isolate having *Avr* genes and considered as race 0 was used in the experiments.

Table 3. Pathogenicity of the applied *P. infestans* isolates

Late blight isolate	Type of avirulence gene	Races of pathogen	Region
Race0	Avr	Avr1,2,3,4,10,11	Keszthely
H12/10	avr	avr1,3,4,7,10,11	Sarmellek
10/2010	avr	avr1,2,3,4,6,7,10,11	Sarmellek

3.1.3. Preparation of inoculums of *P. infestans*

Culture of the H12/10 isolate was grown for one week on tuber slices of the susceptible potato cv. Hópehely, and then it was suspended and sieved through a cheesecloth. The purified suspension was incubated at 25^{o}C for 1 hour to induce sporangium production. Suspension was maintained at 4^{o}C for 2 hours and at room temperature for 20 minutes to induce zoospore formation. The sporangium concentration was determined with a hemocytometer microscope slide and adjusted to 1.5×10^4 spore/ml in a 50 ml total volume. Artificial inoculation of potato clones and cultivars was done in greenhouse and in the laboratory as described below.

3.1.4. Inoculations in greenhouse

Healthy tubers of cultivar White Lady, S440 and of the Mastenbroek R lines were planted into pots and kept in a greenhouse with max 25^{o}C and min 15^{o}C temperature. Four weeks after planting, the plants were sprayed with the suspension of the pathogen. All plants in the pots were sprayed from the abaxial surface. For maintaining the required humidity for infection, plants were covered by net cloth and sprayed every day.

Potato leaflets were sampled from plants at 7th day after inoculation. Water soaked dark grayish lesion appeared on the foliage of plants were assessed in two grade scale: 0—lack of symptoms or non-sporulating lesions, 1—presence of sporulating lesions (Lebecka and Sobkowiak, 2013). Scale 0 and 1were assigned as resistant and susceptible, respectively.

3.1.5. Detached leaf assay

Preparation of the inoculum was the same as described in point 3.1.3. Fully expanded leaves of plants in the beginning of the flowering stage were used and terminal leaflets were detached for inoculation. After rinsing the leaflets with sterile distilled water, 50 µl of sporangia suspension was dropped to the abaxial surface of the leaves. Leaflets treated with sterile distilled water were used as control. All leaves were incubated in humid plastic chambers in a culture room with 16/8 light/dark period at 21^{o}C. The detached leaves were examined six days after inoculation. The detached leaves were examined six days after inoculation.

3.2. Detection of *R* genes in White Lady by specific primers

Existence of *R*-genes in White Lady was tested with specific published primers (Table 4). Genomic DNA of White Lady, S440, Mastenbroek R line and 24 F_1 genotypes of the WL x S440 cross were isolated using the Walbot & Warren protocol (Walbot and Warren, 1988). PCR was performed in an Eppendorf Mastercycler ep384 (Eppendorf, Germany) thermal cycler. PCR reactions were carried out in a total volume of 12 µl, comprising the following reagents: 1.5 µl of the template DNA (100 ng/µl), 1.2 µl of dNTP (0,2 mM), 0.1 µl Dream Taq Polymerase (5 U/µl) , 1.5 µl 10 x Buffer Dream Taq Green Buffer (Fermentas, Lithuania), 1.2 µl (100pmol/µl) of each of primer. PCR profiles were basically the same as in the references for each gene, although some minor optimization was done to get the appropriate amplicons.

Table 4. Characteristics of the *R*-gene specific primers

Name	Sequence	Tm (°C)	Target gene	Product length (kb)	References
76-2SF2	CACTCGTGACATATCCTCACTA	50	*R1*	1.4	(Ballvora et al., 2002)
76-2SR	CAACCCTGGCATGCCACG				
R2F	ATGGCTGATGCCTTTCTATCATTTGC	55	*R2*	2.5	(Kim *et al*., 2012)
R2R	TCACAACATATAATTCCGCTTC				
SHa-F	ATCGTTGTCATGCTATGAGATTGTT	56	*R3a*	0.982	(Huang et al., 2005)
SHa-R	CTTCAAGGTAGTGGGCAGTATGCTT				
R3bF4	GTCGATGAATGCTATGTTTCTCGAGA	55	*R3b*	0.378	(Rietman, 2011)
R3bR5	ACCAGTTTCTTGCAATTCCAGATTG				
184-81F	RRAGATTCAGCCATKGARATTAAGAAA	55	*R8*	0.500	(Jo et al., 2011)
184-81R	ACTCGATTCTCAACCCGAAAG				

3.3. Bulked analysis of transcriptomes captured in multiple time points by next generation sequencing

In order to get a better understanding about the genetic background of pathogen induced resistance response in White Lady a so called RNA-Sequencing (hereafter RNA-Seq) was carried out by next generation sequencing (NGS).

Since preparative parts, like infection of plants, RNA-extraction and related experiments of this research, as well as the RNA-sequencing and some analysis of data was published by Ahmadvand (2013), here (point 3.3) we just briefly summarize the methodology to be able to understand our analyses and results which are based on the transcriptome dataset generated by this RNA-sequencing.

Next generation sequencing of bulked transcriptomes captured in multiple time points was performed to harvest as much as possible molecular information about biotic stress response in tetraploid potato. Two important potato viruses, PVX and PVY, as well as the worldwide possibly most dangerous pathogen of potato, the *P. infestans* were inoculated in the same way as described in point 3.1.4. on White Lady to trigger the resistance response. Post inoculation mRNA from three replications, for the viruses at eleven and for *P. infestans* at eight time points (Table 5.) was isolated with RNAzol (MRC Inc., USA) and mixed into a single bulk so that each individual sample should be represented with the same amount of mRNA. For control in the same time points and also in three replications mock inoculated (for the viruses with healthy tobacco leaf sap, and for *P. infestans* with sterile distilled water) samples were also collected and the purified mRNA was mixed into a single bulk. These two bulks as treated and control were then used for RNA-sequencing with a Life Tech SOLID RNA Sequencing Kit (Life Technologies, USA) and on a 5500 XL SOLiD (Life Technologies) sequencer. Low-quality and broken sequences were removed. After contig assembling and normalization the fold change and the number of reads per thousand bases per million mapped reads (RPKM) (Mortazavi et al. 2008) was analyzed by CLC Genomics Workbench 4.8 (64 bit) software. The ratio of RPKM-treated/RPKM-control value was applied for fold change with the threshold of $\geq + 2$ and ≤ -2, in treated and control samples and was considered for up- and down-regulated genes, respectively. Contigs were mapped to the potato genome sequence: *Solanum tuberosum* L. group Phureja clone DM1-3 516 R44 (hereafter potato-DM) (Xu et al., 2011) and were annotated.

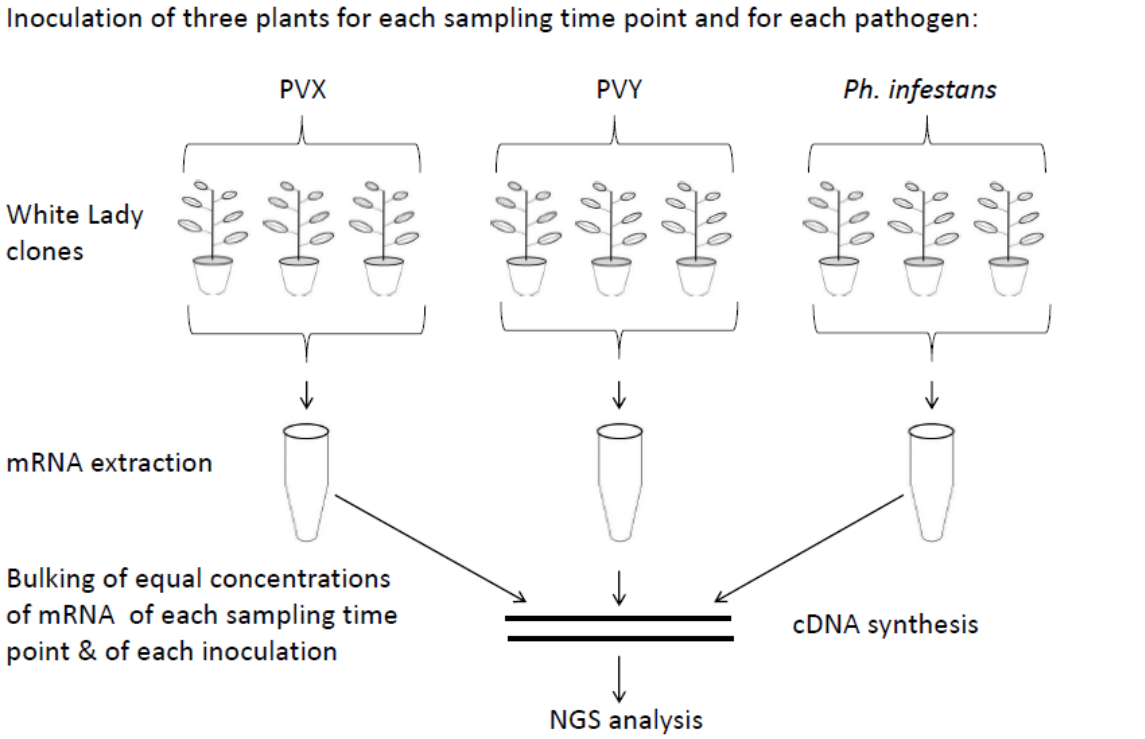

Fig. 5. Experimental design of the bulked analysis of transcriptomes captured in multiple time points.

Table 5. Post inoculation time points when treated and mock inoculated samples were taken

Pathogen	Minute			Hour								Day			Week	
PVX	5	10	30	1	2	4	8	12	-	-	-	2	-	-	1	2
PVY	5	10	30	1	2	4	8	12	-	-	-	2	-	-	1	2
P. infestans	-	-	-	1	-	4	-	-	16	24	30	2	3	6	-	-

It should be emphasized that while important data were expected from this RNA-Seq analysis our main interest was to generate a cumulated dataset for the isolation and subsequent functional analysis of genes with major role in biotic stress response.

3.4. Phylogenetic analysis of *P. infestans* resistance gene homologues

Based on the functional annotation, the TC database was screened for *P. infestans* resistance gene homologs. Selected homologs were identified in the SOL Genomics Network (SGN - http://solgenomics.net/) database, were extracted and blasted in NCBI (National Center for Biotechnology Information, USA). According to arbitrarily chosen thresholds sequences shorter than nine-hundred nucleotides, as well as those with lower than 80% sequence similarity to any of the *P. infestans* resistance genes were excluded from the analysis. After this pre-analysis selected sequences were used for phylogenetic analyses as described in the followings.

3.4.1. Sequence alignment

Multiple sequence assemblages were aligned with MUSCLE (Edgar, 2004) as implemented in the Geneious v.4.8.5 (http://www.geneious.com/) program using default settings. Final datasets were concatenated and formatted to different extension files (FASTA, NEXUS) using the export options of Geneious.

3.4.2. Applied phylogenetic analyzing approaches

3.4.2.1. Parsimony analysis

Phylogenetic analysis with parsimony was performed as an optimality criterion using the program Nona (Goloboff et al., 2008) within a winclada (Nixon, 2002) shell. Five separate analyses (using processor time as a seed to randomize the order of the terminals) were also performed with the following settings: hold 3,000 (holding defined number of trees), 100 replications (search performed with multiple tree-bisection-reconnection algorithm mult*max*), hold/20 (keeping twenty starting trees for each replication). In addition a larger analysis by holding up to 30,000 trees (hold 30,000) but keeping only

two starting trees for each replication (hold/2) was performed too. Jackknife (Farris et al., 1996) support values were calculated using 1,000 replications, with 10 search replications (multi*10) and with one starting tree per replication (hold/1).

3.4.2.2. Maximum Likelihood Analysis

Phylogeny was also inferred using the maximum likelihood (ML) approach implemented in RAxML 7.2.6. (Stamatakis, 2006). All runs were performed with the graphical user interface raxmlGUI 0.93 (Farris et al., 1996). Thorough bootstrap searches (1,000 replicates) were performed under the default general time reversible model of nucleotide substitution with rate heterogeneity following a discrete gamma distribution (GTR+ Γ). RAxML implements only the GTR model and is therefore applied in our analysis. Throughout this paper, 70–84% bootstrap support is considered moderate and 85–100% as strong support. Trees from all analyses were summarized as majority-rule consensus trees and edited with TreeGraph2 (Stöver and Müller, 2010).

3.4.3. Selection test of homologues *P. infestans* resistance genes in TC database

Pairwise alignment of amino acids sequences of the homologues related to different R genes showed that they have undergone more or less multiple variation thought their entire domain. To investigate about the type of diversifying existed in different R gene homologues a selection test was done according to the Kimura model in Mega 5.2 and statistical support were provided for each estimation at probability level of 95% and 99% (Tamura et al., 2011).

The strength of selection was measured for homologues of each gene separately. Amino acid of TC sequences were subjected to the HyPhy program in the Mega 5.2. and numbers of diversed codons in the alignments of TC were measured.

Test of selection on *R* gene homologues in the TC dataset and their correspond known *Rpi* genes was done by using the program to compare the relative abundance of synonymous and nonsynonymous substitutions codons between the gene sequences. For a pair of sequences, this is done by estimating the number of synonymous substitutions

per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN). Null hypothesis (H0: dN = dS) and level of significance in the case of null hypothesis rejection was calculated by using of a Z-test in the program. The level of significance at 5% probability in which the null hypothesis was rejected (alternative hypothesis) and calculation of d_N - d_S determine the type of selection according to the b or c definition as below:

H_0: $d_N = d_S$

HA: (a) $d_N \neq d_S$ (test of neutrality).

(b) $d_N > d_S$ (positive selection).

(c) $d_N < d_S$ (purifying selection).

3.4.4. NBS-LRR motifs in *R* gene homologs alignments

Protein alignments of the *R*-gene homologues were analyzed for the nucleotide binding site (NBS) and leucine rich repeat (LRR) domains. NBS domains were identified with the InterProScan 4 (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and with the Motif-Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) program. LRR domains were identified with the LRR finder program (http://www.lrrfinder.com/lrrfinder.php) and those LRR alignments with significant hits were selected. Protein alignments of TC homologs were screened for the NBS and LRR domains of related R genes. Multiple alignment of these regions of the homologs was done by the Mega 5.2. software.

3.5. Developing of transcriptome-based primers for the identification of *P. infestans* resistance genes and homologs

The biotic stress induced transcriptome (TC) database of White Lady was used for developing of primers from the sequences of *P. infestans* resistance genes and gene homologues. Especially, for the *R1* gene there are many homologues in the TC database which may contribute to the resistance against *P. infestans*.

Three different markering techniques, the intron-targeting, the CAPS (cleaved amplified polymorphic sequence) and the SCAR (sequence characterized amplified region) were applied for primer development. Transcript sequences were extracted with the Tablet

software (Milne et al., 2010) and intron regions of them were determined by the intron finder in the SOL Genomics Network. For those genes where introns identified primers were designed on the adjacent exon regions of the intron using the SIM4 program (Florea et al., 1998). NGS derived SCAR primers were designed for less similar, unique regions of the analyzed sequences. This was true especially for the exon region of *R1* homologs in the TC dataset for which some changes in the site of forward primer were found for the published primer. CAPS primers were designed on the highly similar exon regions of the *R1* homologs. Primer sequences and their characteristics are listed in Table 6.

3.5.1. PCR procedures for the detection of *P. infestans* resistance genes and homologues

Genomic DNA of White Lady, S440, Mastenbroek R1 lines and 24 F_1 population of WL x S440 was extracted using the Walbot & Warren's protocol (Walbot and Warren, 1988). PCR reactions were carried out in a total volume of 12 µl, comprising the followings: 1.5 µl of the template DNA (100 ng/µl), 1.2 µl of dNTP (0.2mM), 0.1 µl Dream Taq Polymerase (5 U/µl), 1.5 µl 10 x Dream Taq Green Buffer (Fermentas, Lithuania) and 1.2 µl (100pmol/µl) of each of primer (Table 6).

For each primer pairs the appropriate annealing temperature was determined by using of gradient PCR on an Eppendorf Mastercycler ep384 (Eppendorf, Germany) thermal cycler (Table 6). All PCR products were separated on 1.5 % agarose gel post stained with ethidium bromide, visualized and analyzed on a Gene Genius (Syngene, UK) gel documentation system.

For the CAPS markers PCR products were digested with restriction endonuclease enzyme *Rsa*I (10 U/µl). The 30 µl reaction mixture (including 18 µl nuclease-free water, 1-2 µl *Rsa*I and 10 µl PCR products) was incubated at 37°C for 14 hours. The enzyme inactivation was done by incubating the mixture at 80°C for 20 minutes. Electrophoresis, staining and gel documentation was performed as described above.

In all experiments electrophoretic pattern was evaluated for the existence/absence of the expected band.

3.5.2. Cloning and sequencing of the amplified fragments

The amplified fragments with the expected size were cloned with a pGEM-T Easy kit (Promega,USA) and plasmids were transformed to JM109 Z-competent cells. Transformed bacteria were cultured in liquid LBA medium for 1.5 hours and 100 µl was spread on solid LBA on which 20µl X-gal (50mg/ml) and 100 µl IPTG (100 mM) was added. White colonies were selected and evaluated by colony PCR for the presence of the expected size band. In colony PCR the M13 plasmid specific primers were used. Colonies with the expected fragments were selected, their plasmid was extracted with the Gene JET Plasmid Miniprep Kit (Thermo Scientific) and prepared for sequencing process with ABI system analyzer 3/10 (ABI PRISM 310 Genetic Analyser, user's manual). Sequences were analyzed in NCBI using the BLASTn function.

Table 6. Characteristics of the transcriptome-based IT primers

Name	Sequence	Tm (°C)	Marker type	enzyme	Target gene (TC)
R1L506-2-F	TCAACTTCATCAACTCGCACTT	56	IT	-	*R1*
R1L506-2-R	CTCAGCAACATATCTACTGTATCACAA				
R1L380-1-F	TCAAAGCAAAGATTCAGGAAAA	55	IT	-	*R1*
R1L380-1-R	TCATTCATCCTCGGAGTCCT				
R1L333-1-F	CCAGAACACAAGGAACAAATAGAA	58	IT	-	*R1*
R1L333-1-R	GCTAGCCTCAATTAAAGCATGA				
RP3a35-1-F	TGAAAATGCTTCACTCCACA	58	IT	-	*RP3a*
RP3a35-1-R	TTGTTCTTTCCGTTTTTCAGTG				
Rp3a80-1-F	TTGGAAATTGATAGTGTAGAGAGTGAA	58	IT	-	*RP3a*
Rp3a80-1-R	CCTCTCTCAGCTGACCATCAT				
Rp3a94-1-F	GGAAGAAGAGCTTCCCTCCT	56	IT	-	*RP3a*
Rp3a94-1-R	GGCAGTGCTGATTCAGAAAG				
R1A4-1F	CCAATACTTTGCCGATCGTCC	57	NGS-caps	RsaI	*R1*
R1A4-1R	TATATCTGGCAGCTGATCTACGC				
R1B23-1F	TAGCCTTCGCAATGAGTACA	57	NGS-caps	RsaI	*R1*
R1B23-1R	GCATCTTCACTTTCTGCGCT				
R1B23-3F	AAGCTGCTCCCCTCTCCTAA	57	SCAR	-	*R1*
R1B23-3R	GCCATCCACGCAAAACCAAT				
R1C3-2F	AAATGCGCTACTGTTCACGC	62	SCAR	-	*R1*
R1C3-2R	CAGTTCCACGGTACAAGGCT				
R1A4-3F	AGCAATGGCAAGTTCCCTCA	58	SCAR	-	*R1*
R1A4-3R	GGCTGACTTGACAACCGACT				

3.6. Quantitative analysis of HR-mediated late blight resistance genes

In order to check the expression level of some genes which may play a role in resistance response to *P. infestans*, biotic stresses induced genes were selected from the

transcriptome dataset and analyzed by qPCR. The criteria for selection were the function and RPKM value of the genes, as well as the type of gene homology in the dataset and sequence homology of these transcripts in NCBI and in SOL Genomics Network. Quantitative analysis of the genes was performed by real-time PCR (RT-PCR) by comparing the expression of the genes between the treated to and mock inoculated control plants. White Lady plants, grown from pathogen-free explants were inoculated with the H12/10 and 10/2010 isolates. The inoculation was done according to the method mentioned in section 3.1.4. Samples for mRNA isolation were collected at eight time points i.e. at 1, 4, 17, 24, 35, 48 and 65 hours post inoculation (hpi) then they were frozen in liquid nitrogen immediately. Samples from mock inoculated plants were collected at the same time points. Isolation of mRNA was done with RNAzol (MRC Inc., USA) according to the recommendations of the producer. The transcribed sequences of selected genes were used for designing specific RT-primer pairs by the primer express software 3 (Life Technologies, USA). The quantitative evaluation was done with a StepOne Real-Time PCR Systems (Life Technologies, USA) machine. Gene expression in this system is analyzed on the basis of monitoring of thermal cycling with a fluorescent chemical reagent, the so called SYBR green binding dye, which is incorporated into the newly amplified DNA fragments (Shepherd et al., 2009). The changes in fluorescence during PCR-reaction was measured by the system and after running the experiment, the output data was analyzed by the StepOne software v2.3 (Life Technologies, USA).

3.6. 1. Preparation of RNA for qPCR

For qPCR analysis mRNA was isolated as above, and was either kept at -80°C until reverse transcription and synthesis of complementary DNA (cDNA) or was immediately used for these processes.

3.6. 2. cDNA synthesis by reverse transcription reaction

In order to quantify the gene expressional changes induced by *P. infestans*, a two-step reaction was used. That involves two separate reactions including the reverse transcription of a poly(A)+ RNA into cDNA and the PCR procedure. The reaction was carried out with High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor

kit, according to the protocol of the manufacturer (life Technologies, USA). This first-strand cDNA synthesis reaction was primed with random primers, oligo(dT), or gene-specific primers (GSPs), but to give an equal representation of all targets in real-time PCR applications and to avoid the 3' bias of oligo (dT) primers as it is advised, random primers were utilized to sythesize cDNA from mRNA by using the Multiscribe Reverse reverse transcriptase in the tests (Life Technologies).

Procedure of reverse transcription was carried out as follows:

1. The reagents of reverse transcription mixture were prepared according to the manual of the 2X RT master mix (High-Capacity cDNA Reverse Transcription Kit) and adjusted the final volume to 10 µl. For prevention of any kinds of RNase contamination of the mastermix, 1 µl RNase inhibitor was added to the mixture.

2. 10 µl of the purified mRNA was added to the mastermix, thus the total volume became 20 µl. The final concentration of mRNA was 1 ng/µl.

3. Pipetting up and down and briefly centrifuge to spin down the content and to remove any bubbles from the reaction solution.

4. Loading the tubes on the thermo-cycler with the following program:

Table 6. Program for reverse transcription of mRNA

Temp. / Time	Step1	Step2	Step3
Temperature	25°C	37°C	85°C	4°C
Time	10min	120min	5min	∞

The cDNA was either directly used for qPCR experiment, or it was store at -20°C.

3.6.3. Designing the RT-PCR primer pairs

Primer pairs for RT-PCR were designed using of Primer Express software version 3.0. (Life Technologies, USA). After uploading the file of gene sequences saved from transcriptome dataset to the software with the finding primer option, candidate primers

and probes were outputted by the program, including some information about the primers, probes and amplicons. Those primers pairs were selected that had a low penalty score and low amplicon length.

3.6.4. QPCR reaction

The quantitative real-time PCR analyses of the genes were performed using the StepOne Real-Time PCR Systems (Life Technologies, USA). For detection of the products the power SYBR green PCR master mix (life Technologies, USA) was used. The master mix contains reagents and enzyme including the fluorescence SYBR green as a reporter dye, highly purified AmpliTaq Gold DNA polymerase, and a proprietary version of ROX dye as passive dye. Besides, RT-primer pairs of the target gene and first-strand cDNA of the transcript as template were added to the PCR master mix according to setup section of the experiment.

The process of qPCR experiment with the real-time machine was carried out in 3 steps:

1. **Setup the experiment:**

   The quantitation type of experiments were set up in the 3-color, 48-well system with comparative CT (ΔΔCT) method. The reagent SYBR green dye was used for fluorescence signaling and cDNA as template. Two targets including a housekeeping gene (ß-tubulin) and the gene of interest were quantified in the range of samples with three replications for each sample. Endogenous control was the housekeeping gene and the reference sample was untreated (control) sample. The qPCR reaction mix contained the followings:

   - 0.45µL of each primer (0.5 µM final concentration)
   - 2.1 µL of cDNA (final concentration = 1 ng/µl)
   - 10 µL of power SYBR green master mix.
   - 7.45 µL of molecular biology water (AccuGENE, Belgium)

2. **Run the experiment:**

   The PCR reaction mixture was loaded on a MicroAmp Fast Optical 48-Well reaction plate (life Technologies, USA), then the plate was sealed with a

Microamp Fast Optical 48-Well adhesive film (Life Technologies, USA) and centrifuged at 400.

For obataining the optimal results, the standard ramp speed (two-hour time) was selected for running the system. The cycling program was as follows:

95°C for 10 min in holding stage, 40 cycles of 95°C for 30s, annealing at the temperature for each primer pairs for 60 s, 95°C for 15 s in cycling stage and 95°C for 15 s, 60°C for 1min and +3 °C in step and hold to 95°C for 15 s during melting curve stage.

3. **Data analysis:**

The comparative Ct method (or ΔCt) was used to assess relative changes in mRNA levels between two or more samples in RT-PCR. The StepOne software v2.3 measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. With calculation of the ΔCt in treatments and control, the software determines the relative quantity of the target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample (Applied Biosystems, life Technologies, USA).

3.6.5. Gel electrophoresis, cloning and sequencing of qPCR amplified fragments

In order to checking the qPCR amplified fragments whether they are at the expected size or not, they were separated on 2% agarose gel at 200 mA direct current, then stained with ethidium bromide and analyzed on a GeneGenius (Syngene, UK) gel documentation system. Cloning and sequencing was performed as described in point 3.5.2.

Alignment and analysis of the amplified qPCR sequences was done with the Mega 5.2 software (Tamura et al., 2011).

3.7. Analysis of the effect of *P. infestans* inoculation on the protein profile of White Lady leaves

The protein profile of treated (*P. infestans* inoculated) and mock inoculated White Lady leaves were analyzed by the SDS-PAGE method. Total extracted proteins were loaded and run on polyacrylamide gel. After de-staining with Methanole-Acetic acid 7%, gels were dried in a gel-drier and the protein profile was scanned.

The protocol of Griga et al. was applied (Griga et al., 2007) with some modification as follows:

- Homogenizing of leaf tissue at a ratio of 100 mg weight to 1 ml extraction buffer with 100 Mm Tris-HCL (PH 6.8) containing SDS 2% and 20% sucrose which was added during mixing
- Sample mixtures were transferred to eppendorf tubes and put on a water bath with 70°C for 5 minutes
- Centrifugation of homogenized samples at 14000 rpm for 15 minutes
- Transferring the 1 ml supernatants in a 1.5 Eppendorf tube and incubating at -20°C for overnight
- Applying of the Acrylamide-Bisacrylamide gel on a vertical cassette and keep it until solidification
- Loading the 60 µl samples + 30 µl dye (BPB+ Mercaptoethanol) per well
- Running the gel with constant current 27 mA until the tracking dye reached the bottom of the gel
- Staining the gel in 0.25% Coomassie Brilliant Blue solution for overnight

The Acryl amide-Bisacrylamide gel was composed of 7.5 ml Tris buffer 0.5 M, 300 µl SDS 10%, 12ml Acrylamaide-bis, 15ml TEMED, 150 µl Aps10% and 10ml H2O. The concentration of protein samples in infected and mock inoculated plant was measured by Nanodrop at A280 nm. The final concentration was determined and adjusted to 1 µg/µl, and then electrophoresis was done with equal volume for each sample

4. Results

4.1. The *R* gene content of White Lady according to inoculation tests

Phenotypic reaction of Mastenbroek differential R lines by isolates of *P. infestans* showed a combination of virulence genes in the genetic composition of isolates H12/10, H10/2010 and WL1 (Table 7). Applying four different isolates with different avirulence gene content in inoculation tests indicated that at least 8 genes, *R1, R2, R3, R4, R6, R7, R10* and *R11* may be present in the genetic background of White Lady (Table 7). The Mastenbroek differential set of R lines was used to assure the avirulence gene content of the isolates. Phenotypic reaction of all R lines was as expected. Frequency of avr*R5*, avr*R8* was low and avr*R9* was not at all present in any isolate of *P. infestans* collected from different locations in Hungary. This may be due to the fact that these avirulence genes are rare in Hungary.

Table 7. Reaction of the Mastenbroek R lines and White Lady (WL) to different *P. infestans* isolates

Isolate	Avirulence gene	Mastenbroek differential R line												WL
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R1,2,3,4	
H12/10	avr1,3,4,7,10,11	+		+	+			+			+	+	-	-
Race 0	AVR1,2,3,4,10,11	-	-	-	-						-	-	-	-
H10/2010	avr1,2,3,4,6,7,10,11	+	+	+	+		+	+			+	+	+	-
WL1*	avr1,2,3,4,5,6,7,10,11	+	+	+	+	+	+	+			+	+	+	+

+: compatible interaction

-: incompatible interaction

***: the results of reaction test on R line and White Lady with this isolate was provided by Istvan Wolf and included in the table for comparison**

4.2. Identification of resistance genes to *P. infestans*

4.2.1. Detection of *P. infestans* major resistance genes by molecular markers

Among the *R* genes, that derive from *S. demissum R1, R2, R3*a and *R3b* have already been cloned (Ballvora et al. 2002, Lokossou et al 2009, Huang et al. 2005, Li et al. 2011) and their presence in White Lady was investigated here with published gene-specific primer pairs.

While the expected products were obtained for the *R2, R3a* and *R3b* genes, the *R1* gene could not be amplified (Fig. 6, 7). The PCR products of each gene were cloned and two clones from each were sequenced. Sequences of each gene were assembled with the Codon Code Aligner program and a contig was constructed for each gene and analyzed in the NCBI database.

With the *R2* specific primers a 2,500 bp contig could be created that showed 97% identity with the *R2* gene in the NCBI. With the *R3a* and *R3b* primers the sequence similarity of the two 1,000 bp and 380 bp size contigs were 98% and 99% in NCBI, respectively (Table 8).

However, inoculation experiments clearly indicated the presence of the *R1* gene in White Lady, this gene could not be amplified by the published (Ballvora et al. 2002) 76-2S specific primers. As shown in Figure 2 with this primer pair the gene could be amplified in the differential *R1* line. Hence, it is suggested that sequence differences between the primers and one or both priming sites could be the reason that no products were obtained with this primer pair.

The primer which is used for R8 gene is for mapping of the genes since this gene wasn't cloned yet. PCR experiment with this anchor marker failed to amplify the fragment containing this gene in the White Lady, however it is not clear that this gene is absent in this cultivar.

Table 8. Similarity of *R*-genes with White Lady contigs of the same loci

Fragment	Primers	Matched homologs in TC	Contig length	Similarity
R2	R2F,R	R2	2500 bp	97%
R3a	SHa-F,R	R3a	1000 bp	98%
R3b	R3bF4R5	R3b	380 bp	99%

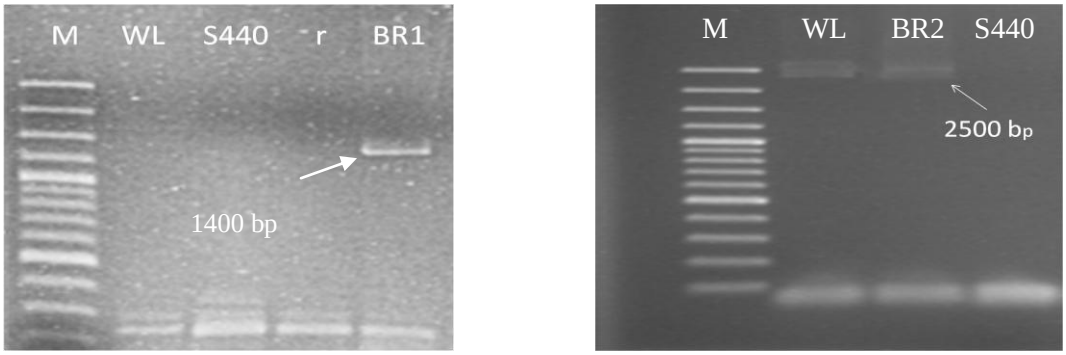

Fig. 6. Results of fragment amplifications with the *R1* specific primer pair 76-2S (left) and with the *R2* specific primer pair R2 (right)

M: 100 bp molecular ladder; WL: White Lady; S440: breeding line; r: Mastenbroek line with recessive allele; BR1, 2: Mastenbroek R-line with dominant allele. The arrow indicates the expected and cloned band.

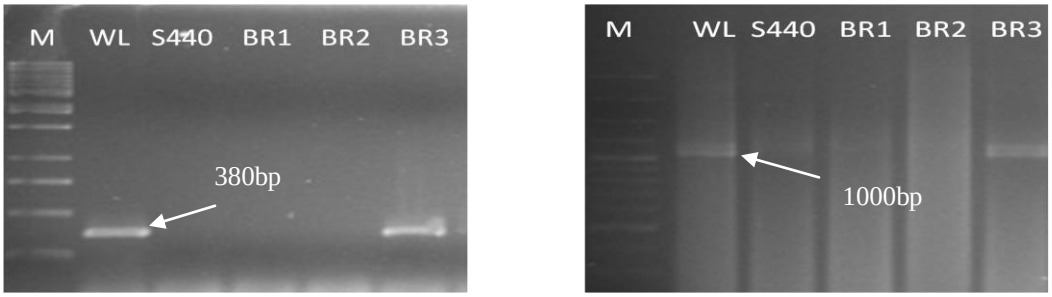

Fig. 7. Results of fragment amplifications with the *R3a* specific primer pair SHa (left) and *R3b* specific primer pair R3b (right)

M: 100 bp molecular ladder; WL: White Lady; S440: breeding line; r: Mastenbroek line with recessive allele; BR1, 2, 3: Mastenbroek R-line with dominant allele. The arrow indicates the expected and cloned band.

4.2.2. Results of transcriptome (TC) analysis

Since main data of this TC analysis have already been published by Ahmadvand (2013), here in point 4.3 we just cite some of those data which are necessary for understanding our results of further analyses of that huge TC dataset and of consecutive experiments based on the TC dataset. Published results of Ahmadvand (2013) are indicated with a ***.**

For transcriptome analysis a so called RNA-sequencing (RNA-Seq) strategy was used that belongs to the next generation sequencing (NGS) technologies. The purpose of this RNA-Seq analysis was to generate a huge dataset about biotic stress induced gene expressional changes in tetraploid potato. RNA-sequencing of transcriptomes captured at multiple time points of the treated (inoculated with PVX, PVY and *P. infestans*) sample resulted in 12,060,751* and of the control in 9,861,651* reads. Reads were assembled into contigs and were mapped to the doubled haploid potato-DM reference genome. By this, out of the 39,031 known protein-coding genes of the doubled haploid potato-DM reference genome 38,062* and 37,926* expressed genes could be identified in the treated and control samples, respectively. In total, only 358 genes of the reference genome were either not present or were not expressed in the treated as well as in the control sample. Almost 60% of these (211 out of 358) were genes of unknown function.

4.2.3. Copy number and gene regulation

Mapped reads for each transcript were normalized and expression of them was defined by the RPKM value (Mortazavi et al., 2008) (Fig. 8). By comparing the fold change of the treated to control transcripts 8,464* up-regulated as well as 4,671* down-regulated genes were identified.

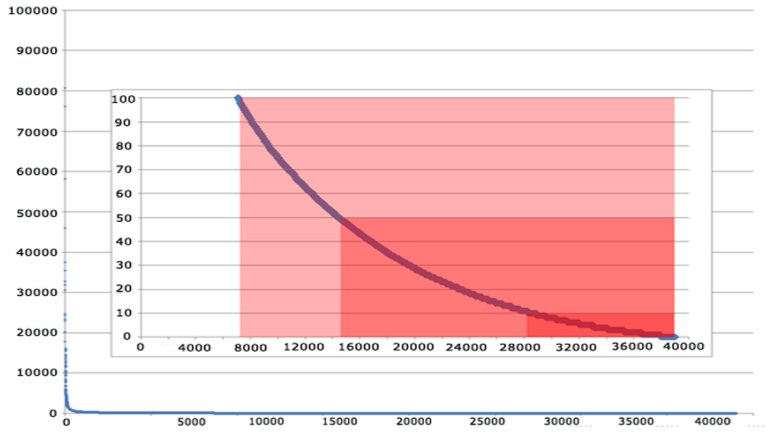

Fig. 8. Number of reads of the mapped genes in the treated sample. Y-axis: number of reads; X-axis: number of mapped genes

As illustrated in Figure 5, while the copy number in the 51-100 read spectrum was almost the same in the control (18,9%) and treated (19) samples, a decreasing of contribution of lower copy number reads and a corresponding increasing contribution of higher copy number reads could be observed in the treated sample (Fig. 9).

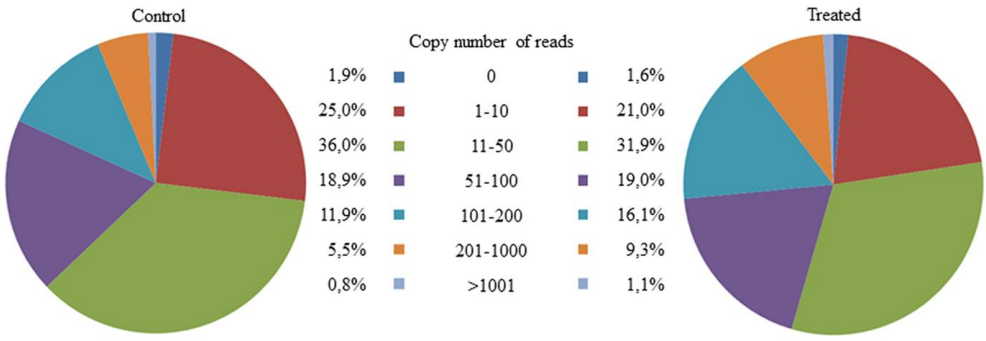

Fig. 9. Distribution of the copy numbers of the reads in the control and treated samples.

For approximately one third of both the up-regulated and down-regulated genes of mapped sequences annotation was not possible, since these sequences matched proteins of unknown function. There were 747* up-regulated genes in the treated sample which were detected in the control, indicating that those genes were activated by the stress response. On the other hand, 611 genes were found to be inactivated in the treated sample, indicating that the pathogen attack had induced strong genome-wide expressional modification. According to their annotations, 747 resistance genes and analogues were identified: of these, 385 were NBS and/or LRR domain containing genes, 142 were late blight resistance genes and homologs, and the remainder was resistance response genes and analogs.

4.2.4. Heat map analysis

Out of the 747 annotated resistance genes, 11 were expressed only in the control sample, while 13 were found to be expressed only in the treated sample. These later genes expressed just in the treated sample included 3 NBS type, 1 LRR type, 3 late blight resistance and 6 other resistance genes. The highest read number 232,365 was observed for a conserved gene of unknown function in the treated sample.

A scale of spectra for fold change of transcripts was used to construct a heat-map to visualise the rate of up-regulation and down regulation of some genes. Fold changes were determined with the Log_2 RPKM values in a range between -2 to 2 (Fig. 10).

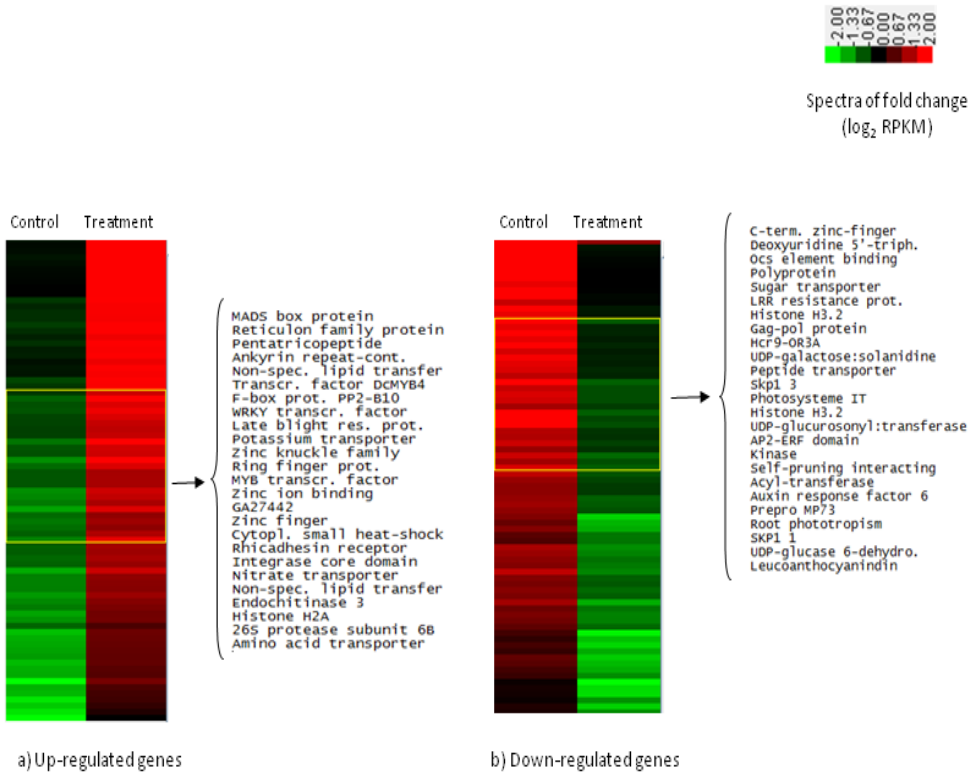

Fig. 10. Heat-map of some of the most up-, and down-regulated genes. Log_2 of the RPKM values was used as a scale to reveal color intensity. Red color indicated higher RPKM values compared to the green with lower values. The heat map was created by the Java tree view software (Saldanha, 2004).

4.2.5. Identification of resistance genes to *P. infestans* in the transcriptome dataset

Based on the functional annotation a total of 142 *P. infestans* resistance genes and homologs were expressed in the treated sample. In the White Lady genome the presence of the *R1*, *R2*, *R3a* and *R3b* genes was proved either by sequence amplification or phenotypic reaction test. In the transcriptome dataset only the *R1*, *R2*, and *R3a* genes could be identified by annotation. Since, as described above the presence of *R3b* could be revealed in White Lady, it is suggested that possibly due to sequence similarity with the *R3a* gene or with other homologs, this gene could be either miss-annotated, or reads of it are present in the unmapped bulk of reads. For the remaining 139 sequences it is concluded that these are *Rpi* homologs or other non-isolated *R* genes.

4.2.6. Selection test of different *R* gene homologs

Selection test with amino acid alignment of the resistance genes *R1*, *R2*, *R3a*, *R3b*, *Rpi-blb1*, *Rpi-blb2* and *Rpi-vnt1* of the TC database reveal different types of diversification for each gene homolog. As a model for the test, synonymous/non synonymous substitutions were evaluated by the Kimura method (Kimura, 1980) with the MEGA5 software for each gene separately and positively selected sequences of homologs were determined (Appendix 1). The probability level for all *R* gene homologs in the TC was significant. Results of selection showed that *R1* homologs positively diversified in the selection test with probability level higher than 95% (Table 9). Analysis of sequences revealed seven diversified homologs in this gene homologs with significant level. Not only gene sequences of *R2* in the TC showed the highest convergence with the related gene in NCBI, but also show the highest level of synonymous amino acid between them. Although the calculated means of values of dN > dS for *R1* homologs was more than other *R*-gene homologs, but only one, the *Rpi-blb2* homolog in the TC showed positive diversity to the accession no. DQ122125 and most of the *Rpi-blb2* homologs showed purifying with synonymous substitution in the test in the test (Table 9). Among different *R* genes identified in the TC, and have a main role in resistance to late blight disease *R2* and *Rpi-blb2* gene homologs were more convergent to their cloned resistance genes and *R2* homologs were accounted as purifying with the synonymous substitution of amino acid.

Table 9. Evolutionary analysis of codon-based test of positive selection on sequences of *Rpi* gene homologs in TC

R-gene	No. of diversified homologs[a]	Positive selection of paired sequences	
		Probability[b]	Value (dN-dS)
R1	7	0.014 , 0.000 , 0.001 , 0.011 0.015, 0.048 , 0.002	2.213, 3.376, 3.134, 2.336, 2.193, 1.683, 3.015
R2	1	0.023	2.020
R3a	2	0.015, 0.022	2.187, 2.045
R3b	2	0.030, 0.018	1.90, 2.128
Rpi-blb1	2	0.026, 0.010	1.964, 2.353
Rpi-blb2	1	0.002	2.893
Rpi-vnt1	1	0.010	1.477

a: Z-test of selection for measuring number of positively diversified homologs on the basis of synonymous/non synonymous substitutions per site

b: Probability level of diversified ones with hypothesis rejection at 5% level

4.2.7. Phylogenetic analyses of the *Rpi* homologs

To study the relationship of the Rpi homologs of White Lady phylogenetic trees were constructed from the nucleotide alignment.

Pre-analysis revealed massive size differences and a high degree of sequence difference among the homologs. Therefore arbitrary thresholds were chosen and sequences shorter than 900 nucleotides, as well as those with less than 80% similarity to their homologous Rpi gene were excluded from the analysis. Using this approach, 82 sequences were included in the final phylogenetic analysis with 21 being *P. infestans* resistance genes. The *I2* gene of tomato was included also, which is similar to the *R3* genes, and used the *Bs2* gene of pepper as an out-group.

Phylogenetic analysis was performed using maximum parsimony and maximum likelihood (Appendix 2 and 3). Both approaches revealed large phylogenetic distance

among the analyzed sequences. While with maximum likelihood analysis members of the *R1* gene group grouped together, they separated in the parsimony analysis. Excepting this, the results of the two analyses were almost identical.

The main clades were formed by known *Rpi* genes and matching homologs. The gene *R2* (including also *Rpi-abpt, Rpi-hjt, Rpi-snk* and *Rpi-blb3* genes) represented a separate clade, and separated well from the groups formed by *R1* and *Rpi-blb2*. *Rpi-blb1* (including *Rpi-sto* and *Rpi-bt1*) and *Rpi-vnt* genes formed separate clades, while *R3a* with *R3b* was part of a larger clade with 12 homologs. In the maximum likelihood analysis 12 homologs didn't grouped with any *Rpi* gene.

Out of the 82 homologs in the treated sample, 17 were up-regulated, while 15 were down-regulated. Interestingly, the 8 adjacent homologs of the *R2* block of *Rpi* genes and 5 homologs in the *Rpi-blb2* sub-clade showed significant expressional changes, while in the *R1* sub-clade pathogen inoculation did not induce expressional changes, except in one homolog. Sequence analysis of the *Rpi* homologs indicated that except five, all of them belong to the NB-LRR family of resistance genes (data not shown).

4.2.8. NBS-LRR alignments of *R*-gene homologs of the TC dataset

It was hypothesized that the NBS and LRR regions of the *R* gene homologs may contain conserved sequence motifs. To test the hypothesis the nucleic acid sequence of all homologs with an NBS and/or LRR motif were translated to amino acid sequence with the Expasy program and the NBS and LRR regions of them were selected and aligned with the MEGA5.2 program. These two regions of the *P. infestans* resistance genes (*R* genes and *Rpi* genes) were also used in the analysis. According to the clustering in the phylogenetic analysis amino acid sequence of the homologs of the *R1*, *R2*, *R3a*, *R3b*, *Rpi-bt1*, *Rpi-blb2* and *Rpi-vnt1* genes were aligned in separate groups. Both the NBS and the LRR alignments showed similarity between the appropriate *R* gene and the homologs of it, but to different extent. Except short identical sequences or single amino acids in given positions, hardly any homogeneous regions could be detected which are characteristic for groups of the homologs. In contrast to this, in those clusters which contained more than

one *R* gene, extensive sequence similarity could be observed in both regions among the *R* genes. Sequence similarity is especially high among the *R* genes of the *R2* cluster (Appendix 4). Although, many LRR regions were determined which exhibit more changes than the NBS region of the same homolog, but the magnitude of changes in residues depends on the type of diversity of the *Rpi*-gene homologs

4. 3. Development of intron-targeting primers for the detection of the R1 gene

Different published and analyzed homologs of *R1* including *R1A4, R1B23, R1C3, R1-B16* and *R1-Tken* (Kuang et al., 2005) could be identified in the TC dataset. A study on homologs of three haplotypes of *R1* including *-A, B* and *C* (Kuang et al., 2005) showed that some of these including *R1A4, R1B23, R1C3* are located in the fast evolving regions of *R1* which exhibit high level of sequence similarity.

Based on the TC data and on genebank (NCBI) data of the *R1* gene, three intron-targeting primers were designed on the intron flanking exon regions. Among them, the R1L333 primer pair amplifyed a 380 bp sequence in White Lady. This band was polymorphic in an F_1 population of 24 genotypes derived from a cross of White Lady and the susceptible breeding line S440. This band was amplified in White Lady, in resistant cultivars and in resistant F_1 genotypes (Figure 11 and 12), was extracted from the gel, cloned and sequenced.

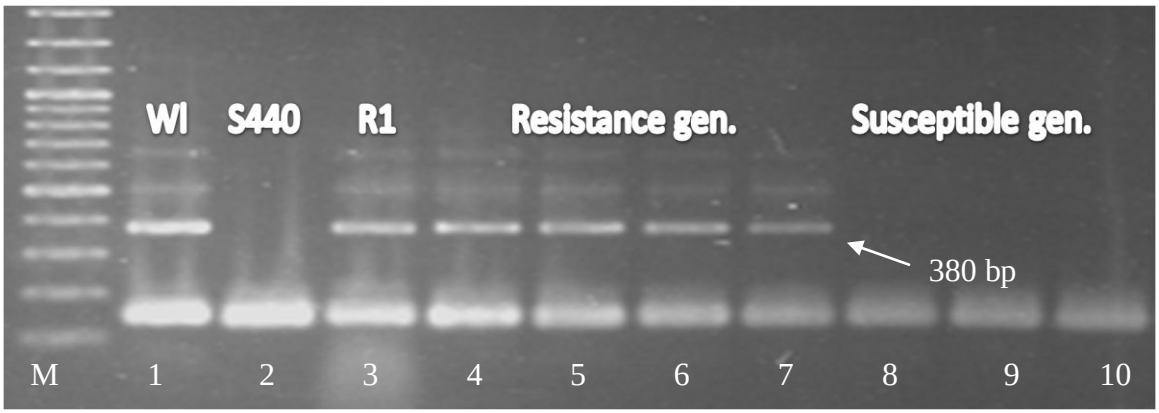

Fig. 11. The R1L333 IT marker of the *R1* gene in White Lady (lane 1), in S440 (lane 2), in R1 (lane 3), in resistant (lane 4-7) and in susceptible (lane 8-10) F_1 genotypes. Lane M is 100 bp molecular ladder.

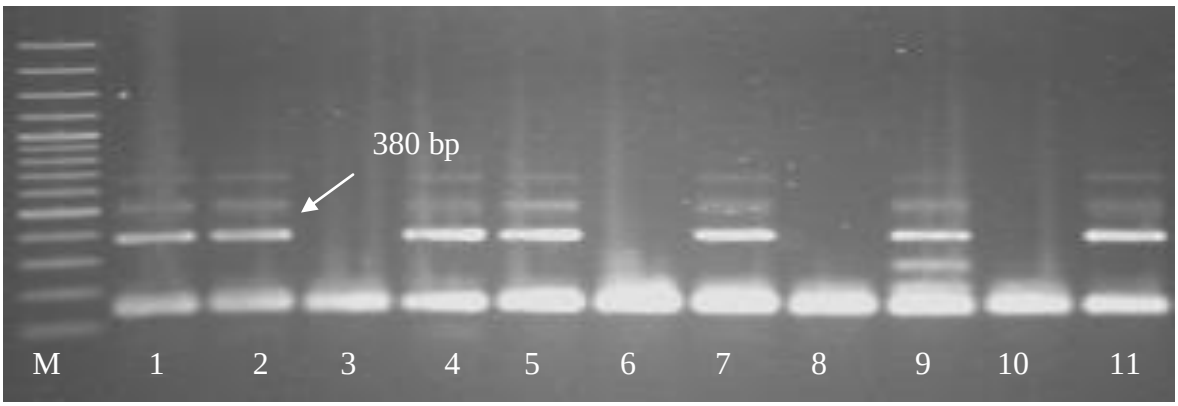

Fig. 12. Amplified band in PCR by using of IT primer in different potato cultivars. Lane 1 - White Lady (resistant), lane 2 - Venus gold (resistant), lane 3 - Desiree (susceptible), lane 4 - Cara (resistant), lane 5 Luca (resistant), lane 6 - Russet Burbank (susceptible), lane 7 - Victoria (resistant), lane 8 - Demon (susceptible), lane9 - Sante (resistant), lane 10 - Agria (susceptible), lane 11 - Rosita (resistant). Lane M is 100 bp molecular ladder.

The 380 bp amplified band obtained with the R1L333 IT-primer was extracted from several resistant cultivars and two resistant F_1 genotypes of the White Lady x S440 cross, and was sequenced and blast analysis was performed in NCBI an in SGN databases. We found that this fragment has high degree of similarity (98%) with the *R1* gene originating from *S. demissum*, although this sequence is located in an intron region in some haplotypic homologs of this gene including R1A4 R1C3, R1B23 which homologs were also identified in our TC database and according to Kuang et al. (2005) these homologs are located in the proximal region of the gene. Other homologs of the *R1* gene like R1B16 and R1-Tken have not this intron sequence (Table 10). Sequence analysis of the homologs of *R1* with the vector NTI software (Lu and Moriyama, 2004) showed that the amplified fragment of White Lady is in the first intron region of the main open reading frame (ORF) on the forward strand of each homolog.

Table 10. Results of pairwise alignment of the R1L333 marker of White Lady and published *R1* homologs

Fragment	Sequence coverage (%)	E-value	Identity (%)
R1-A4	95	4e-111	89%
R1-C3	95	4e-100	87%
R1-B23	69%	4e-86	91%
R1-B16	-	-	-
R1-Tken	-	-	-

4.3.1. Analysis of IT-amplified fragment in gene databases

In order to study the similarity of the R1L333 IT marker in both the NCBI and SGN databases, sequences of the amplified fragments of each genotype were blasted both in these databases (Table 11). All amplified fragments showed high similarity with the *R1* gene or with *R1* homolog. Interestingly, all sequences showed an 87-96% similarity with the same sequence in SGN. Sequence similarity of the same amplified fragments was in the range between 90-98% in NCBI where different accessions of the *R1* gene derived from *S. tuberosum* clones were in data output.

Table11. Blasting results of the R1L333 IT marker in SGN and NCBI

Genotype (cultivar*, line**)	SGN BLAST					NCBI BLAST				
	Seq ID	Function	Coverage (%)	E-Value	Identity (%)	Accession no.	Function	Coverage (%)	E-Value	Identity (%)
White Lady	PGSC0003DMG400003380	late blight resistance protein homolog R1	95.41	1.8e-49	89.42	AY547666.1	*Solanum tuberosum* clone 1327 *R1* (*R1*) gene, partial cds	95%%	2e-152	98%
Venus gold	PGSC0003DMG400003380	late blight resistance protein homolog R1	82.51	1.7e-46	88.52	AY547666.1	*Solanum tuberosum* clone 1145 *R1* (*R1*) gene, partial cds	91%	2e-103	91%
Cara	PGSC0003DMG400003380	late blight resistance protein homolog R1	84.08	3.5e-47	87.19	XM_006363186.1	*Solanum tuberosum* putative late blight resistance protein homolog *R1*A-4 (LOC102594522), mRNA	90	2e-102	92
Sante	PGSC0003DMG400003380	late blight resistance protein homolog R1	72.42	1.1-45	87.87	AY547596.1	*Solanum tuberosum* clone 1215 *R1*-like (R1) gene, partial sequence	90	8e-102	90
Luca	PGSC0003DMG400003380	late blight resistance protein homolog R1	100	7.7e-24	96.53	XM_006363186.1	*Solanum tuberosum* putative late blight resistance protein homolog *R1*A-4 (LOC102594522), mRNA	97%	2e-59	97%
85	PGSC0003DMG400003380	late blight resistance protein homolog R1	84.31	3.5e-47	87.19	AY547583.1	*Solanum tuberosum* clone 1245 *R1*-like (*R1*) gene, partial sequence	92	4e-101	90
467	PGSC0003DMG400003380	late blight resistance protein homolog R1	84.22	6.2e-49	88.36	AY547583.1	*Solanum tuberosum* clone 1245 *R1*-like (*R1*) gene, partial sequence	92	3e-107	91

*: resistant potato cultivars against late blight pathogen **: resistant F_1 genotypes of WL X S440

4.4. Quantitative analysis

4.4.1. Protein analysis of *P. infestans* inoculated potato leaves

Total protein was extracted from White Lady leaves at different time points after inoculation with *P. infestans*. The samples were separated in a polyacrylamide gel (Figure 9). Differences in the banding pattern of the first two samples which were taken one (lanes 1 and 2), four (lanes 3 and 4) and seventeen (lanes 5 and 6) hours after inoculation indicated a strong band in the 50-60 kDa interval and 80-150 kDa in the inoculated compared to the mock inoculated sample (Fig. 13), although there was a difference between lane 13 and 14 at sixty five hours after inoculation. Obvious differences in banding patterns during the first time points after inoculation imply that more proteins were expressed in this period in the treated plants which may be involved in resistance to the pathogen.

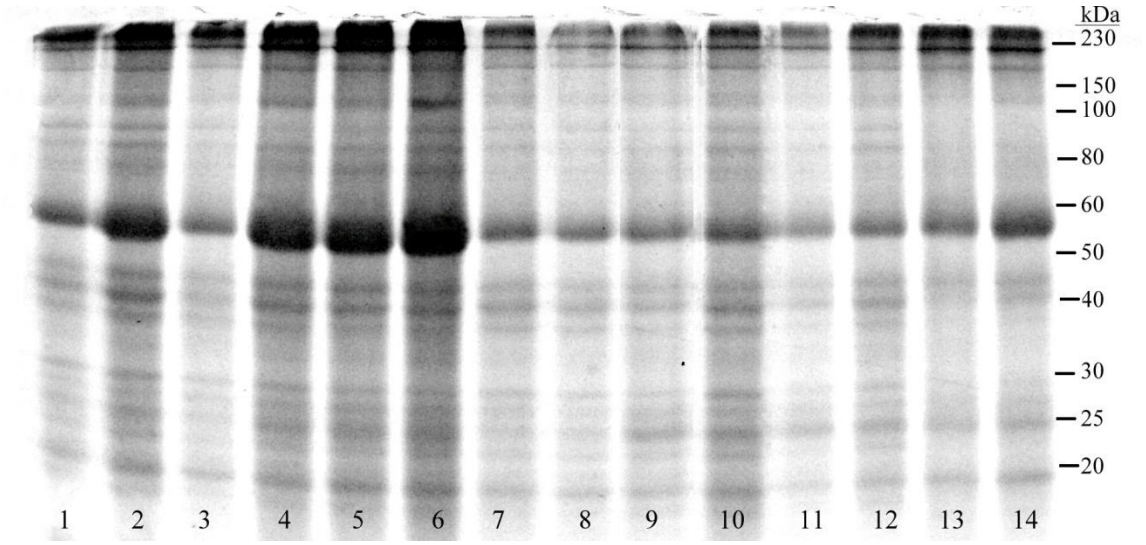

Fig. 13. Protein profile of mock inoculated (lanes: 1, 3, 5, 7, 9, 11 and 13) and *P. infestans* inoculated (lanes: 2, 4, 6, 8, 10, 12 and 14) samples

Post inoculation sampling time points were: 1 hpi (lanes 1 + 2), 4 hpi (lanes 3 + 4), 17 hpi (lanes 5 + 6), 24 hpi (lanes 7 + 8), 31 hpi (lanes 9 + 10), 48 hpi (lanes 11 + 12), 65 hpi (lanes 13 + 14).

4.4.2. Gene expression analysis of biotic stress response genes by qPCR

To characterize the *P. infestans* inoculation triggered expression of biotic stress response genes during a successful resistance response, genes of several resistance gene families were selected from the transcriptome dataset and were analyzed. To this end, leaves of White Lady plants were inoculated with the H12/10 and 10/2010 isolates and for expression tests samples were collected in the following time points: just before infection (for control), then 1, 4, 17, 24, 35, 48 and 65 hours post inoculation (hpi). RNA was extracted and quantitation of the transcripts for each time point was done by qPCR. Genes for analysis were chosen from the NGS generated transcriptome dataset. From among the more than 38,000 transcriptomes of the TC dataset biotic stress response genes were chosen for quantitation according to their RPKM (reads per kilobase per million mapped reads) value, which indicates the pathogen inoculation induced changes in the copy number of a transcriptome (Table 12). Sequence similarity and homology was also considered in selection, especially in such cases as for example the respiratory burst oxidase family (*Rboh*) which has different homologs, distinguished as A, B and C. These homologs were identified in the TC dataset as inoculation induced genes, hence they were used in the analysis.

Different types of protease inhibitor genes were identified in the transcriptome dataset. Among them four up-regulated homologs of different types including aspartic protease inhibitor, cysteine protease inhibitor, Kunitz-type inhibitor and serine protease inhibitor were evaluated quantitatively.

It should be noted that reason of the present expressional analysis was also to determine if the observed up-regulation of the selected genes of the TC dataset was really caused by the *P. infestans* inoculation, or possibly by the other two pathogens.

As indicated in Table 12, in total four different protease inhibitors, four genes belonging to the reactive oxygen species (ROS), two pathogenesis related protein (PR) genes, one immune receptor gene, one *P. infestans* resistance gene and four NBS-LRR type *P.*

infestans resistance gene homologs were selected and analyzed by qPCR. The stably expressed beta-tubulin gene was applied as housekeeping gene in the analysis.

Among different homologs of pathogenesis related proteins (PR-protein) one up-regulated homolog, the PR1 (PGSC0003DMG400037874) was expressed only in the treated sample. Since PR10 proteins are also involved in the resistance response, the only PR10 protein of the TC dataset, the PGSC0003DMG402001494 was also used in the expressional analysis, although, it showed no significant expressional changes in the transcriptome analysis (Table 12).

In selection of the *P. infestans* resistance gene homologs results of the phylogenetic analysis were also considered. Among the 82 homologs used in the phylogenetic analysis 17 were up-regulated, while 15 were down-regulated, and expression of the remaining homologs doesn't changed over the RPKM criteria. The most pronounced expressional changes were found among the homologs of the *Rpi-blb2* and *R2* gene clusters. The fewest changes were observed in the *R1* sub-clade where among the nine homologs, except one the pathogen inoculation didn't induced expressional changes.

According to the phylogenetic trees, besides eight *Rpi*-genes 26 homologs clustered together with the *R2* gene. Among them, 11 homologs showed significant expressional changes with five up-regulated and six down-regulated genes.

Among the eight homologs of the *Rpi-blb2* sub-clade four were up-regulated and one was down-regulated.

After screening of originally 22 homologs of *Rpi-bt1* found in the White Lady TC dataset, 14 were selected and used for phylogenetic analysis. Except two, 12 homologs grouped to the same clade together with the *Rpi-bt1, Rpi-sto1*and *Rpi-pta1* genes. In this clade the PGSC0003DMG401007609 homolog showed the highest fold change (Fig. 14).

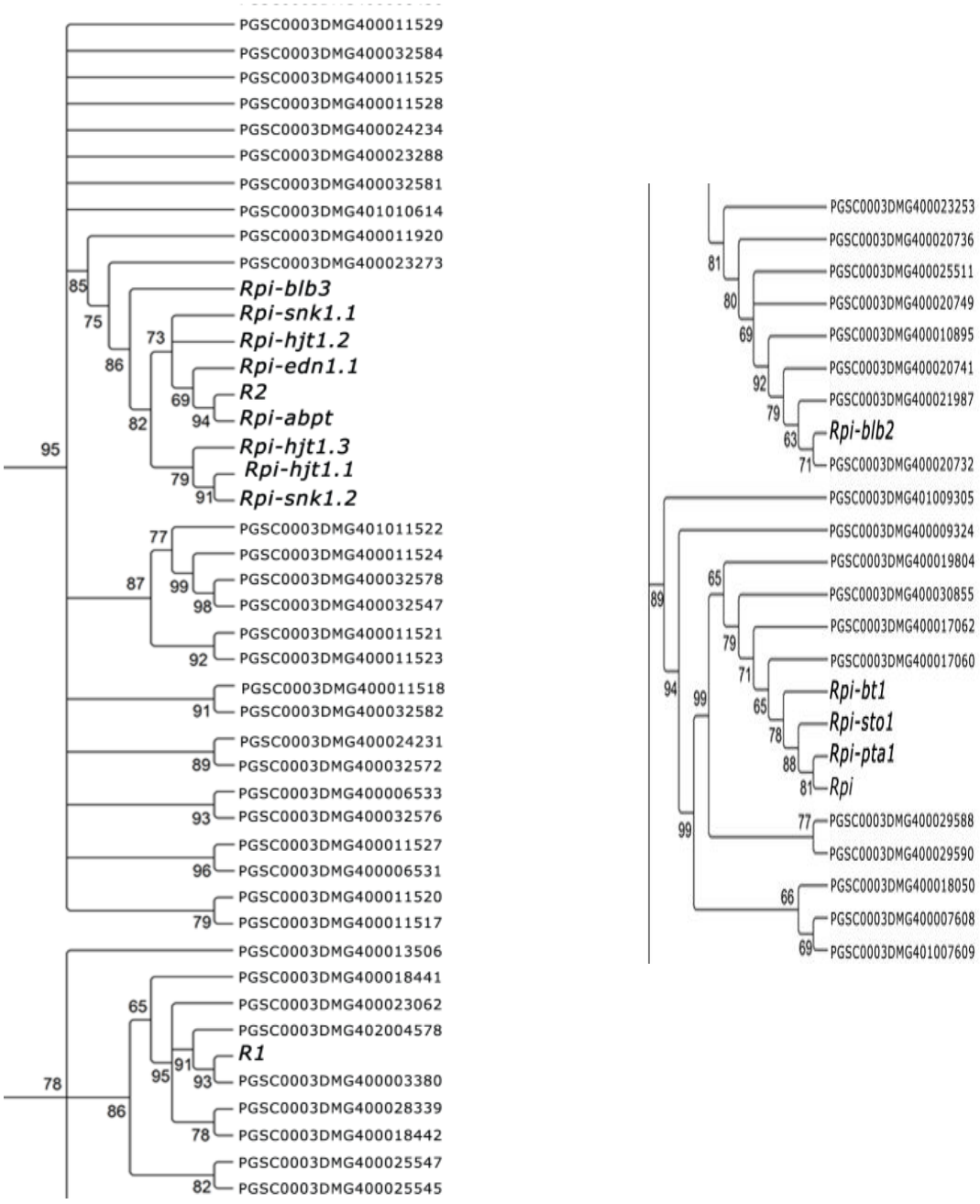

Fig.14. Partial dendrogram of the maximum parsimony tree showing the clusters of the *R1, R2* (right) and *Rpi-bt1* and *Rpi-blb2* (left) genes. (The complete dendrogram can be found in Appendix 3.)

Table 12. Characteristics of genes identified in the transcriptome dataset and analyzed by qPCR

	Gene family	Gene function	Transcript*	SGN ID**	RPKM-Control	RPKM-Treated
1	Protease inhibitors	Aspartic protease inhibitor (*AspI*)	3	PGSC0003DMG400009513	1.48	10.42 up
2		Cysteine protease inhibitor (*CyspI*)	5	PGSC0003DMG400010134	0.66	9.46 up
3		Kunitz-type protease inhibitor (*KtpI*)	6	PGSC0003DMG400015267	3.89	6.00
4		Serine protease inhibitor (*SerpI*)	7	PGSC0003DMG400026953	3.12	6.99 up
5	Respiratory burst oxidase homologs	*RbohC*	1	PGSC0003DMG400014168	5.82	11.10
6		*RbohA*	1	PGSC0003DMG400012316	2.54	3.43
7		*RbohB*	1	PGSC0003DMG400024754	1.66	3.12
8	Antioxidant	Glutathione peroxidase (*Glutp*)	4	PGSC0003DMG402004978	2.99	18.01 up
9	*PR*-proteins	*PR1*	9	PGSC0003DMG400037874	0	2.43 up
10		*PR10*	1	PGSC0003DMG402001494	2.93	2.96
11	Immune receptor	*PPR1*	16	PGSC0003DMG400020554	3.26	18.52 up
12	*R*-genes & homologs from *S. demissum*	*R1* homolog	9	PGSC0003DMG400025545	1.54	2.23
13		*R2* homolog	26	PGSC0003DMG401011522	2.42	8.30 up
14		*R3a* gene	12	PGSC0003DMG402030235	2.14	5.10 up
15	*Rpi*-homologs from *S. bulbocastanum*	*Rpi-bt1* homolog	11	PGSC0003DMG401007609	1.22	6.69 up
16		*Rpi-blb2* homolog	9	PGSC0003DMG400023253	2.97	8.02 up

*: Number of transcripts in the TC dataset for that type of gene

**: Potato gene identification numbers of the SOL Genomics Network (SGN) database according to which transcripts of White Lady were annotated

up: Up-regulated genes according to the >+2 RPKM value criterion

Based on the selection criteria, 16 homologs of 6 gene family groups were selected for RT-PCR studies. As a criterion for resistance against *P. infestans*, *PR1* gene which proved to have expressional changes in resistant potato cultivar treated with the late blight during the first time point (Orłowska et al., 2011), was selected and evaluated with other gene homologs in these experiments.

4.4.2.1. Expression analysis of protease inhibitor genes

Among the protease inhibitors three different types, the aspartic-, the cysteine and the serine protease inhibitors with different numbers of homologs were expressed in White Lady. No expression could be detected for the Kunitz-type protease inhibitor. Since the level of expression of the selected aspartic- and cysteine protease inhibitor gene are almost similar they are shown in the same figure (Fig. 15). Nevertheless, there is a strong difference in expressional peak of the two genes. The aspartic protease inhibitor is as its maximum at the 1st hour after inoculation with a 7 times increase, thus the inoculation triggers an immediate up-regulated of this gene. Then, its expression decreases rapidly. The cysteine protease inhibitor was expressed at low level and had the maximal up-regulation with 12.25 times fold change at 31 hours after inoculation, and it then decreased again in the following hours post inoculation (Fig. 15).

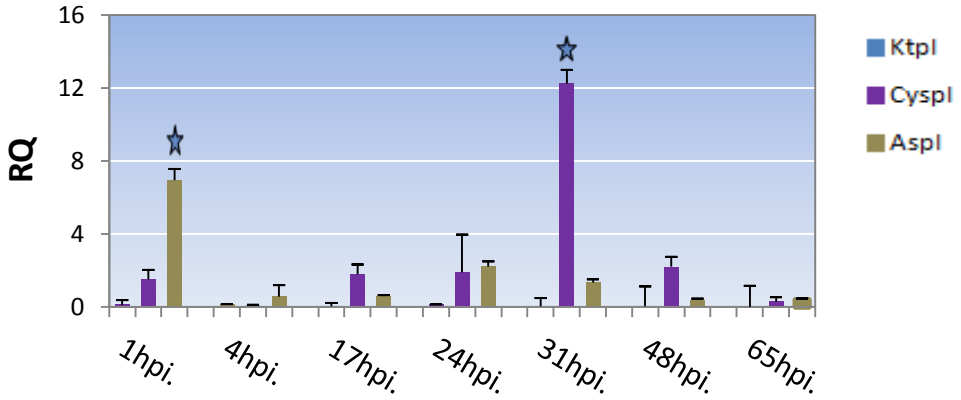

Fig. 15. Expression profile of the Kunitz-type protease inhibitor (*Ktpi*), of the asparatic- (*Aspi*) and cysteine (*Cyspi*) protease inhibitor genes in seven different post inoculation (hpi) time points

RQ: Relative quantitation

The serine protease inhibitor showed a gradual increase of expression and reached its maximum at 17 hours post inoculation with more than 180 times copy number increase (Fig. 16). Comparison between relative quantitation of different protease inhibitors at the peak point presented significantly higher expression levels for the serine protease inhibitor with a 26.76 and 15.13 times more than for the aspartic protease inhibitor and cysteine protease inhibitor, respectively.

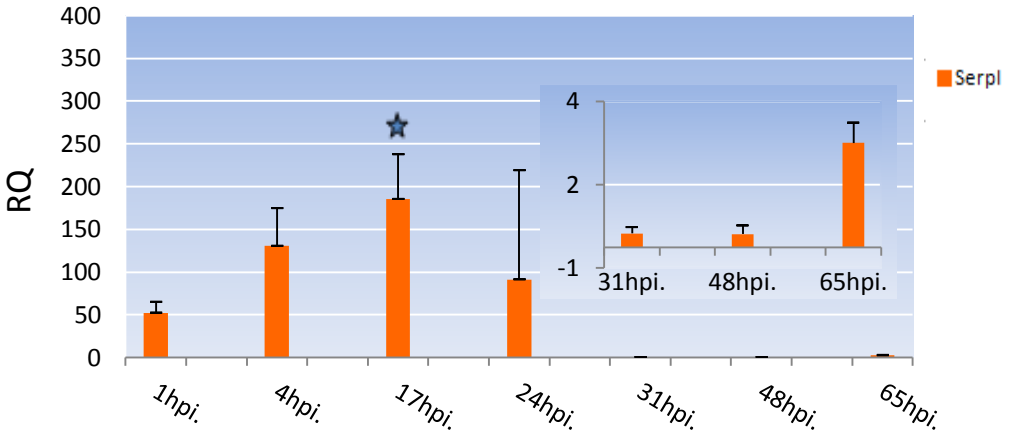

Fig. 16. Expression profile of the serine protease inhibitor gene (*Serpi*) in in seven different post inoculation (hpi) time points

RQ: Relative quantitation

4.4.2.2. Expression analysis of reactive oxygen species

All respiratory burst oxidase homologs were significantly induced after inoculation. Respiratory burst oxidase homologs (*Rboh*) are assigned as the main sources of reactive oxygen species and play a critical role in the signal transduction pathway and programmed cell death in the physiological process during HR (Thoma et al., 2003; Mur et al., 2008b).

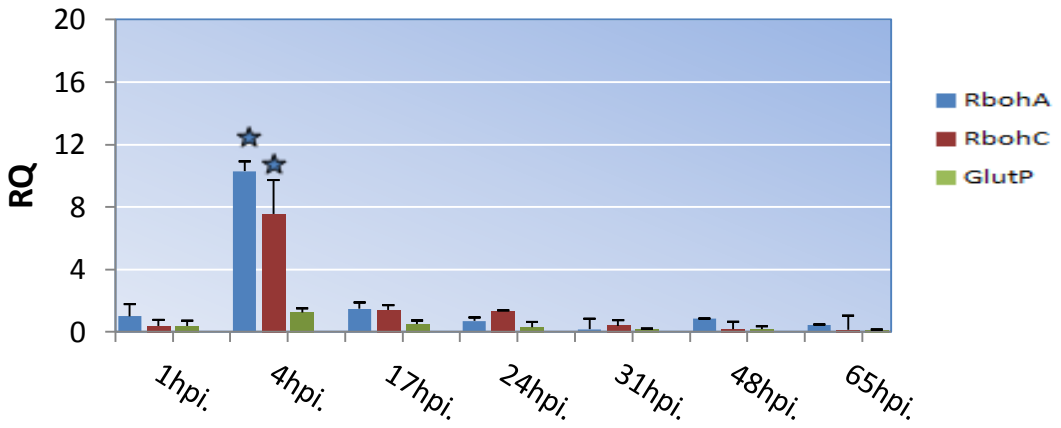

Fig. 17. Expression profile of respiratory burst oxidase inhibitors type A (*RbohA*) and C (*RbohC*) and of the glutathione peroxidase gene (*GlutP*) in seven different post inoculation (hpi) time points

RQ:Relative quantitation

Expression levels of *Rboh* type *A* and *C* increased only at four hours post inoculation with a maximum 10 fold change in *RbohA* (Fig. 17), while for *RbohB*, the gene was rapidly activated and reached its maximum with ~180 fold change at four hpi, that is 24 and 17.9 times more expressional change than for *RbohA* and *RbohC* at 4 hpi (Fig. 18).

The analyzed glutathione peroxidase homolog was not significantly induced, it implies that this gene may not be induced in the early period of the succesful defense response to *P. infestans*.

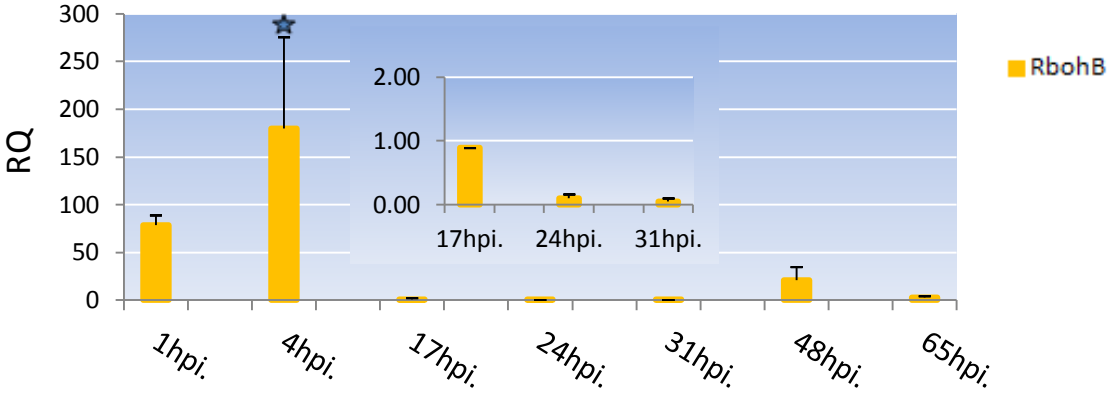

Fig. 18. Expression profile of respiratory burst oxidase inhibitors type B (*RbohB*) in seven different post inoculation (hpi) time points

RQ: Relative quantitation

4.4.2.3. Expression analysis of PR proteins and immune receptor genes

Both pathogenesis-related proteins in this study have different activation ranges. For *PR1* the expression maximum was at 4 hpi and it then decreased rapidly, while *PR10* activation peaked at 31 hpi when the *PR1*was almost inactive. (Fig. 19)

The only plant immune receptor homolog which showed a high fold change of RPKM value in the transcriptome dataset expressed maximum at 4 hpi (Fig. 19).

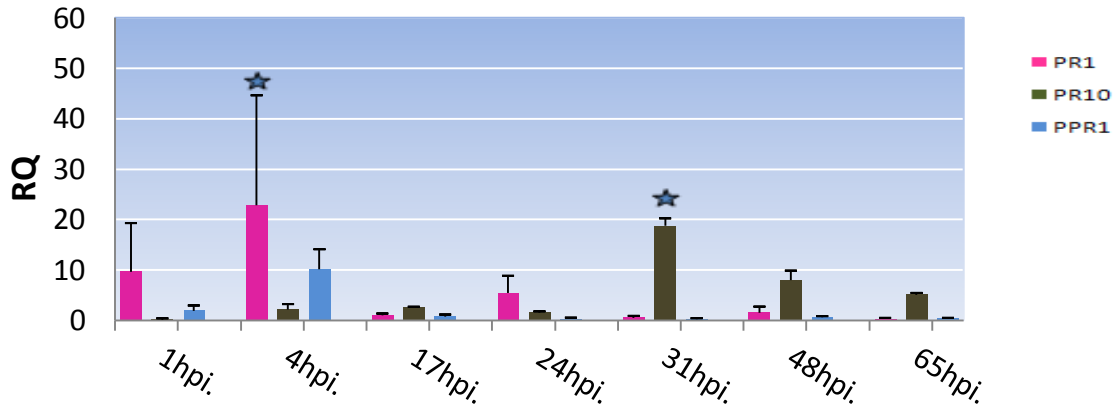

Fig. 19. Expression profile of pathogenesis related proteins (*PR1* and *PR10*) and of the immune receptor gene (*PPR1*) in seven different post inoculation (hpi) time points

RQ: Relative quantitation

4.4.2.4. Expression analysis of NB-LRR genes

The *R1* and *R2* homologs as well as the *R3a* gene showed a low constituent expression during the examined time period, but at 65 hpi the *R2* homolog got a very strong up-regulation (169 fold change) and the *R1* homolog a much less however significant (15.4 fold change) up-regulation, while the *R3a* gene expression showed no change at all (Fig. 20).

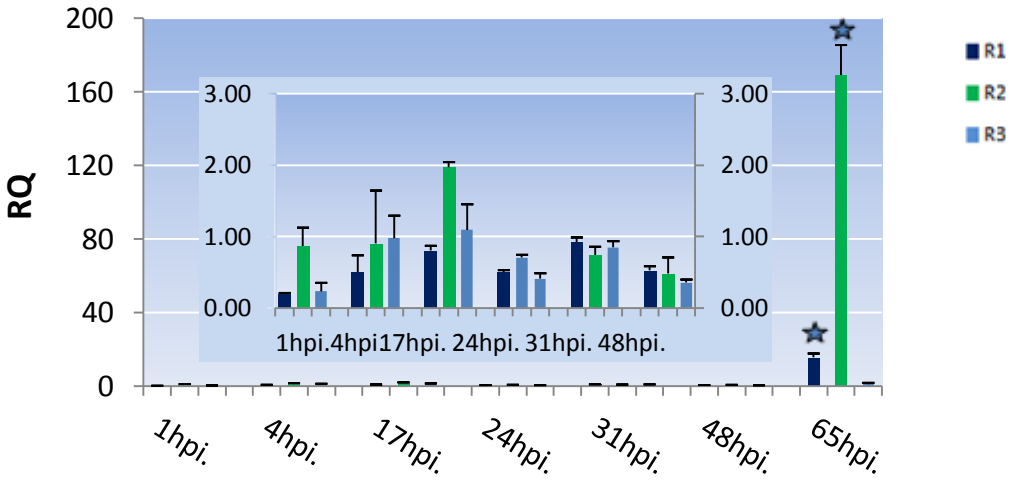

Fig.20. Expression profile of *R* gene (*R1, R2 and R3a*) derived from *S. demissum* in seven different post inoculation (hpi) time points

RQ: Relative quantitation

Among the *Rpi*–gene homologs which show similarity with *S. bulbocastanum* derived *Rpi* genes the *Rpi-bt1* homolog was activated by the inoculation and had an expression maximum (~21 fold change) at 4 hpi and it then was inactivated rapidly. The *Rpi-blb2* homolog showed a very low expression level during the examined period, which is somewhat surprising since for this gene much higher up-regulation was registered in the TC dataset. If this up-regulation is due to the infection of the other pathogens, it needs further examinations. (Fig. 21).

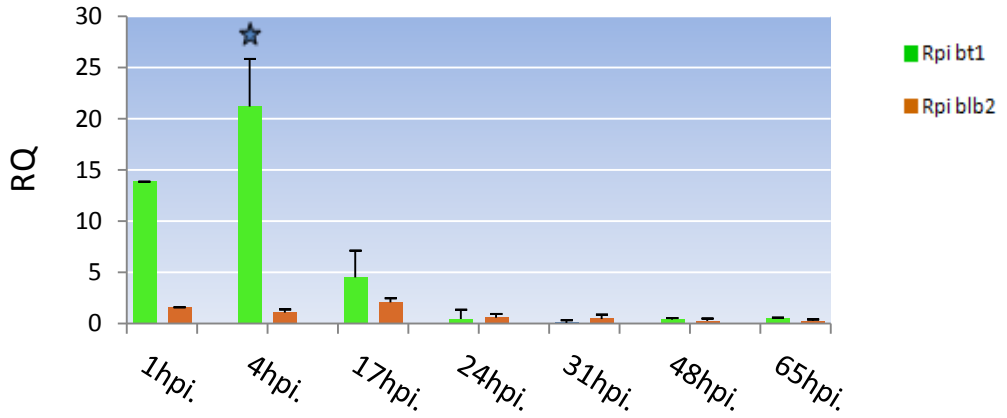

Fig. 21. Expression profile of *Rpi* genes (*Rpi-bt1* and *Rpi-blb2*) derived form *S. bulbocastanum* in seven different post inoculation (hpi) time points

RQ: Relative quantitation

4.4.3. Gel electrophoresis and sequence analysis of qPCR amplified fragments

Electrophoretic pattern of qPCR amplified fragments of all analyzed genes showed the expected size which is shown in Appendix 5. Each band was cloned and sequenced. Sequencing of the amplified genes proved that the true genes have been analyzed by qPCR (Fig. 22).

*Lane 1 in all electrophoretic patterns is beta-tubulin (ß -tub) as a housekeeping gene. The arrangement of the profile band in each pattern is as below:

Protease inhibitors: aspartic protease inhibitor (AspI), cysteine protease inhibitor (*CyspI*) and serine protease inhibitor (*SerpI*); Rboh-s and Glutathion peroxidase genes: *Rboh A*, *B*, *C*, glutathione peroxidise (*GlutpI*); PR-proteins and immune receptor gene: *PR1*, *PR10* and *PPR1*; Rpi-genes: *Rpi-bt1* homolog, *Rpi-blb2* homolog, *R1*, *R2* homologs and *R3a* gene

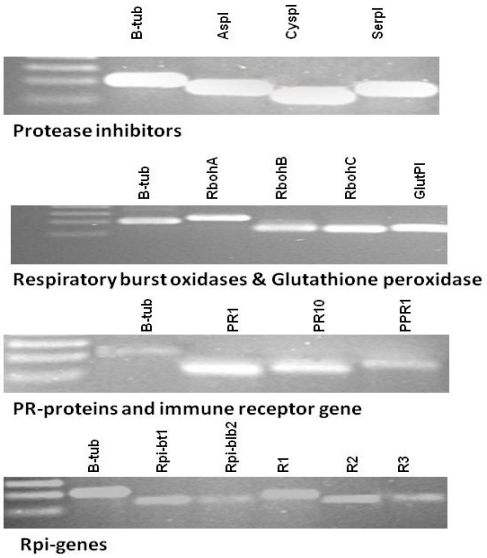

Fig. 22. Gel electrophoresis pattern of the genes analyzed by qPCR

5. DISCUSSION

5.1. *Rpi*-genes, importance and struggle for resistance against *P. infestans*

It is strongly believed that stacking of multiple *R*-genes in plants delays the onset of the pathogen invasion and potentially provide enhancement of durability against it.

High rate of recombination and sexual diversity of the pathogen stimulate evolution in potato to overcome and defeat the new races of the pathogen genetically. Variation in resistance gene resources to late blight may take place either by allele changes or segmental gene duplications during recombination. In general clustering of functional genes for qualitative and quantitative resistance to various pathogens suggests their evolution from common ancestors by local gene duplication followed by functional diversification (Friedman and Baker, 2007). There are at least 21 cloned major *P. infestans* resistance genes in potato and related *Solanum* species, while there is a supposedly large reservoir of up-to-now undetected genes that may also be utilized in cultivar improvement against this pathogen. Two important factors including pathogen effectors and gene nature during evolution have driven the evolution of gene variation. Race specific resistance genes generally have many homologs in the same *Solanum* species or different orthologs in other *Solanaceae* which may play a role in late blight resistance.

Phenotypic studies using also the Mastenbroek R lines revealed the existence of some *S. demissum* originating *R* genes in the genetic background of the Hungarian potato cultivar White Lady. This cultivar has high tolerance to *P. infestans* races presently widespread in Hungary, but gene compositions involved in resistance to late blight haven't been identified yet. Newly, multifunctional high throughput sequencing techniques called next generation sequencing offer novel, rapid ways for genome-wide characterization and profiling among others of mRNAs, small RNAs, transcription factor regions, chromatin structure and DNA methylation patterns, in any organism or in metagenomics studies (Ansorge, 2009). This technique is expected to create condition for identification of R genes and further accelerates their cloning (Vleeshouwers et al., 2011b). Implementation of next generation sequencing on biotic stress induced mRNA samples of White Lady

revealed the existence of a number of gene homologs of different race-specific -genes and broad spectrum *P. infestans* resistance genes in this cultivar. From a total of 142 *P. infestans* resistance gene homologs identified in this cultivar, more than 60 belong to race-specific *R*–genes, while the others were homologs of broad spectrum resistance genes (*Rpi*-genes) of *S. bulbocastanum* and other *Solanum* species. Out of these genes, *R1, R2, R3a* and *R3b* which have already been cloned (Ballvora et al., 2002; Huang et al., 2005; Lokossou et al., 2009; Li et al., 2011) were detected also by specific primers in the cultivar White Lady in this study.

5.2. Phylogenetic relationship of *P. infestans* resistance genes and gene homologs identified in the TC dataset

In the present analysis, 142 annotated late blight resistance gene homologs for 21 of the above *P. infestans* resistance genes (*R* genes and *Rpi* genes) were found, and except for five homologs, all of them proved to be of the NB-LRR type and could be identified among the potato-DM NB-LRR list of Jupe et al. (2012).

Recently, broad spectrum *P. infestans* resistance genes (Rpi) were identified and cloned of the diploid wild potato species *S. bulbocastanum* Dunal. These isolated genes are the *Rpi-blb1* (Van Der Vossen et al., 2003) also known as *RB* (Song et al., 2003), the *Rpi-blb2* (Vossen et al., 2005), the *Rpi-blb3* (Lokossou et al.,2009) and the *Rpi-bt1* (Oosumi et al., 2009). An *Rpi*-gene which possibly derives from *S. bulbocastanum* is the *Rpi-abpt*, (Lokossou et al.,2009) that was isolated from a complex quadruple hybrid of *S. acaule, S. bulbocastanum, S. tuberosum* Group *Phureja* and *S. tuberosum* (Park et al., 2005b). Further late blight resistance genes were cloned from *S. stoloniferum* (Vleeshouwers et al., 2008), from *S. edinense* Berthault, *S. hjertingii* Hawkes, *S. schenckii* Bitt., (Champouret, 2010), and from *S. venturii* Hawkes & Hjert. (Foster et al., 2009). From each of the four later species several *Rpi*-genes were cloned. Late blight resistance genes from *S. okadae* Hawkes & Hjert., and *S. mochiquense* Ochoa (Jones et al., 2013) were isolated and patented. While in White Lady only *S. demissum* derived *R* genes could be detected, interestingly, many *Rpi* gene homologs of different *Solanum* species, such as S. *bulbocastanum, S. hjertingii, S. schenkii, S. stoloniferum,* and *S. venturii* were found in

the TC dataset. It is suggested that there was a common ancestor of potato which contain some resistance gene homologs for any kinds of biotic stresses including *P. infestans.* During speciation, many of these genes have undergone diversification which led to emergence of new variants of the genes in a number of different species and their derivative cultivars.

In our phylogenetic analyses based on nucleotide sequences large distances were observed, which could mainly be attributed to large differences appearing in the sequences. Interestingly, we observed both homologous and non-homologous blocks corresponding to different regions of the *P. infestans* resistance genes. Most of the non-homologues regions were unalignable forming autoapomorphic changes being characteristic to separate clades of the phylogenetic tree (Appendix 1, 2). Clades formed from the 82 homologues and the 21 *Rpi* genes corresponded to those found in the potato-DM NB-LRR tree of Jupe et al. (2012), which was based on analysis of the amino-acid chain of NB-LRR motifs of 438 genes were analyzed (Jupe et al., 2012). In the present phylogenetic analysis 50 late blight resistance gene homologs did not show expressional changes. These annotated late blight resistance genes were not affected by the inoculations, indicating that they have other roles in the genome. Nevertheless, the function of these 50 annotated genes is not clarified. It can be hypothesized that these homologs are also involved in the evolution of biotic stress response genes. The evolution of disease resistance genes is dynamic involving divergent selection and birth-and-death processes (Michelmore and Meyers, 1998).

Resistance genes are frequently clustered in the genome, and show significant similarities in sequence homology and structural motifs resulting from sequence exchanges among paralogs which generate novel chimeras (Kuang et al., 2005). To clarify the role of the 17 up-and of the 15 down-regulated genes in late blight resistance needs further investigation.

Gao et al. (2013) studied the interaction of potato tubers and *P. infestans* on the non-transformed late blight susceptible cultivar Russet Burbank, as well as on a transformed late blight resistant Russet Burbank line carrying the *RB* (*Rpi-blb1*) late blight resistance gene (Gao et al., 2013). They identified ~30,000 potato genes by 36 RNA-Seq reactions

of samples taken at different time points (0, 24 and 48 hours post inoculation) and found expressional changes in 2,531 genes during this period of time. They concluded that time and treatment (pathogen treatment vs. and mock inoculation) have larger influences than the genotype on overall transcriptome differences. The *RB* gene confers resistance to all known *P. infestans* strains, while the White Lady cultivar carries strain specific late blight resistance genes and was challenged with an isolate containing six different avirulence genes. In our study leaves and three different pathogens were used, the post inoculation sampling time was much longer, and more sampling times were included. It is proposed that any or all of these factors could explain the observed changes affecting a much higher number of genes.

Structure based prediction of the function of genes lacking functional information is challenging, since homologous proteins often have distinct and sometimes multiple functions (Alberts, 2002). It would be very important to determine the role of the genes with unknown functions in the pathogen-potato interaction in order to utilize key resistance response genes in marker assisted potato breeding.

5.3. Intron targeting marker development for the detection of the *R1* gene

The presence of the *R1* gene in White Lady was identified by phenotypic tests and highly similar transcripts of this gene were found in the TC dataset. Among the different *R1* primers the intron primers which were developed in this analysis resulted only the expected marker. Interestingly, it was found that this intron sequence of the *R1* homologs has significantly higher nucleotide similarity than the flanking exon sequences which are usually highly conserved sequences. The relatively high nucleotide identities for the intron sequences were not caused by the frequent sequence exchanges, and homogenized intron sequences among the *R1* homologues at the proximal sub-cluster suggest that they are type I resistance genes. So they haven't diverse region in this homologues genes as it is for coding region (Kuang et al., 2005).

IT-primers were designed for the flaking exon regions of the homologous sequences of the *R1* gene. One IT-primer showed polymorphic bands between White Lady and the late blight susceptible S440 breeding line. Sequence analysis of the amplified intron fragment

in the NCBI database showed that it has highest similarity with the *R1* gene of *S. demissum*.

Pairwise alignment of the amplified intron region of White Lady with different homologues showed that it covers the first intron of the *A4*, *B23* and *C3 R1* homologs , but doesn't gives any product in *B16* that is another *R1* homolog (Fig. 23). Although the region of this marker is an intron but this region was embedded in two exon regions with around 2600 bp length (Fig 23).

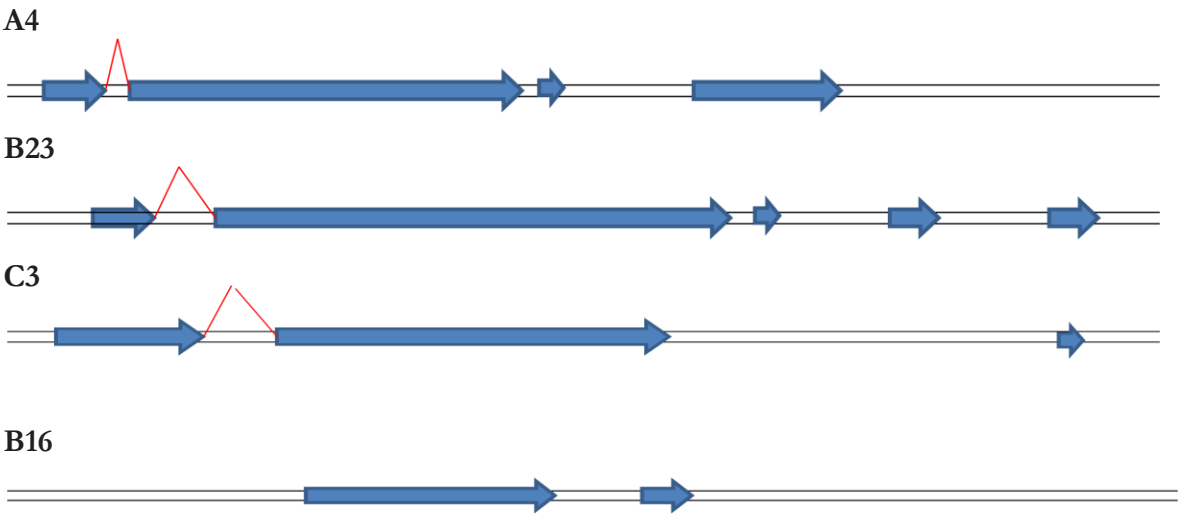

Fig.23. The intron region detected with the R1l333 IT primer pair in the *R1* homologs

A4: R1-A4 homolog, B23: R1-B23 homolog, C3: R1-C3 homolog, B16: R1-B16 homolog

Inverted red colored V shape shows the intron region of the A4, B23 and C3 R1 homologs.

First and second blue arrows are exon regions of the main ORF of the R1 homologs A4, B23 and C3.

5.4. *P. infestans* inoculation induced expressional changes as revealed by qPCR

The highly sensitive real-time PCR enables the quantification of gene expression changes. The results of quantification by this method are invaluable in the evaluation of the role of genes at the early stage of biotic stress response. Our study of the selected genes which are known to be involved in biotic stress responses revealed that *Aspi*, *Serpi*, *Rboh A, B, C, PR1* and *PPR1* are expressed more significantly at the biotrophic phase of infection with *P. infestans*. The first two time points, the 1 and 4 hours post inoculation showed up-regulation in most of these cases. These genes are involved in suppression of the pathogen by encoding of antimicrobial products, inhibitor proteins or controlling host cell death at the site of infection. It is clearly perceived that pathogens at biotrophic phase require living host plant cells for their survival and suppress the defense responses (Heath, 2000; Vleeshouwers et al., 2000c). A comparison between observed detached leaves and whole Sarpo Mira plants after inoculation with *P. infestans* showed a significant expression of some important genes including *PR1*, *Chitinase A*, *Chitinase B* and *patatin-like*. They were expressed more in leaves of whole plants, than in detached leaves at 1 hpi (Orłowska et al., 2012). It is strongly believed that very early expression has a great impact on resistance to the pathogen which is in relation to systemic acquired resistance (SAR) characterized by coordinated activation of a specific set of genes in both local and distal tissues (Durrant and Dong, 2004). Disruption of the SAR phenomena by leaf detachment deprives it of early induced response genes and leads to susceptibility (Orłowska et al., 2012). Among five transcript derived fragments (TDFs) which significantly up-regulated in the resistant potato cultivar Sarpo Mira compared to susceptible Bintje at 1 hour after inoculation with *P. infestans*, four genes showed different expression profiles throughout the whole infection process between both cultivars, hence they are considered to be involved in the resistance response to the pathogen (Orłowska et al., 2011).

5.4.1. Expression pattern of non-specific resistance genes to *P. infestans*

During the infection process, the *Phytophthora* produces enzymes that target host cells. In return plants encode secreted proteins that inhibit enzymatic activities of the pathogen. SDS-PAGE banding patterns of total leaf protein obtained at different time points after inoculation showed in some cases stronger bands in the treated samples. In a research study Fernandez et al. (2012) performed a densitometric analysis of SDS-PAGE bands of apoplastic hydrophobic proteins that were extracted from tubers of resistant and susceptible potato cultivars 24 hours after inoculation with *P. infestans*. The results showed higher amount of aspartic-, serine- and cysteine protease inhibitors in resistant samples compared to the control (mock-inoculated) and susceptible tubers (Fernández et al., 2012). Nevertheless, for the Kunitz-type protease inhibitors they didn't found expressional changes. Among different protease inhibitors in this study, Kunitz-type protease inhibitor with Id homolog PGSC0003DMG400010129 didn't express significantly in the treated White Lady over control. In a study, *Kunitz PIs* gene homologs were analyzed quantitatively in a 24 hpi treated resistant potato to control (sterile water). Among different homologues, only *Kunitz type PIs* didn't show any expressional changes (Fernández et al., 2012).

More abundant protease inhibitors which have been linked to plant defense response against pathogens are the trypsin and chemotrypsin inhibitors. Increased levels of these compounds correlated with the plants resistance to the pathogen (Kim et al., 2009) They belong to the serine protease type that inhibits the enzyme activity of the pathogen. Many different types of these molecules including *BTI-1*, *BTI-2*, *PT-1* have been found to play a role in resistance against pathogens in potato (Tian et al., 2004; Kim et al., 2005; Fernández et al., 2012). Sequence identity of one type of serine protease inhibitor in the test which was expressed more than other protease inhibitors was done by using of potato genomics resources database. The function of this gene was determined as '*BTI-2* trypsin inhibitor'.

Highest level of expression for all *Rboh* genes occurred at the 4th hour post inoculation. ROS signaling in the plant cell has a dynamic and rapid nature. This event is a consequence of contrasting processes between ROS production and scavenging. To reach the pinnacle of balance between scavenging and production rate, rapid alteration of ROS levels occur in plant cells (Mittler et al., 2011). Therefore it is possible that in a resistant potato plant like White Lady upon the pathogen attack, a leap in value of ROS followed by activation of NADPH oxidase *Rboh* genes are triggered by different stimuli.

The expressional changes of Rboh homologs showed a high fold changes of RbohB compared to other homologs in the test. Co-infiltration of *Nicotiana benthamiana* by Agrobacterium-containing *StCDPK5VK* gene originated from potato lead to activate four homologues of *Strboh* including A, B, C and D. By measuring the relative chemiluminescence intensity of *Strboh* homologs induced in transgenic plants highest intensity was measured for *StrbohB* (Kobayashi et al., 2012).

The results of this study as well as other similar studies indicate, *PR1* is an antimicrobial compound which is expressed mostly at the first time points after inoculation with the pathogen (Orłowska et al., 2011; Orłowska et al., 2012). This protein has an inhibitory effect on zoospore germination and mycelial growth (Niderman et al., 1995). At the early stage after inoculation, the sprayed sporangia and germinating zoospores of the fungi are present on the surface of the leaves and penetration process of infection and creation of haustoria develop. Production of *PR1* with its antimicrobial activity in the early stage of infection which is the biotrophic phase of pathogen would be a good criterion for resistance against *P. infestans*. Therefore White Lady with around 10 and 23 relative quantitation values at 1 and 4 hours after inoculation respectively assigned as a resistant cultivar to the pathogen. Another antifungal pathogenesis-related protein is *PR10*. Only one homologue of this gene was found in the TC dataset of White Lady. Unlike previous studies which indicates that *PR10* had no effect on *P. infestans* (Constabel et al., 1993), in our study proved that it has a role in resistance against this pathogen, however PR10 was expressed later than *PR1*.

Some plant resistance proteins have been found that could make physical interaction with pathogen effectors. These interactions agree with the model of effector-triggered immunity following direct recognition of effectors like *ATR1* by plant immune receptors *PPR1* (Postma et al., 2012). Sixteen homologues of plant immune receptors *PPR1* were identified in our TC dataset, and interestingly 10 of them were up-regulated and the most up-regulated was analyzed by qPCR. Nevertheless, our results indicated much less up-regulation of this PPR1 gene, indicating, that possibly either or both of the other two pathogens (PVX and PVY) may have triggered stronger up-regulation of this gene.

5.4.2. qPCR analysis of race-specific and broad spectrum resistance genes to *P. infestans*

Some homologs of race-specific (*R*) and broad spectrum (*Rpi*) resistance genes were found in the TC dataset that were significantly up-regulated after the inoculations. In the examined time period only the *R1* and *R2* homologs showed up-regulation but only at 65 hpi, while for the *R3a* gene no expressional change was observed. Interestingly the *R2* homolog had the highest expression level among the analyzed resistance gene homologs. Our results of the *R1* homolog are in accordance with the results of Ross et al. (2004) who found that changes in expression of the *R1* gene occurred only at the third day post inoculation. Our results on the *R3a* gene are somewhat astonishing, since in the TC dataset significant up-regulation was registered for this gene. Nevertheless, Huang et al. (2005) found that the *R3a* gene is a constitutively expressed gene which is in accordance with our qPCR results. The observed up-regulation in the TC analysis for this gene may be considered as an experimental mistake or a mistake of the assembling of the reads, where small homologous reads from different genomic regions amplified the signal of the *R3a* gene.

QPCR of the *Rpi-bt1* gene homolog showed a rapid and extended expression in the first half of the examined period. This gene is constitutively expressed in the wild *S. bulbocastum* in which it was identified, but introgression into different genetic backgrounds may affect both basal expression as well as the transcription activation after infection with late blight pathogen (Kramer et al., 2009).

In general, quantitative analysis of NGS-identified genes involved in resistance by qPCR contributes to our understanding about their role in resistance. The qPCR analysis revealed up-regulation of 11 genes from the analyzed 16 genes, indicating their role in the resistance response to *P. infestans* and determining the homologues of the target gene for resistance in biotic and abiotic stresses. In this study, some non-specific resistance gene homologues including *SerpI* with *BTI-2* trypsin inhibitor function and *RbohB* have higher expression value than other analyzed genes. On the other hand, some genes belong to NB-LRR including *R1* gene homolog with a high expression level and *Rbt1* with a high and extended level of expression, were identified which can be used in functional analysis studies. Since White Lady is a valuable source of different resistances and is used in our breeding programs, evaluation of the role of the above genes contribute to our efforts in developing biomarkers, which could be applied in resistance breeding to *P. infestans*.

LIST OF NEW FINDINGS

1) Identification of the *R1, R2, R3a* and *R3b* race-specific *P. infestans* resistance genes in cultivar White Lady by infection tests and/or by specific primers.

2) By analysis of the transcriptome dataset new data on pathogen inoculation induced genome-wide expressional changes have been obtained, which created new opportunities to design further genomics researches in the field of biotic stress response of potato. (These researches were partially realized in the present study.)

3) By phylogenetic analysis of *P. infestans* resistance gene homologs it was revealed that not only *S. demissum* derived *R*-gene homologs are present in White Lady, but also homologs of broad spectrum resistance (*Rpi*) genes of such species which are not present in the genetic background of this cultivar. This indicates the common ancestral origin of *P. infestans* resistance genes in potato and sheds light on their evolution.

4) Development of an intron targeting marker for the *R1* gene based on sequence analysis of the transcriptome dataset of White Lady. The usefulness of this marker in the analysis of *R1* homologs was proven. Since the *R1* sequence in the NCBI for that region that was analyzed in the present study is somewhat different, it is suggested that the White Lady either has an allelic version of that *R1* sequence or a functional homolog of this gene.

5) By quantitation analysis of biotic stress response genes eleven genes could be identified which were up-regulated by the *P. infestans* inoculation. The expression of these genes was characterized in seven different time points during the early period of the successful resistance response.

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7. Ahmadvand, R., **Hajianfar, R**., Polgár, Z, and Taller, J. (2013). Transcriptome and functional marker study in potato. A Pannon Növény-Biotechnológiai Egyesület konferenciája PhD hallgatók számára, 2013. Május 15

8. Ahmadvand, R.**, Hajianfar, R**., Mousapour Gorji, A., El-Banna, A., Polgár, Z, and Taller, J. (2013). Development of intron-targeting markers as a tool for molecular breeding in response to pathogens. Proceeding of 8th plant breeding international conference, 6-7 May, Egypt.

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1. Hajianfar, R .and Zarbakhsh, A. 2010.Study on reaction of tomato genotypes to *Alternaria* stem canker. Seed and plant Improvement Journal. 26, No. 4. (in Persian).

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Appendixes

Appendix 1. Codon-based Test of Positive Selection for analysis between sequences of each clade of *R*-gene homologs of the TC data set of White Lady

Title: fasta file
Description: *R1* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 1127
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

[1] #PGSC0003DMG400028339
[2] #PGSC0003DMG400003380
[3] #PGSC0003DMG400018441
[4] #PGSC0003DMG400018442
[5] #PGSC0003DMG400025547
[6] #PGSC0003DMG400025545
[7] #PGSC0003DMG402004578
[8] #PGSC0003DMG400023062
[9] #PGSC0003DMG400013506
[10] #R1_gene(AF447489.1)

	1	2	3	4	5	6	7	8	9	10
[1]		[2.569]	[3.329]	[2.944]	[1.227]	[3.086]	[1.862]	[2.283]	[1.353]	[2.213]
[2]	0.006		[2.209]	[1.974]	[1.756]	[2.225]	[1.484]	[0.972]	[2.013]	[0.868]
[3]	0.001	0.015		[1.650]	[1.591]	[3.128]	[2.651]	[2.953]	[1.821]	[3.376]
[4]	0.002	0.025	0.051		[1.831]	[1.851]	[2.147]	[2.346]	[1.738]	[3.134]
[5]	0.111	0.041	0.057	0.035		[0.752]	[1.522]	[2.280]	[2.616]	[2.336]
[6]	0.001	0.014	0.001	0.033	0.227		[1.802]	[-0.484]	[0.532]	[1.160]
[7]	0.033	0.070	0.005	0.017	0.065	0.037		[1.882]	[2.729]	[2.193]
[8]	0.012	0.167	0.002	0.010	0.012	1.000	0.031		[2.598]	[1.683]
[9]	0.089	0.023	0.036	0.042	0.005	0.298	0.004	0.005		[3.015]

[10] 0.014 0.194 0.000 0.001 0.011 0.124 0.015 0.048 0.002

Title: fasta file
Description: *R2* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 754
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

[1] #PGSC0003DMG400006531
[2] #PGSC0003DMG400006533
[3] #PGSC0003DMG400011517
[4] #PGSC0003DMG400011518
[5] #PGSC0003DMG400011520
[6] #PGSC0003DMG400011521
[7] #PGSC0003DMG401011522
[8] #PGSC0003DMG400011523
[9] #PGSC0003DMG400011524
[10] #PGSC0003DMG400011525
[11] #PGSC0003DMG400011527
[12] #PGSC0003DMG400011528
[13] #PGSC0003DMG400011529
[14] #PGSC0003DMG400011920
[15] #PGSC0003DMG400032572
[16] #PGSC0003DMG400032576
[17] #PGSC0003DMG402032547
[18] #PGSC0003DMG400032578
[19] #PGSC0003DMG400032581
[20] #PGSC0003DMG400032584
[21] #PGSC0003DMG400024231
[22] #PGSC0003DMG400024234
[23] #PGSC0003DMG400032582*
[24] #PGSC0003DMG400023288
[25] #PGSC0003DMG401010614
[26] #PGSC0003DMG400023273
[27] #R2_gene_(FJ536325)
[28] #Rpi_protein_gene_ABPT(FJ536324.1)
[29] #Rpi-blb3_(FJ536326.1)

*: Only homolog no. 23 has significant diversified codons in the analysis of positive selection

Title: fasta file
Description: *R3* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 1055
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

[1] #PGSC0003DMG400007344
[2] #PGSC0003DMG400014047
[3] #PGSC0003DMG400018570
[4] #PGSC0003DMG400018574
[5] #PGSC0003DMG402018576
[6] #PGSC0003DMG403019668
[7] #PGSC0003DMG400018694
[8] #PGSC0003DMG401030236
[9] #PGSC0003DMG400030238
[10] #PGSC0003DMG400030239
[11] #R3a_gene|AY845382|
[12] #R3b_gene|JF900492|
[13] #AF118127.1_L.esculentum_-I2_gene

[	1	2	3	4	5	6	7	8	9	10	11	12	13
[1]		[0.962]	[2.928]	[-0.412]	[1.618]	[2.774]	[-1.412]	[2.260]	[1.299]	[2.440]	[2.187]	[1.905]	[2.784]
[2]	0.169		[-0.474]	[0.585]	[-0.673]	[0.626]	[-0.088]	[-0.501]	[-1.431]	[0.155]	[-0.995]	[-1.304]	[-0.576]
[3]	0.002	1.000		[1.773]	[-0.859]	[0.025]	[1.652]	[2.182]	[-0.248]	[1.597]	[-0.090]	[0.512]	[1.653]
[4]	1.000	0.280	0.039		[0.056]	[1.457]	[-0.751]	[1.243]	[0.619]	[1.425]	[0.822]	[0.845]	[1.553]
[5]	0.054	1.000	1.000	-21.000		[0.145]	[1.271]	[-2.364]	[-2.656]	[-2.160]	[-1.138]	[-2.285]	[-1.193]
[6]	0.003	0.266	0.490	0.074	0.443		[.486]	[-0.186]	[-0.954]	[0.257]	[0.142]	[-0.239]	[0.296]
[7]	1.000	1.000	0.051	1.000	0.103	0.070		[1.659]	[0.172]	[1.826]	[2.045]	[2.128]	[2.135]
[8]	0.013	1.000	0.016	0.108	1.000	1.000	0.050		[-0.633]	[1.121]	[0.262]	[0.543]	[1.496]
[9]	0.098	1.000	1.000	0.268	1.000	1.000	0.432	1.000		[-1.521]	[-0.562]	[-0.671]	[-0.066]
[10]	0.008	0.438	0.056	0.078	1.000	0.399	0.035	0.132	1.000		[0.417]	[-0.010]	[0.969]
[11]	0.015	1.000	1.000	0.206	1.000	0.444	0.022	0.397	1.000	0.339		[-1.150]	[-0.431]
[12]	0.030	1.000	0.305	0.200	1.000	1.000	0.018	0.294	1.000	1.000	1.000		[-0.025]
[13]	0.003	1.000	0.050	0.062	1.000	0.384	0.017	0.069	1.000	0.167	1.000	1.000	

Title: fasta file
Description: *Rpi-blb1* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 875
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

[1] #PGSC0003DMG400009324
[2] #PGSC0003DMG400019804
[3] #PGSC0003DMG400030855
[4] #PGSC0003DMG400017060
[5] #PGSC0003DMG400017062
[6] #PGSC0003DMG400018050
[7] #PGSC0003DMG402009305
[8] #PGSC0003DMG400007608
[9] #PGSC0003DMG401007609
[10] #PGSC0003DMG400029588
[11] #PGSC0003DMG400029590
[12] #S._bulbocastanum_Rpi-bt1(FJ188415)
[13] #Rpi-pta1(EU884422)
[14] #S._bulbocastanum_RPI_gene(AY426259)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1]		[-1.016]	[0.304]	[1.418]	[2.370]	[0.113]	[-0.888]	[0.697]	[1.030]	[0.064]	[-0.050]	[1.964]	[0.938]	[0.789]
[2]	1.000		[-0.399]	[-0.286]	[0.013]	[-2.324]	[-0.426]	[-1.600]	[-1.249]	[-0.349]	[-1.604]	[0.420]	[-0.818]	[-0.886]
[3]	0.381	1.000		[0.057]	[1.242]	[-0.546]	[1.189]	[-0.799]	[-0.534]	[-0.168]	[-0.720]	[0.438]	[-0.003]	[-0.237]
[4]	0.079	1.000	0.477		[0.559]	[0.013]	[0.649]	[-0.483]	[-0.012]	[-0.646]	[-2.178]	[2.353]	[1.531]	[1.505]
[5]	0.010	0.495	0.108	0.289		[0.441]	[-0.111]	[0.192]	[0.943]	[-0.612]	[-1.152]	[1.500]	[0.926]	[0.867]
[6]	0.455	1.000	1.000	0.495	0.330		[0.660]	[-1.776]	[-2.389]	[-1.287]	[-2.021]	[-0.521]	[-0.822]	[-0.704]
[7]	1.000	1.000	0.118	0.259	1.000	0.255		[1.165]	[0.501]	[-0.735]	[-0.328]	[-0.265]	[-0.173]	[-0.173]
[8]	0.244	1.000	1.000	1.000	0.424	1.000	0.123		[-0.027]	[-1.168]	[-2.387]	[0.033]	[-0.525]	[-0.590]
[9]	0.153	1.000	1.000	1.000	0.174	1.000	0.309	1.000		[-1.168]	[-2.710]	[0.248]	[-0.328]	[-0.382]
[10]	0.475	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		[0.000]	[-0.157]	[-1.093]	[-1.093]
[11]	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		[-1.787]	[-2.298]	[-2.459]
[12]	0.026	0.338	0.331	0.010	0.068	1.000	1.000	0.487	0.402	1.000	1.000		[0.390]	[0.546]
[13]	0.175	1.000	1.000	0.064	0.178	1.000	1.000	1.000	1.000	1.000	1.000	0.348		[0.815]

[14] 0.216 1.000 1.000 0.067 0.194 1.000 1.000 1.000 1.000 1.000 1.000 0.293 0.208

Title: fasta file
Description: *Rpi-blb2* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 1023
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

[1] #PGSC0003DMG400023253
[2] #PGSC0003DMG400020736
[3] #PGSC0003DMG400025511
[4] #PGSC0003DMG400020749
[5] #PGSC0003DMG400010895
[6] #PGSC0003DMG400020741
[7] #PGSC0003DMG400021987
[8] #PGSC0003DMG400020732
[9] #Rpi-blb2_(DQ122125)

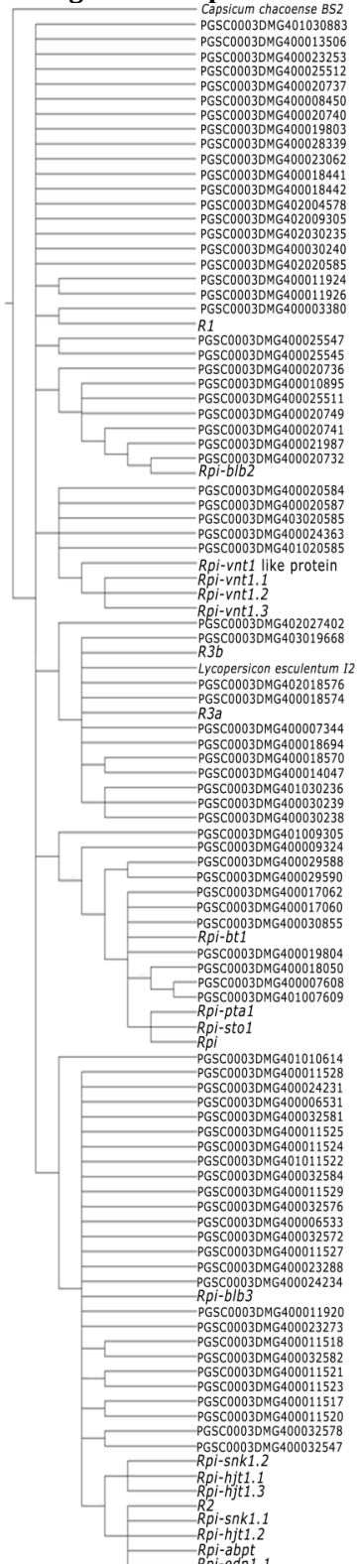
[	1	2	3	4	5	6	7	8	9
[1]	[0.896]	[0.408]	[-0.916]	[-0.080]	[-0.407]	[0.525]	[-0.903]	[0.307]	
[2]	0.186	[3.006]	[0.417]	[4.861]	[2.117]	[2.318]	[1.775]	[2.893]	
[3]	0.342	0.002	[-0.659]	[2.288]	[-0.664]	[0.199]	[0.468]	[-0.151]	
[4]	1.000	0.339	1.000	[0.492]	[-0.168]	[-1.066]	[-1.065]	[-1.162]	
[5]	1.000	0.000	0.012	0.312	[-0.128]	[1.859]	[1.556]	[1.353]	
[6]	1.000	0.018	1.000	1.000	1.000	[1.056]	[-1.551]	[-1.210]	
[7]	0.300	0.011	0.421	1.000	0.033	0.146	[-0.751]	[-0.494]	
[8]	1.000	0.039	0.320	1.000	0.061	1.000	1.000	[-0.372]	
[9]	0.380	0.002	1.000	1.000	0.089	1.000	1.000	1.000	

Title: fasta file
Description: *Rpi-vnt1.1* homologs of TC
Analysis
Analysis ---------- Z-test of Selection
Scope ---------- In Sequence Pairs
Test Hypothesis (HA: alternative) --- Positive selection (HA: dN > dS)
Estimate Variance
Variance Estimation Method ---------- Bootstrap method
No. of Bootstrap Replications ------- 500
Substitution Model
Substitutions Type ---------- Syn-Nonsynonymous
Model/Method ---------- Kumar method (Kimura 2-para)
Data Subset to Use
Gaps/Missing Data Treatment --------- Pairwise deletion
No. of Sites : 812
Prob (black) : Probability computed (must be <0.05 for hypothesis rejection at 5% level [yellow backgroud])
Stat (blue) : Statistic used to compute the probability (blue).

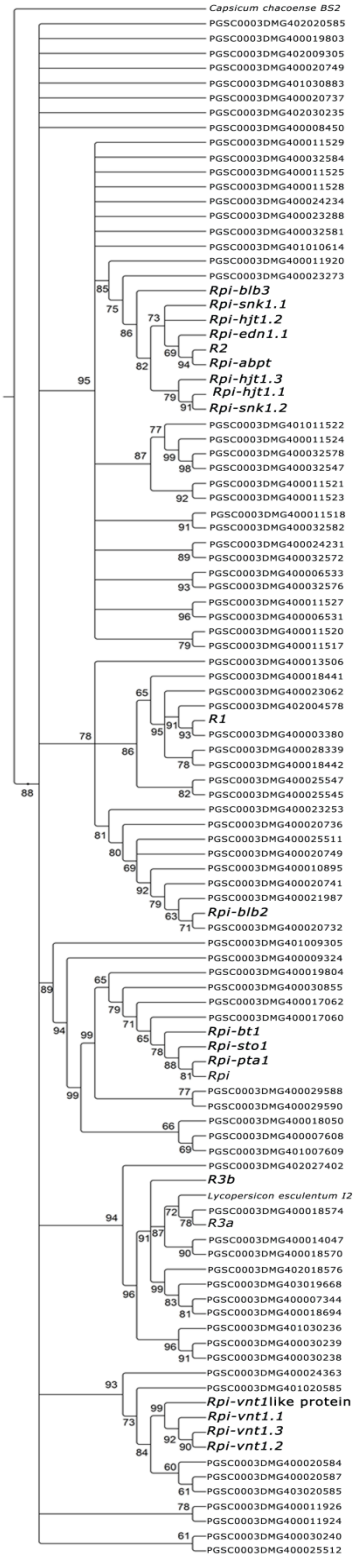
[1] #PGSC0003DMG400024363
[2] #PGSC0003DMG401020585
[3] #PGSC0003DMG400020584
[4] #PGSC0003DMG400020587
[5] #PGSC0003DMG403020585
[6] #Rpi-vnt1.1(FJ423044)
[7] #Rpi-vnt1.2(Fj423045)
[8] #Rpi-vnt1.3_(FJ423046)
[9] #Rpi-vnt1_like_protein(oka_745-1)

	1	2	3	4	5	6	7	8	9
[1]		[0.528]	[1.793]	[1.645]	[1.494]	[0.814]	[0.899]	[0.899]	[0.905]
[2]	0.299		[1.845]	[0.601]	[-0.378]	[0.304]	[0.542]	[0.542]	[0.892]
[3]	0.038	0.034		[1.877]	[1.084]	[0.045]	[0.544]	[0.544]	[1.252]
[4]	0.051	0.275	0.032		[1.308]	[2.169]	[2.350]	[2.350]	[1.477]
[5]	0.069	1.000	0.140	0.097		[1.561]	[1.566]	[1.566]	[0.786]
[6]	0.209	0.381	0.482	0.016	0.061		[-0.536]	[-0.536]	[0.315]
[7]	0.185	0.294	0.294	0.010	0.060	1.000		[0.000]	[0.540]
[8]	0.185	0.294	0.294	0.010	0.060	1.000	1.000		[0.540]
[9]	0.184	0.187	0.106	0.071	0.217	0.377	0.295	0.295	

Appendix 2: Most parsimonious tree found by the phylogenetic analysis of the late blight resistance gene homologues of the potato cultivar White Lady*



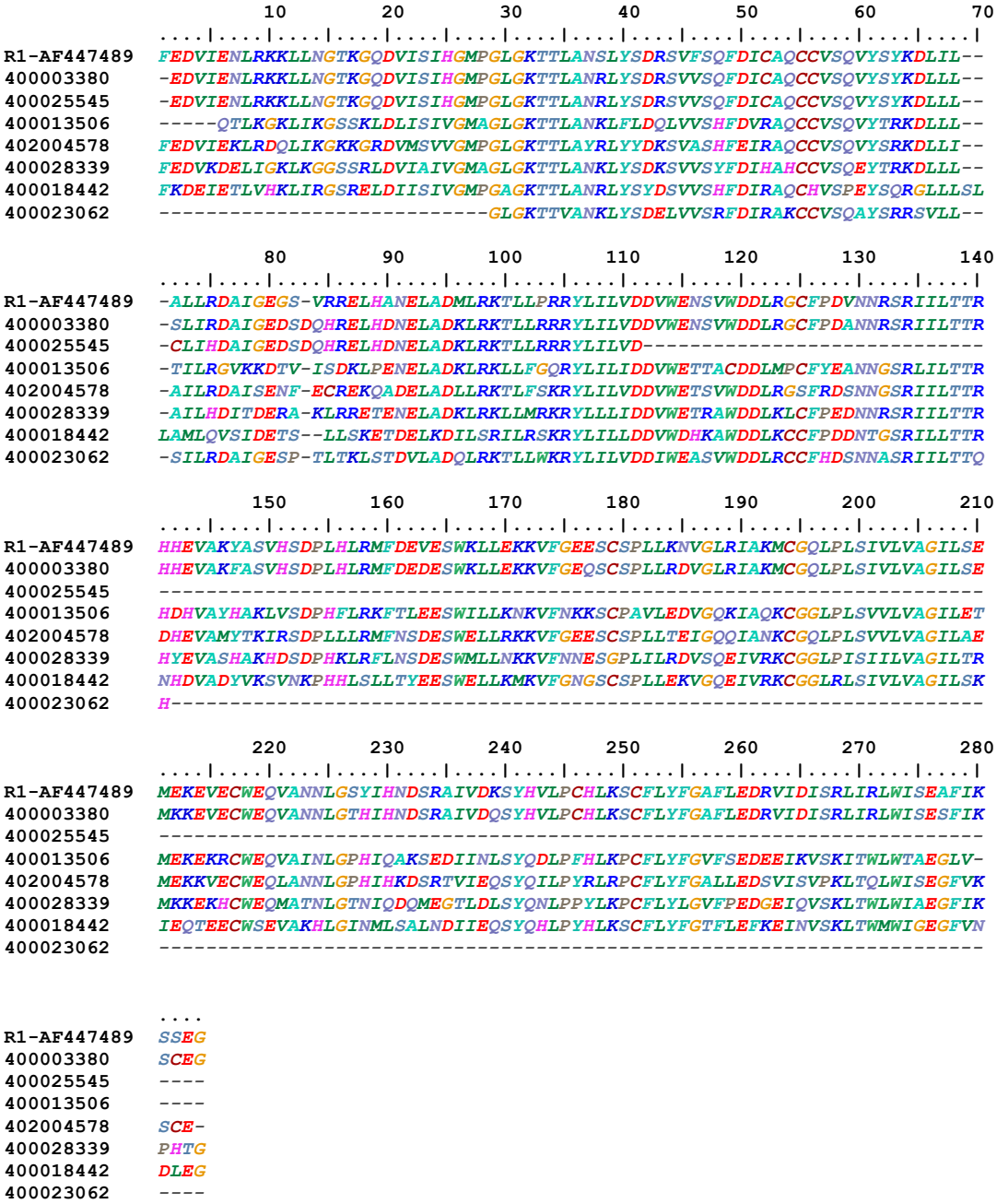
Appendix 3: Maximum likelihood (ML) majority rule consensus tree of the late blight resistance gene homologues of the potato cultivar White Lady*



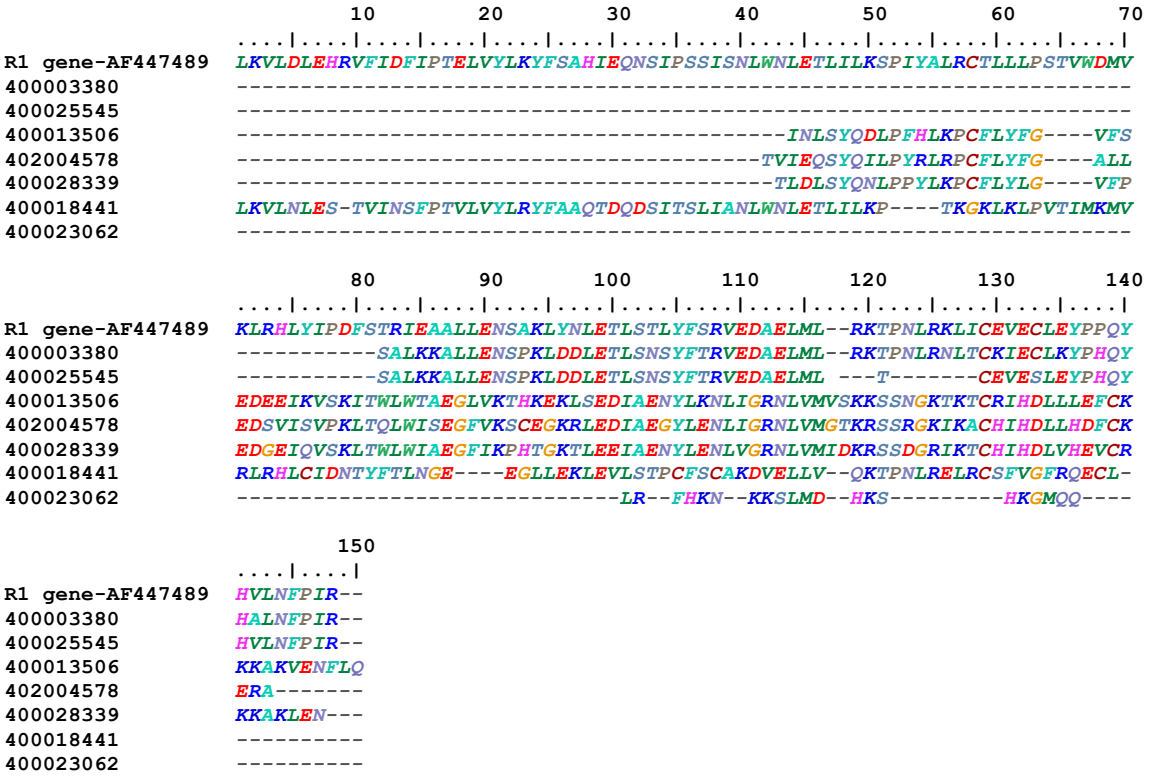
*: Late blight resistance genes are abbreviated as in NCBI and are written in bold-italics.

Appendix 4: NBS-LRR region alignments of *R*-gene homologues of White Lady

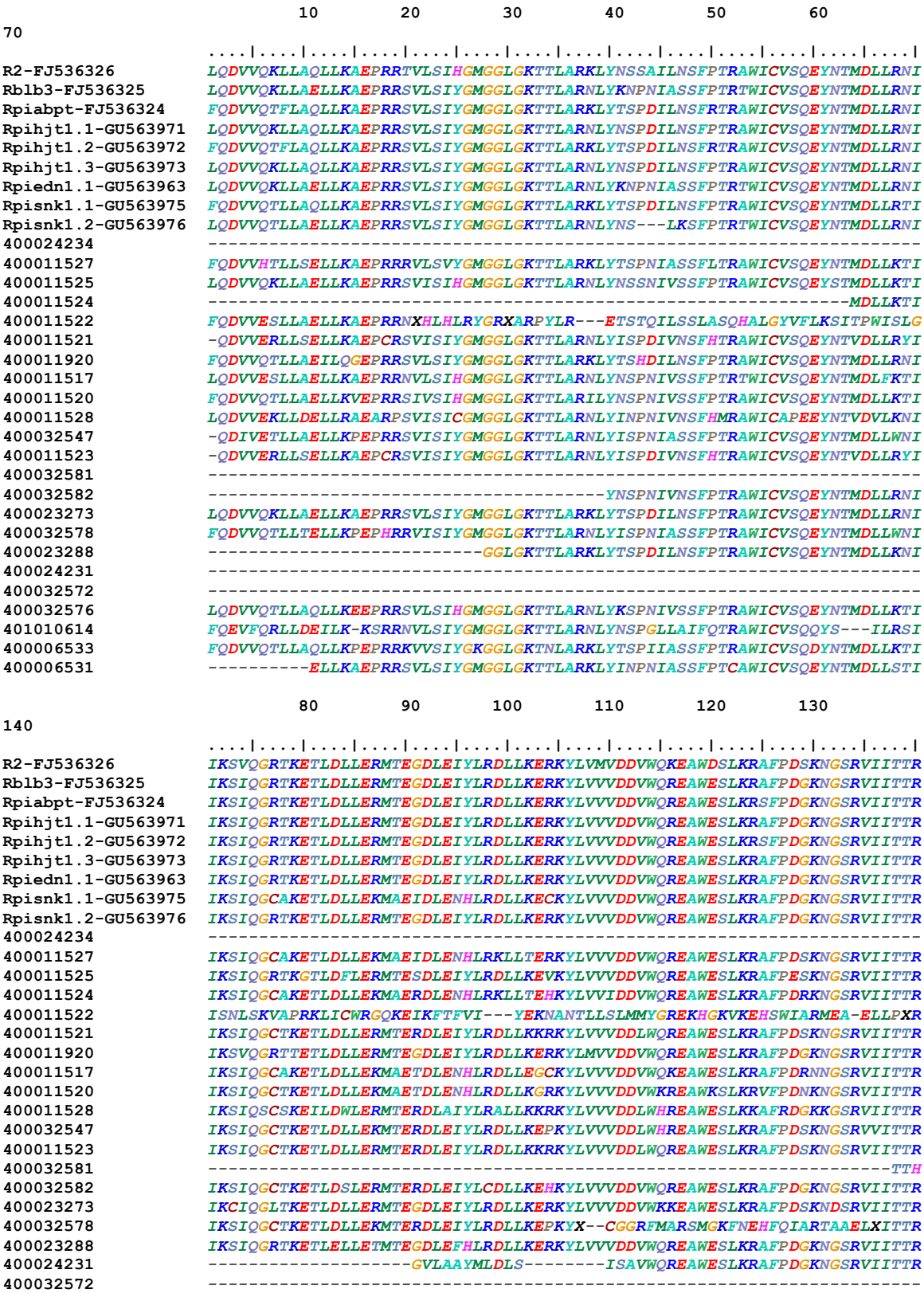
Protein sequence alignment of the NBS region of *R1* homologs

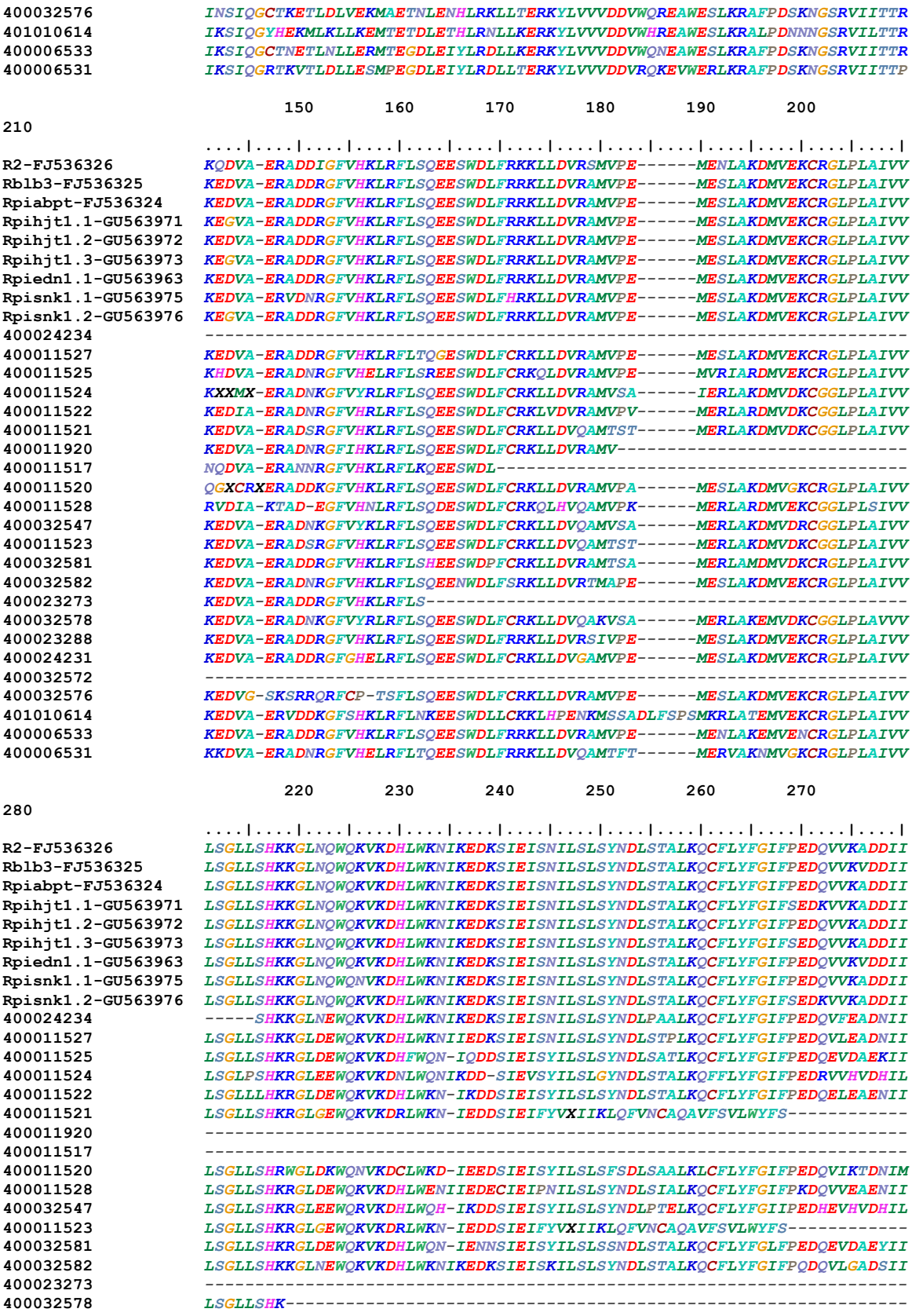


Protein sequence alignment of the LRR region of *R1* homologs



Protein sequence alignment of the NBS region of *R2* homologs

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400006533 IKSIQGCTNETLNLLERMTEGDLEIYLRDLLKERKYLVVVDDVWQNEAWESLKRAFPDSKNGSRVIITTR
400006531 IKSIQGRTKVTLDLLESMPEGDLEIYLRDLLTERKYLVVVDDVRQKEVWERLKRAFPDSKNGSRVIITTP
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210
R2-FJ536326 KQDVA-ERADDIGFVHKLRFLSQEESWDLFRKKLLDVRSMVPE------MENLAKDMVEKCRGLPLAIVV
Rblb3-FJ536325 KEDVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpiabpt-FJ536324 KEDVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpihjt1.1-GU563971 KEGVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpihjt1.2-GU563972 KEDVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpihjt1.3-GU563973 KEGVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpiedn1.1-GU563963 KEDVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpisnk1.1-GU563975 KEDVA-ERVDNRGFVHKLRFLSQEESWDLFHRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
Rpisnk1.2-GU563976 KEGVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
400024234 ----------
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400011522 KEDIA-ERADNRGFVHRLRFLSQEESWDLFCRKLVDVRAMVPV------MERLARDMVDKCGGLPLAIVV
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400011920 KEDVA-ERADNRGFIHKLRFLSQEESWDLFCRKLLDVRAMV----------
400011517 NQDVA-ERANNRGFVHKLRFLKQEESWDL----------
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400032547 KEDVA-ERADNKGFVYKLRFLSQEESWDLFCRKLLDVQAMVSA------MERLAKDMVDRCGGLPLAIVV
400011523 KEDVA-ERADSRGFVHKLRFLSQEESWDLFCRKLLDVQAMTST------MERLAKDMVDKCGGLPLAIVV
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400032582 KEDVA-ERADNRGFVHKLRFLSQEENWDLFSRKLLDVRTMAPE------MESLAKDMVEKCRGLPLAIVV
400023273 KEDVA-ERADDRGFVHKLRFLS----------
400032578 KEDVA-ERADNKGFVYRLRFLSQEESWDLFCRKLLDVQAKVSA------MERLAKEMVDKCGGLPLAVVV
400023288 KEDVA-ERADDRGFVHKLRFLSQEESWDLFRRKLLDVRSIVPE------MESLAKDMVEKCRGLPLAIVV
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400032572 ----------
400032576 KEDVG-SKSRRQRFCP-TSFLSQEESWDLFCRKLLDVRAMVPE------MESLAKDMVEKCRGLPLAIVV
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400006531 KKDVA-ERADNRGFVHELRFLTQEESWDLFRRKLLDVQAMTFT------MERVAKNMVGKCRGLPLAIVV
220 230 240 250 260 270
280
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Rblb3-FJ536325 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFPEDQVVKVDDII
Rpiabpt-FJ536324 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFPEDQVVKADDII
Rpihjt1.1-GU563971 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFSEDKVVKADDII
Rpihjt1.2-GU563972 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFPEDQVVKADDII
Rpihjt1.3-GU563973 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFSEDQVVKADDII
Rpiedn1.1-GU563963 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFPEDQVVKVDDII
Rpisnk1.1-GU563975 LSGLLSHKKGLNQWQNVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFPEDQVVKADDII
Rpisnk1.2-GU563976 LSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLYFGIFSEDKVVKADDII
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400011527 LSGLLSHKKGLDEWQKVKDHLWKNIIEDKSIEISNILSLSYNDLSTPLKQCFLYFGIFPEDQVLEADNII
400011525 LSGLLSHKRGLDEWQKVKDHFWQN-IQDDSIEISYILSLSYNDLSATLKQCFLYFGIFPEDQEVDAEKII
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400011517 ----------
400011520 LSGLLSHRWGLDKWQNVKDCLWKD-IEEDSIEISYILSLSFSDLSAALKLCFLYFGIFPEDQVIKTDNIM
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400032572          ----------------------------------------LSTVLKQCFLYFGIFPEDQEVDAEKII
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                            290
                   ....|....|....|
R2-FJ536326        RLWMAEGFIPRGEE-
Rblb3-FJ536325     RLWMAEGFIPRGEE-
Rpiabpt-FJ536324   RLWMAEGFIPRGEE-
Rpihjt1.1-GU563971 RLWMAEGFIPRGEE-
Rpihjt1.2-GU563972 RLWMAEGFIPRGEE-
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400011521          ---------------
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400011528          RLWMAEXF-------
400032547          WLWMAEGFIPTGEE-
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400032581          WLWMAEGFIPNGEE-
400032582          RLWMVEGFTPIGEE-
400023273          ---------------
400032578          ---------------
400023288          HLWMAEGFIPRGEE-
400024231          RLWMAEGFVPNGDE-
400032572          LLWMAEGFIPNGEE-
400032576          WLWMAEGFVPRGEE-
401010614          RLWLAEGFIPRIEH-
400006533          RLWMAEGFIVPRGEE
400006531          RLWMAEGFIPRGEE-
```

Protein sequence alignment of the LRR region of *R2* homologs

```
                            10        20        30        40        50        60
70
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|
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Rpiblb3-FJ536326   SNILSLSYNDLSTALKQCFLYFGIFPEDQVVKVDDIIRLWMAEGF-IPRGEERMEDVAEGFLNELIRRSL
Rpiabpt-FJ536324   SNILSLSYNDLSTALKQCFLYFGIFPEDQVVKADDIIRLWMAEGF-IPRGEERMEDVADGFLNELIRRSL
Rpihjti.1-GU563971 SNILSLSYNDLSTALKQCFLYFGIFSEDKVVKADDIIRLWMAEGF-IPRGEERMEDVAEGFLNELIRRSL
Rpihjt1.2-GU563972 SNILSLSYNDLSTALKQCFLYFGIFPEDQVVKADDIIRLWMAEGF-IPRGEERMEDVADGFLNELIRRSL
Rpihjt1.3-GU563973 SNILSLSYNDLSTALKQCFLYFGIFSEDQVVKADDIIRLWMAEGF-IPRGEERMEDVAEGFLNELIRRSL
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Rpisnk1.2-GU563976 SNILSLSYNDLSTALKQCFLYFGIFSEDKVVKADDIIRLWMAEGF-IPRGEERMEDVAEGFLNELIRRSL
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400011517          --------LXLSNSKLRSIMFFDPHICN-VFQHIDVFRHIYVLYLDIDYGKVIPDAIGSLYNLKLLRLSV
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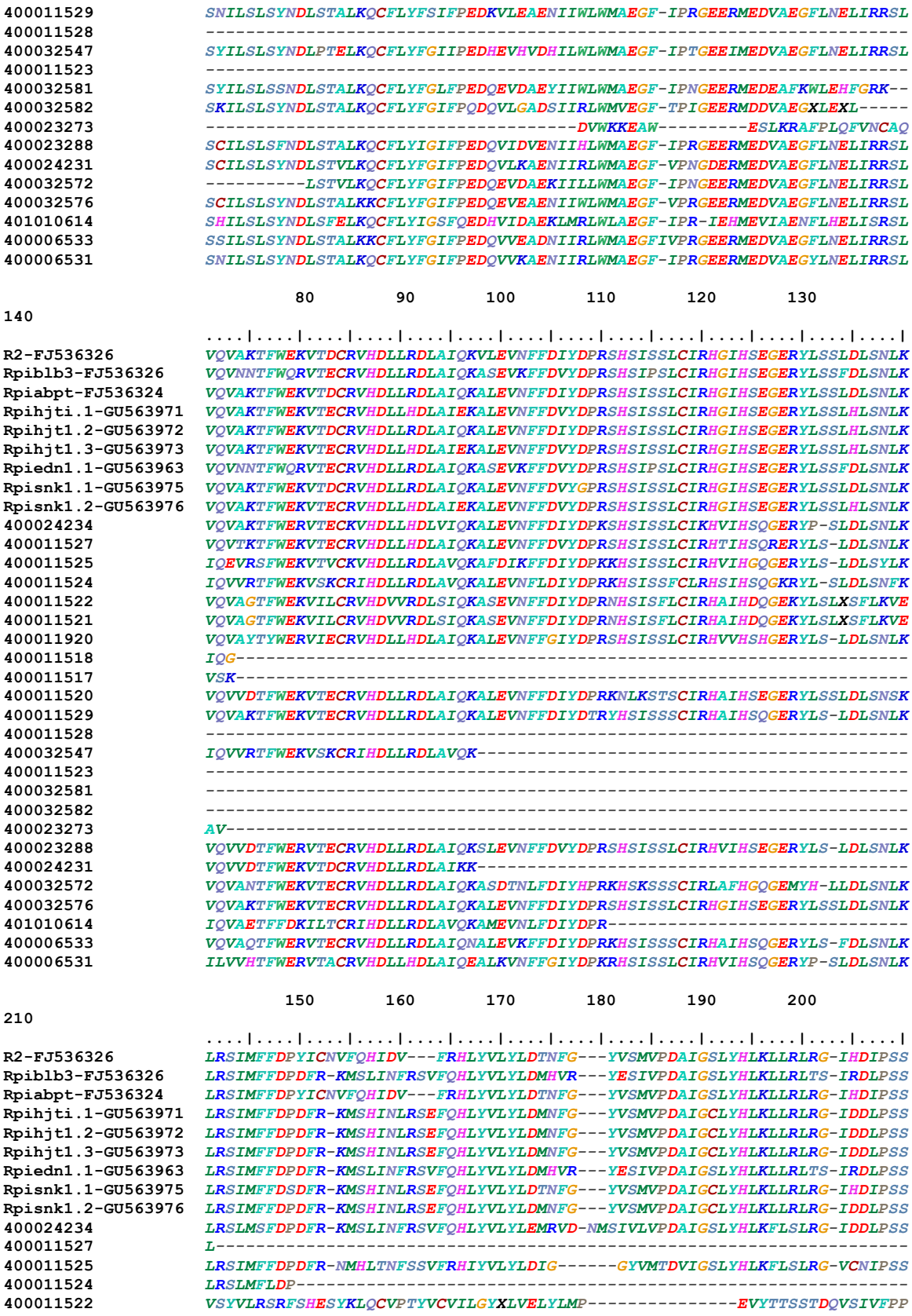
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400032572 ----------LSTVLKQCFLYFGIFPEDQEVDAEKIILLWMAEGF-IPNGEERMEDVAEGFLNELIRRSL
400032576 SCILSLSYNDLSTALKKCFLYFGIFPEDQEVEAENIIWLWMAEGF-VPRGEERMEDVAEGFLNELIRRSL
401010614 SHILSLSYNDLSFELKQCFLYIGSFQEDHVIDAEKLMRLWLAEGF-IPR-IEHMEVIAENFLHELISRSL
400006533 SSILSLSYNDLSTALKKCFLYFGIFPEDQVVEADNIIRLWMAEGFIVPRGEERMEDVAEGFLNELIRRSL
400006531 SNILSLSYNDLSTALKQCFLYFGIFPEDQVVKAENIIRLWMAEGF-IPRGEERMEDVAEGYLNELIRRSL
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140
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Rpiblb3-FJ536326 VQVNNTFWQRVTECRVHDLLRDLAIQKASEVKFFDVYDPRSHSIPSLCIRHGIHSEGERYLSSFDLSNLK
Rpiabpt-FJ536324 VQVAKTFWEKVTDCRVHDLLRDLAIQKALEVNFFDIYDPRSHSISSLCIRHGIHSEGERYLSSLDLSNLK
Rpihjti.1-GU563971 VQVAKTFWEKVTECRVHDLLHDLAIEKALEVNFFDVYDPRSHSISSLCIRHGIHSEGERYLSSLHLSNLK
Rpihjt1.2-GU563972 VQVAKTFWEKVTDCRVHDLLRDLAIQKALEVNFFDIYDPRSHSISSLCIRHGIHSEGERYLSSLHLSNLK
Rpihjt1.3-GU563973 VQVAKTFWEKVTECRVHDLLHDLAIEKALEVNFFDVYDPRSHSISSLCIRHGIHSEGERYLSSLHLSNLK
Rpiedn1.1-GU563963 VQVNNTFWQRVTECRVHDLLRDLAIQKASEVKFFDVYDPRSHSIPSLCIRHGIHSEGERYLSSFDLSNLK
Rpisnk1.1-GU563975 VQVAKTFWEKVTDCRVHDLLRDLAIQKALEVNFFDVYGPRSHSISSLCIRHGIHSEGERYLSSLDLSNLK
Rpisnk1.2-GU563976 VQVAKTFWEKVTECRVHDLLHDLAIEKALEVNFFDVYDPRSHSISSLCIRHGIHSEGERYLSSLHLSNLK
400024234 VQVAKTFWERVTECKVHDLLHDLVIQKALEVNFFDIYDPKSHSISSLCIKHVIHSQGERYP-SLDLSNLK
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400011525 IQEVRSFWEKVTVCKVHDLLRDLAVQKAFDIKFFDIYDPKKHSISSLCIRHVIHGQGERYLS-LDLSYLK
400011524 IQVVRTFWEKVSKCRIHDLLRDLAVQKALEVNFLDIYDPRKHSISSFCLRHSIHSQGKRYL-SLDLSNFK
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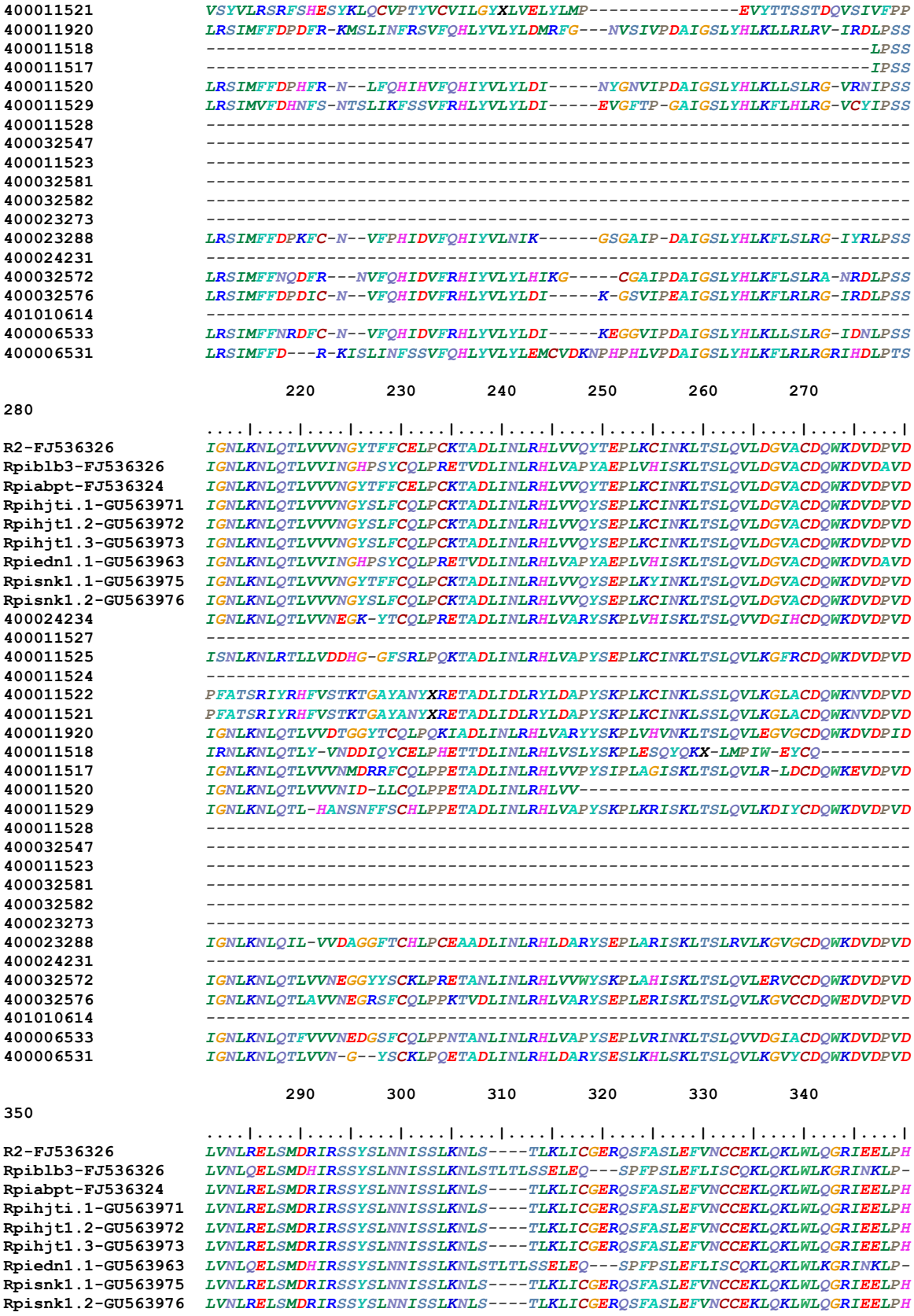
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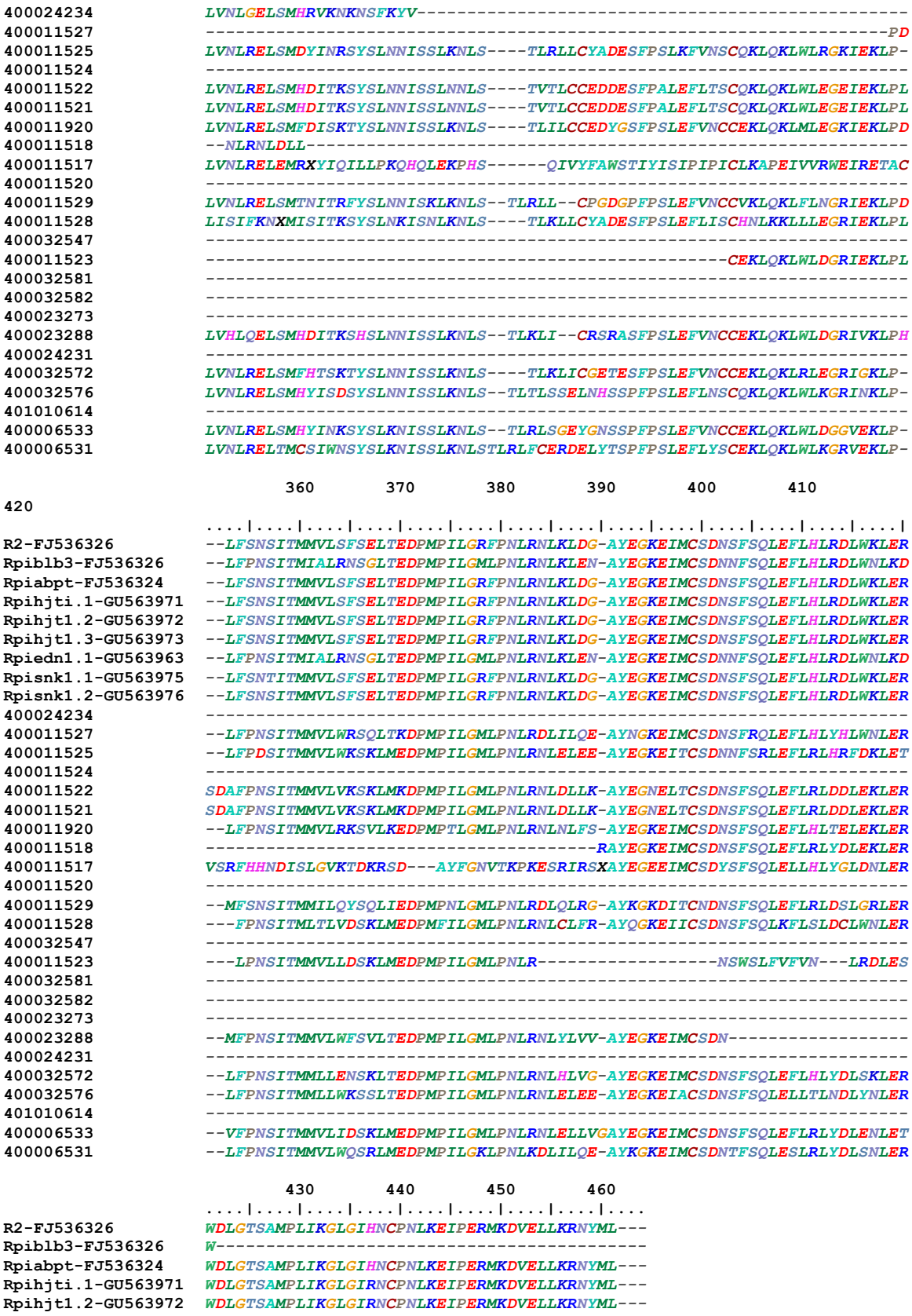
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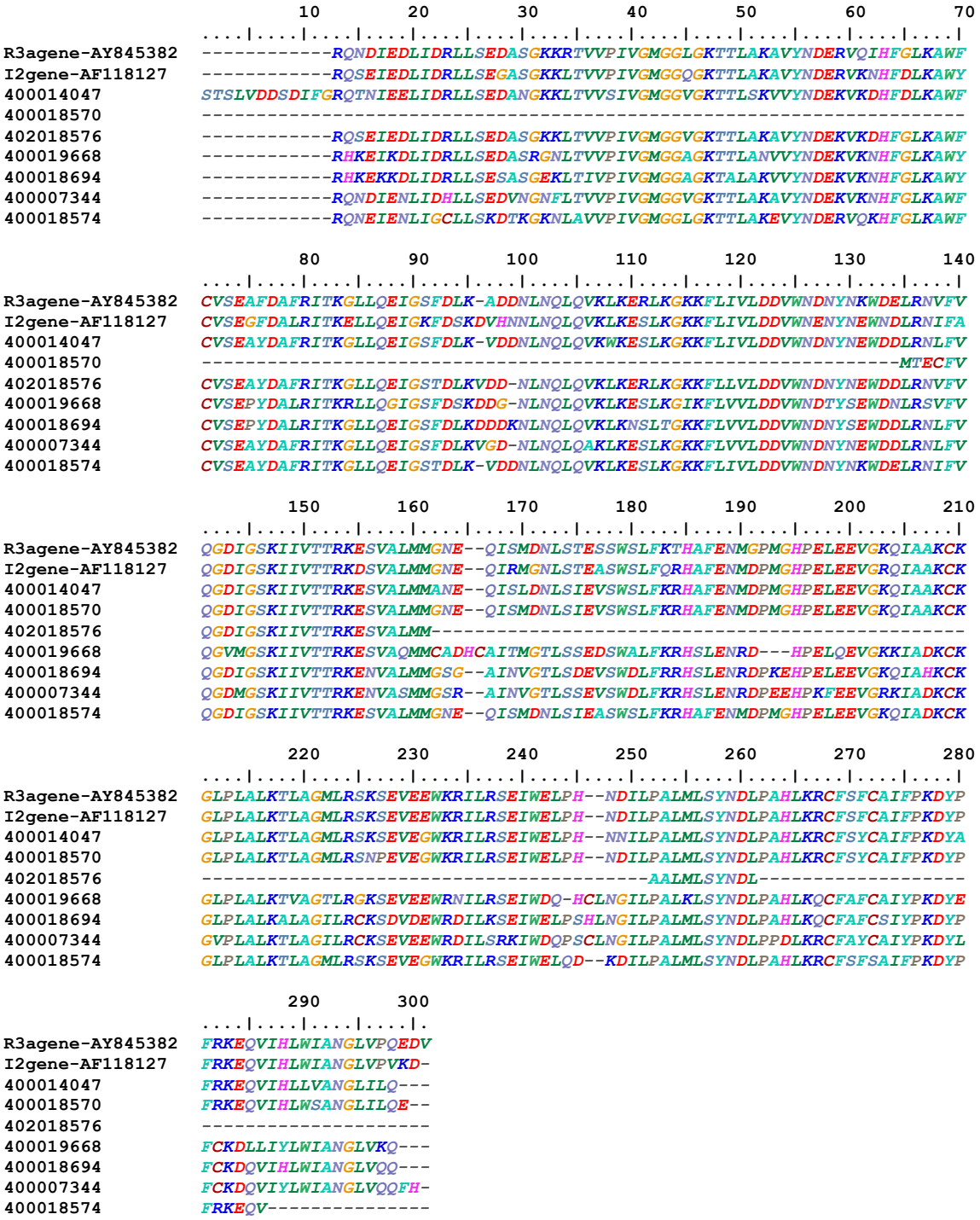
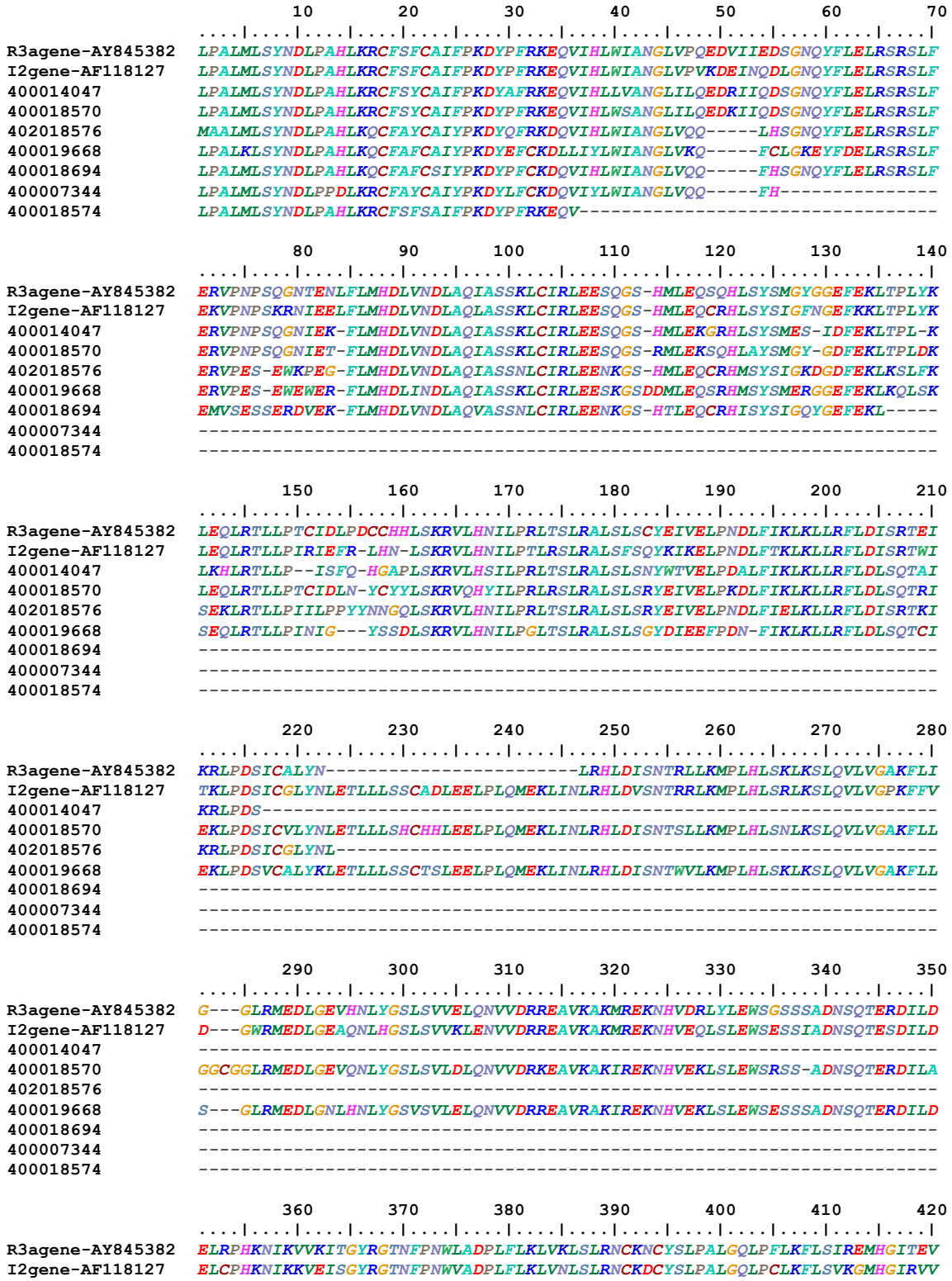
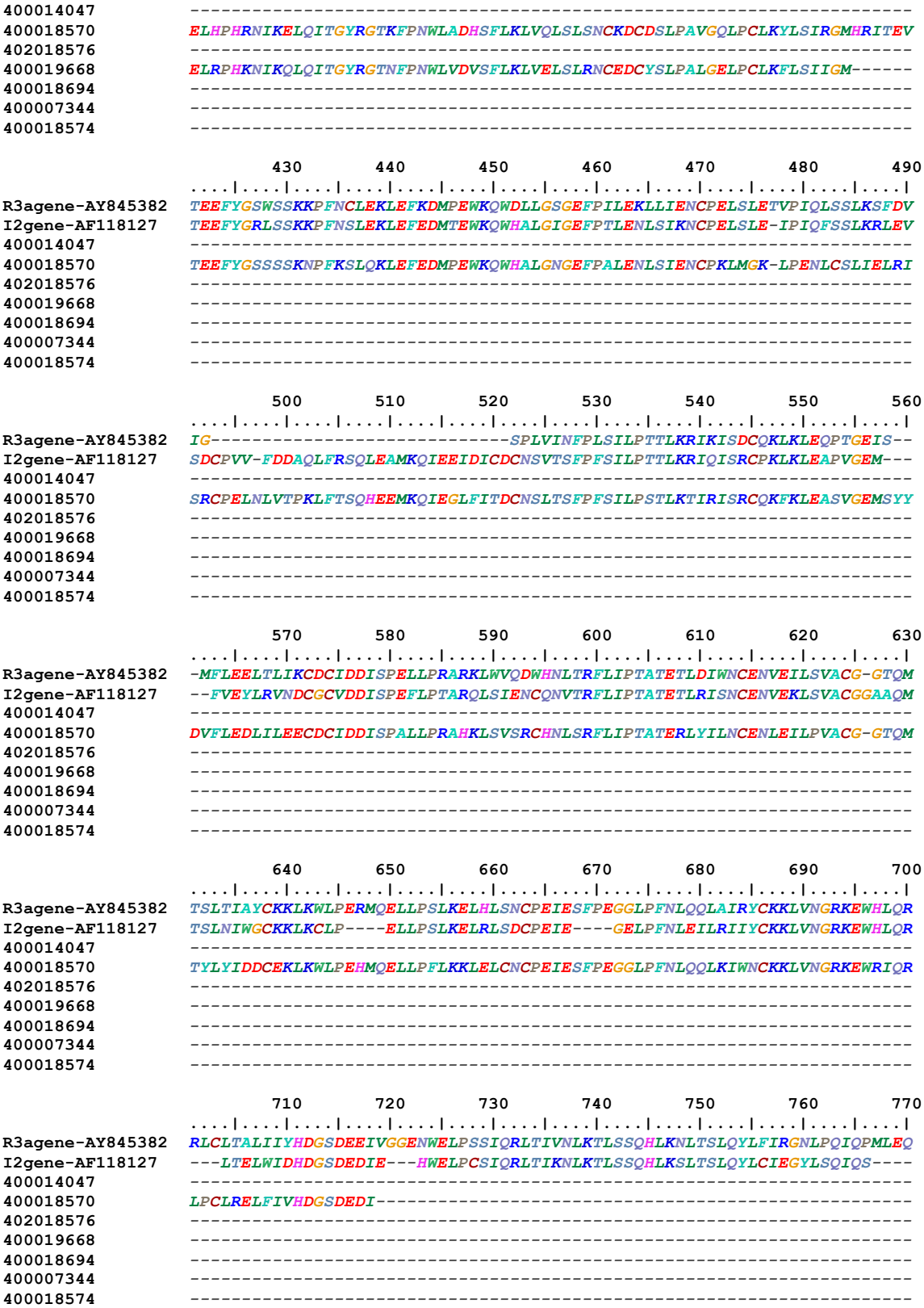
Protein sequence alignment of the NBS region of *R3a* homologs



Protein sequence alignment of the LRR region of *R3a* homologs

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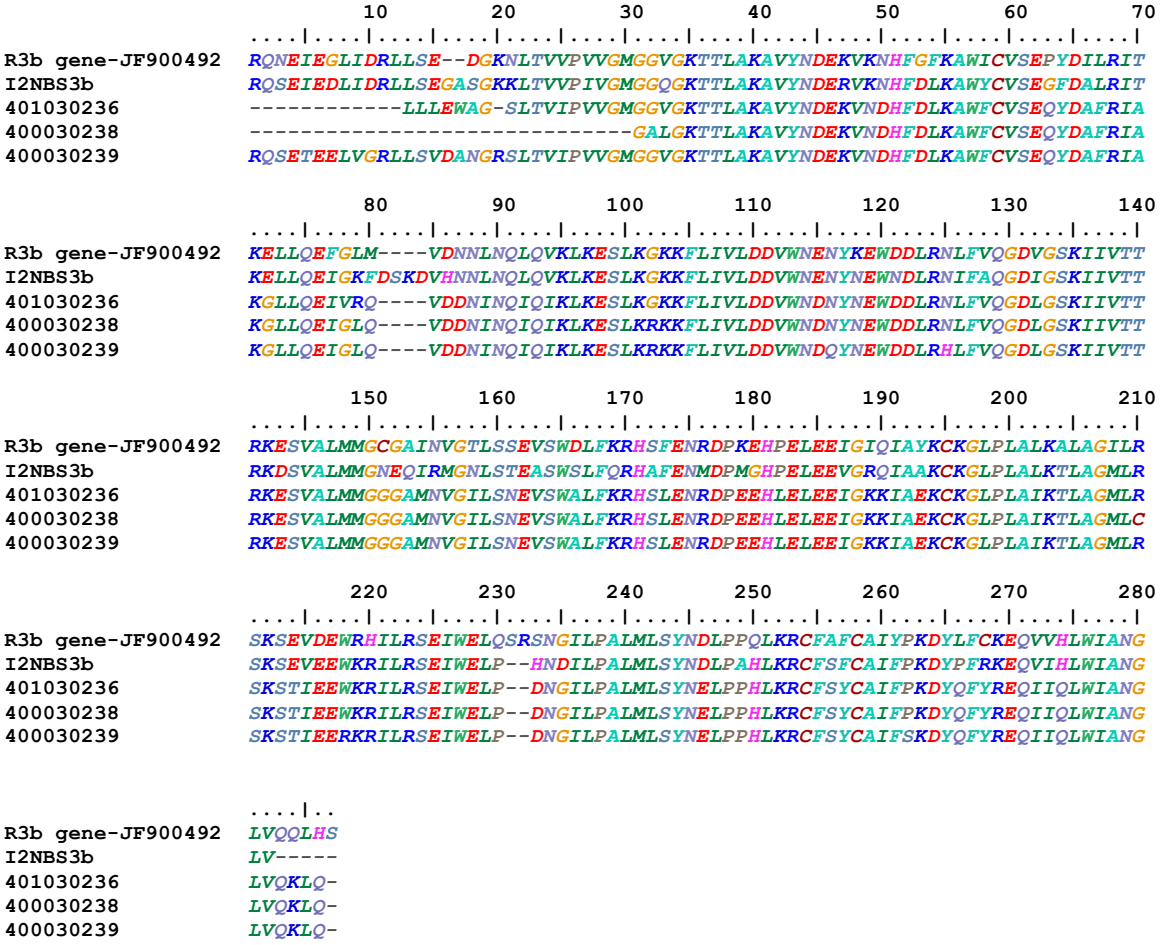
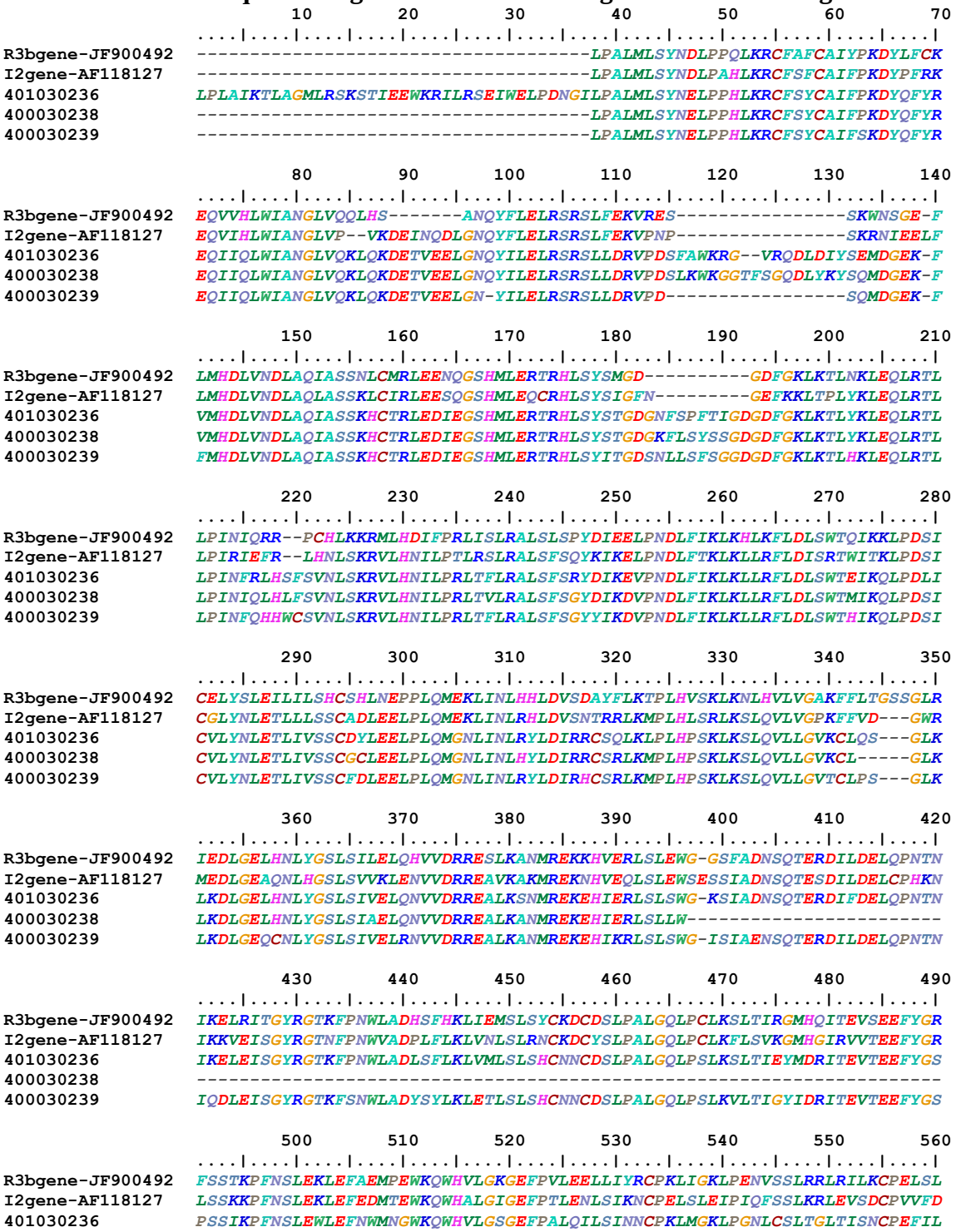
Protein sequence alignment of the NBS region of *R3b* homologs



Protein sequence alignment of the LRR region of *R3b* homologs



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I2gene-AF118127    D------------------------AQLFRSQLEAMKQIEEIDICDCNSVTSFPFSILPTTLKRIQISR
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R3bgene-JF900492   CGELKLEASMNAMFLEKLSLVKCD-----SPELVPRARNLSVRSCNNLTRLLIPTATERLSIRDYDNLEI
I2gene-AF118127    CPKLKLEAPVGEMFVEYLRVNDCGCVDDISPEFLPTARQLSIENCQNVTRFLIPTATETLRISNCENVEK
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400030238          ------------------------------------------------------------------------
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I2gene-AF118127    LSVACGGAAQMTSLNIWGCKKLKCLP----ELLPSLKELRLSDCPEI----EGELPFNLEILRIIYCKKL
401030236          LLVASRTPTLR-NLHIHSCKKLKSLPKHMQELLPSLNYLNLYDCPEIKSFPEGGLPFSLEVLQIWNCEKL
400030238          ------------------------------------------------------------------------
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400030238          ------------------------------------------------------------------------
400030239          ENDRKEWHLQRLPCLRKLHILHDGSDE----GIHWELPCSIRKLLV------------------------

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401030236          ------------------------------------------------NLPQIQSLIEEG---------
400030238          ------------------------------------------------------------------------
400030239          ------------------------------------------------------------------------

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R3bgene-JF900492   ------------------------------------------------------
I2gene-AF118127    IQDCPNLQSLPFKGMPSSLSKLSIFNCPLLTPLLEFDKGEYWPQIAHIPIINID
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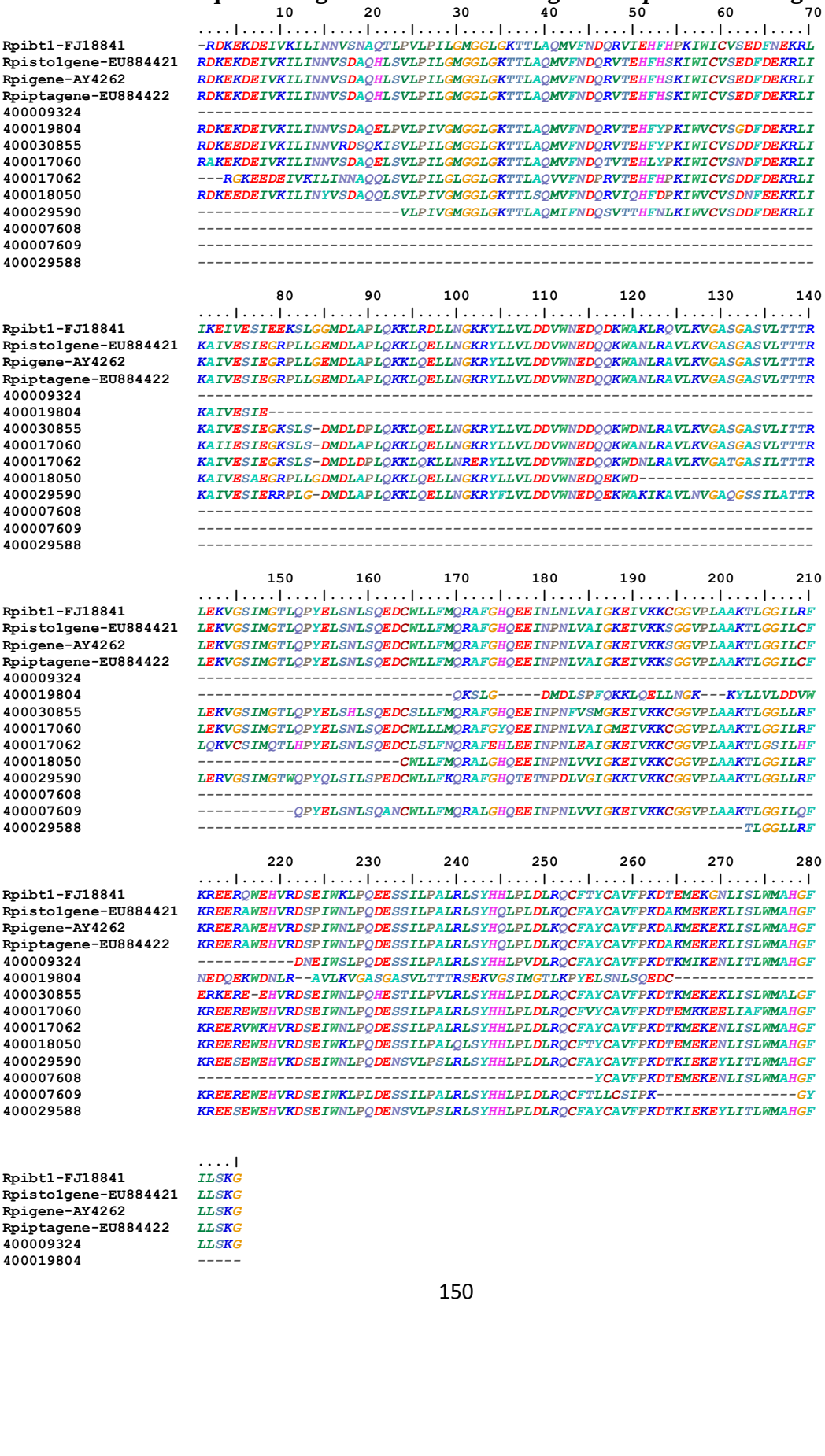
Protein sequence alignment of the NBS region of *Rpi-bt1* homologs



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400017062            LSSKG
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400029590            L----
400007608            LLSKG
400007609            -----
400029588            LLSKG
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Protein sequence alignment of the LRR region of *Rpi-bt1* homologs

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Rpisto1-EU884421     LRVLNLG--DSTFNKLPSSIGDLVHLRYLNLYGS-GMRSLPKQLCKLQNLQTLDLQYCTKLCCLPKETSK
Rpigene-AY4261       LRVLNLG--DSTFNKLPSSIGDLVHLRYLNLYGS-GMRSLPKQLCKLQNLQTLDLQYCTKLCCLPKETSK
Rpipta1 gene-EU884422 LRVLNLG--DSTFNKLPSSIGDLVHLRYLNLYGS-GMRSLPKQLCKLQNLQTLDLQYCTKLCCLPKETSK
400009324            LRVLNLS--YSKLEQLPSSIGDLLHLRYLDLSRN-NFRSLPERLCKLQNLQTLDVHNCYSLNCLPKQTSK
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Rpigene-AY4261       AKEANLSAKGNLHSLSMSWNNF-GPHIYESEEVKVLEALKPHSNLTSLKIYGFRGIHLPEWMNHSVLKNI
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400007609            ------------------------------------------------------------------------
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400029590            ------------------------------------------------------------------------

                                220       230       240       250       260       270       280
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Rpipta1 gene-EU884422 VSILISNFRNCSCLPPFGDLPCLESLELHWGSADVEYVEEVDIDVHSGFPTRIRFPSLRKLDIWDFGSLK
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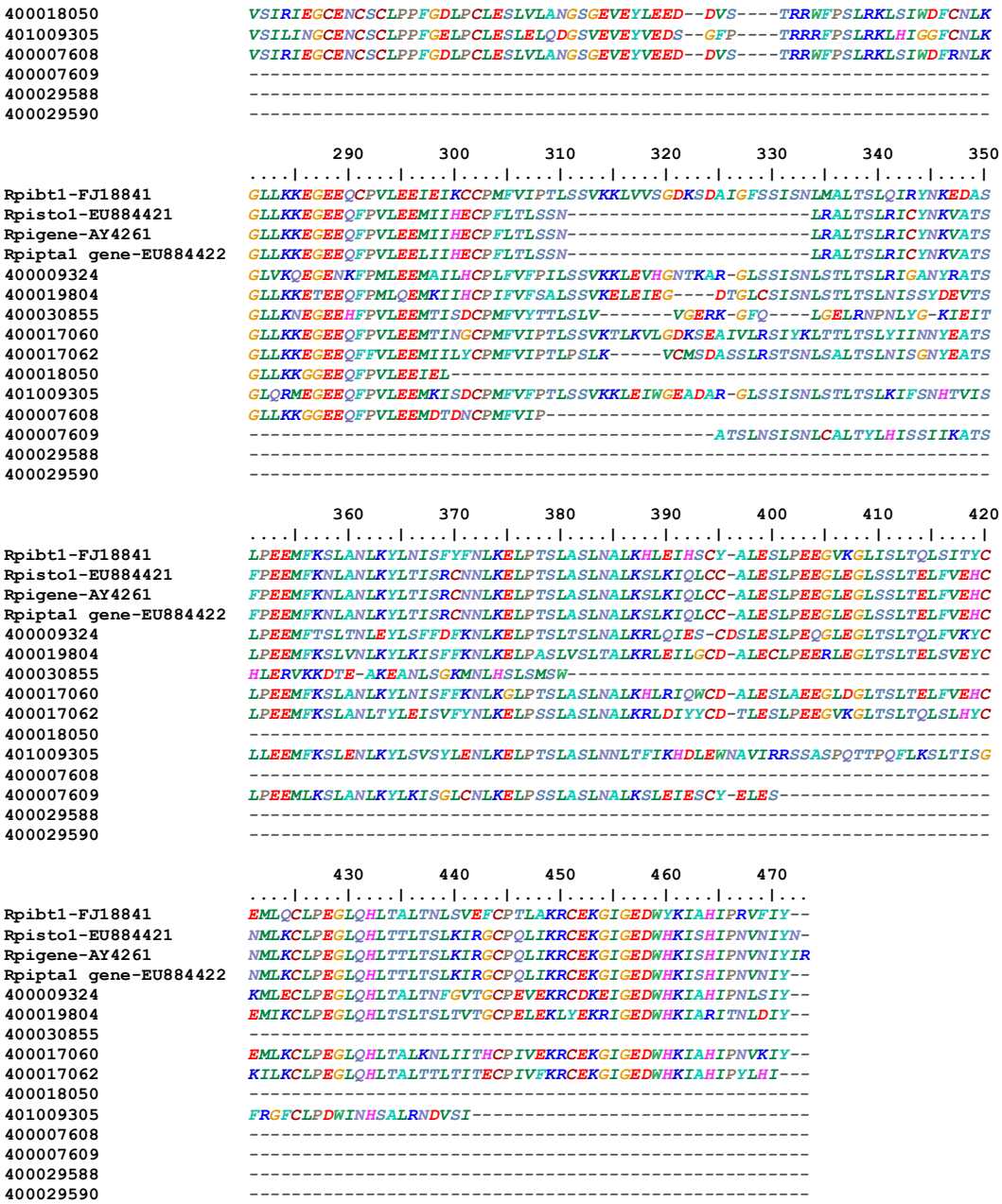
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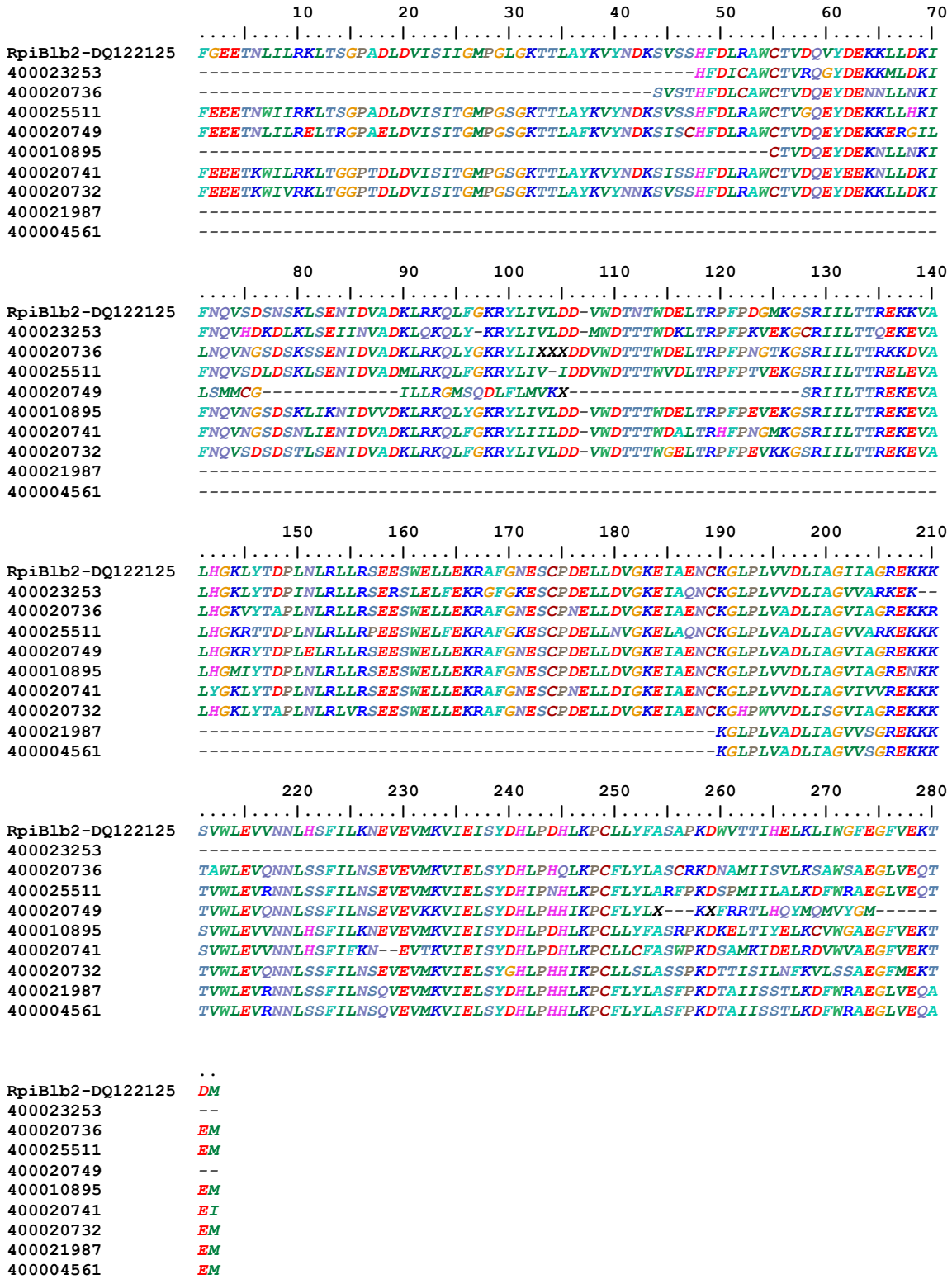
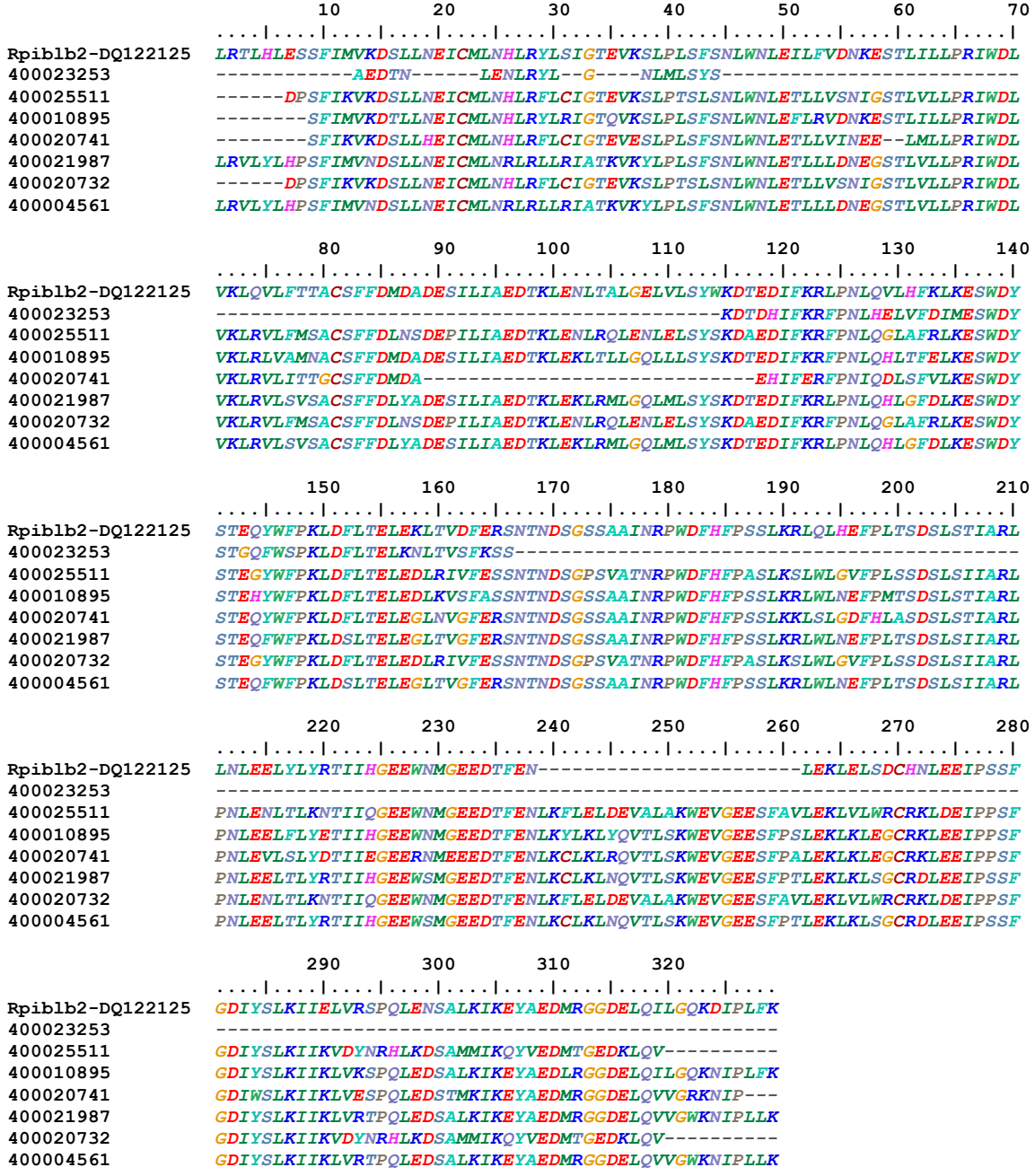
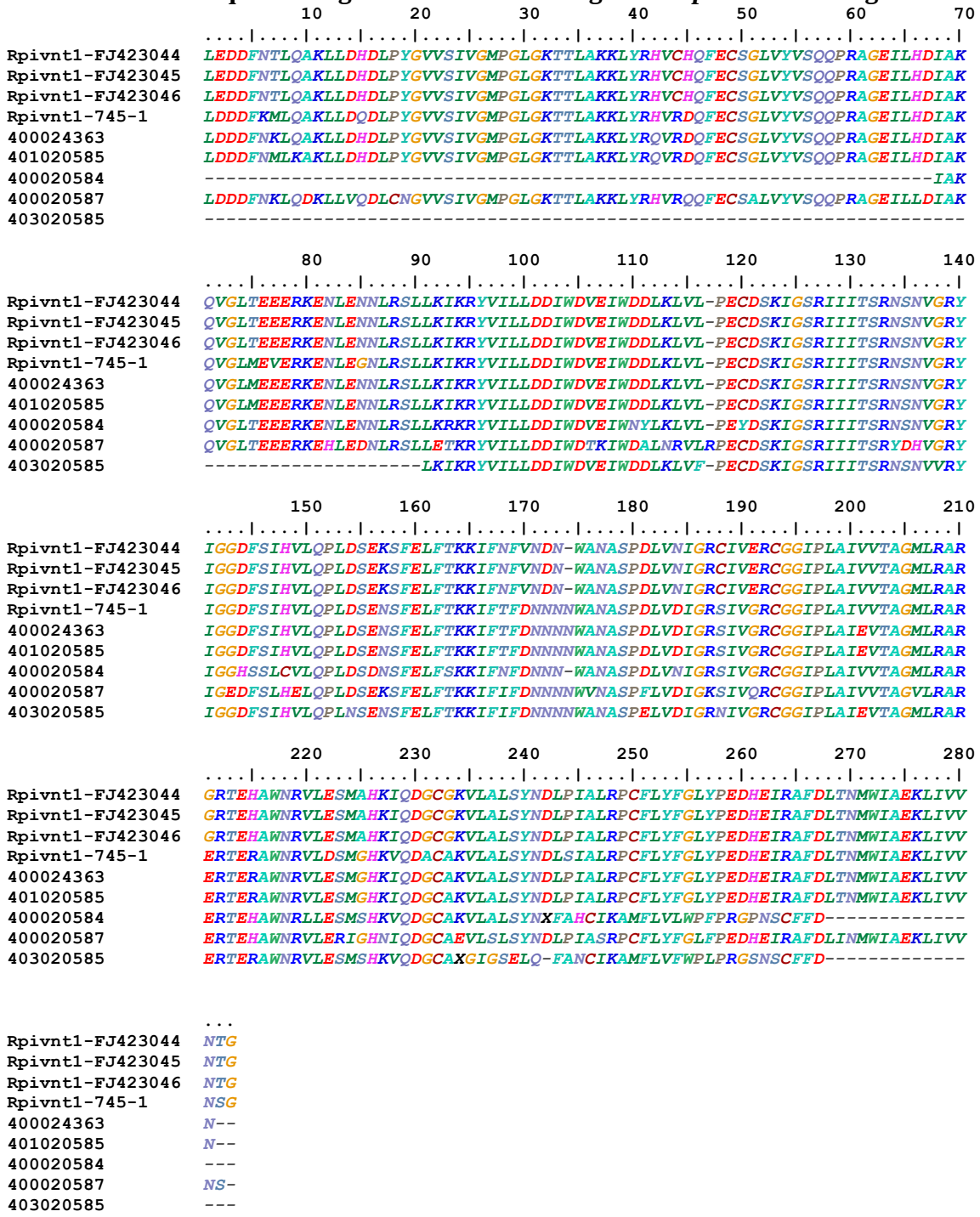
Protein sequence alignment of the NBS region of *Rpi-blb2* homologs



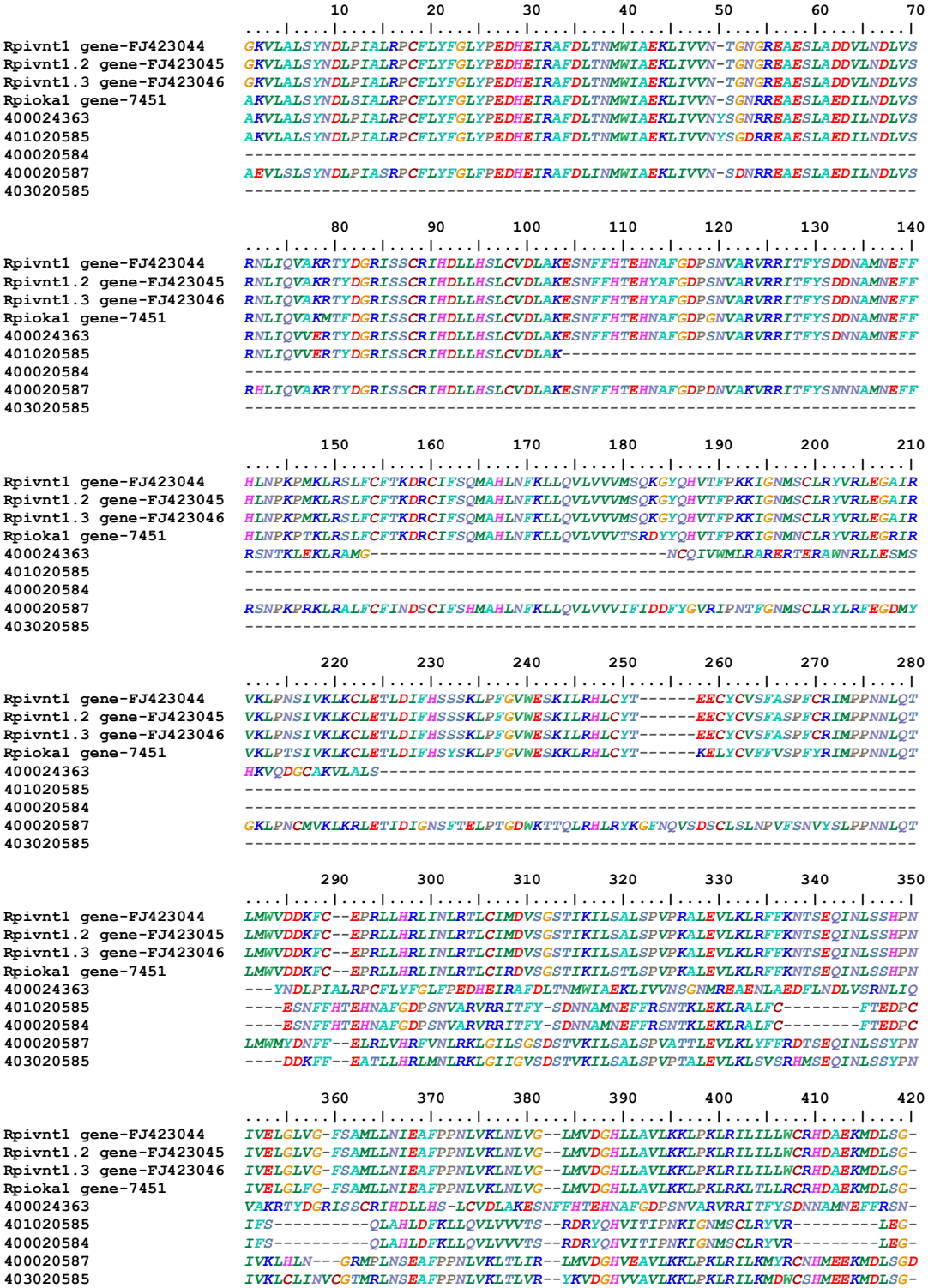
Protein sequence alignment of the LRR region of *Rpi-blb2* homologs



Protein sequence alignment of the NBS region of *Rpi-vnt1* homologs



Protein sequence alignment of the LRR region of *Rpi-vnt1* homologs



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Rpivnt1.3 gene-FJ423046-DSFPQLEVLYIEDAQGLSEVTCMDDMSMPKLKKLFLVQGPNISPISLRVSERLAKLRISQVL
Rpioka1 gene-7451      -DSFPQLEVLHIEDAHGLSEVTCMDDMSMPKLKKLLLVQGPIISPISLRVSERLAKLRISQVL
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400020584              --------RIRVKLPNSIVKLKCLETL-----------------------------------
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Appendix 5. Characteristics of qPCR primer pairs of gene homologs selected from the TC database

Code	Homolog ID	Function	Primer	Size (bp)	Tm (°C)	GC	Amp Tm (°C)	Amp length (bp)
22GH2	1011522	HJRTR2GH1	TCCATTTGCAACCTCAAGAATTTA	24	59	33	78	64
			GGCATAAGCGCCCGTTT	17	58	59		
45B23	25545	R1B-23	CAGTGATCAAATGGCTAACCTTCAG	25	59	45	77	76
			ATCTAGTATTGGCAGATGAATGAGATTG	28	59	40		
Bt609	7609	Rpi-bt1	TTCACACCGCTTTTCCAGTGT	21	59	48	79	63
			GCACCTAACAGCCCTCACAAG	21	58	57		
Bo54	24754	Rboh-B	AATGTCGACGCCACTCTTAGC	21	58	52	80	63
			GCCCGGTCTGCACTAATCAC	20	59	60		
RbohA	12316	Rboh-A	TCTTCTCCTTATTTTCTACACGGTTTCT	28	59	36	72	53
			CCTTGTGATGGAAAAGAGTTTGTTT	25	59	36		
RbohC	14168	Rboh-C	CCAAGTCCAATGACGATGCTT	21	59	48	79	58
			AGCCACAGAGTCTTCACGAACA	22	59	50		
P10-94	2001494	PR10-protein	CGTTTTAATTACGAGCGCAGTTC	23	59	43	78	65
			GCGCAAGAAGGTTTGAAGCT	20	58	50		
235A3	2030235	R3-TC	TCTATTAGAGCTATGCATTTACGAGTGA	28	58	36	76	71
			TCAAGGACCAAGGAGAATGGA	21	58	48		

Code	Homolog ID	Function	Primer	Size (bp)	Tm (°C)	GC	Amp Tm (°C)	Amp length (bp)
Pp54	20554	PPR1 protein	TGTCTAAGTTGGGAGGTGATATGC	24	58	46	77	70
			TTCGAGCCTCTTCTGAAATCCT	22	58	45		
53Bl2	23253	Rpi-blb2	TTCCATTCTCCCCCCTGAAT	20	59	50	78	64
			GCGAGACTGCCCAACTTGA	19	59	58		
glup78	2004978	Glutathione peroxidase	GGACATAAAAGTGCTGTTGGGAAT	24	59	42	78	70
			CGAAATCGATCCACATTGTTGA	22	59	41		
P1-74	37874	PR1-protein	GGCTTTCCCCGCACTCA	17	59	65	85	57
			TGCTTCTCATCGACCCACATC	21	52	52		
Ktp67	15267	Kunitz-type protease inhib	GGGTATCCACAACCAGGCTATC	22	58	55	77	72
			TGGCAGGCTTTGGTCATAATAA	22	58	41		
Aspi	10129	Aspartic protease inhibitor 10	CGGCGTGTTCCGTTACG	17	58	65	84	61
			TCTGCCACCCCTAGGCCTAT	20	60	60		
Serpi	26953	Serine protease inhibitor 1	TTTTGGTTGCATGAAACAAGCT	22	58	36	77	56
			TCTGTCTCTCATCTCCTTCTTTTCC	25	58	44		
Cyspi	10134	Cysteine protease inhibitor 1	CCAAACGCCGTGTTGCA	17	60	59	80	59
			GGGTGTGCCTTTTCCCATAA	20	58	50		