Molecular Genetic and Functional Characterization of candidate loci for controlling quantitative resistance to the oomycete *Phytophthora infestans*

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To my family

Table of contents

Abbreviations

Chapter1: Introduction 1

1.1 Importance and origin of the potato 1

1.2 The late blight pathogen *P. infestans* 2

1.2.1 The disease symptoms 2

1.2.2 The life cycle, biology and genome of *Phytophthora infestans* 3

1.2.3 Migration and genome of *P. infestans* 4

1.3 Breeding for resistance to *Phytophthora infestans* 6

1.4 Prospects for durable resistance 7

1.5 Plant Disease Resistance Genes 9

1.5.1 Major classes, Structure and Function 9

1.6 Qualitative versus quantitative resistance traits 12

1.6.1 Overview of QTLs to *P. infestans* in potato 12

1.6.2 Major *R*-gene clusters, mapped and cloned *R*-genes in potato for resistance to *P. infestans* 15

1.7 Single nucleotide polymorphism markers (SNPs) 16

1.8 The concept of linkage disequilibrium in plants 16

1.9 Candidate gene approach versus Genome-Wide analysis 18

1.10 Expression studies in potato 19

1.11 Goal of the thesis 20

Chapter 2: Materials and Methods 21

2.1 Plant material 21

2.1.1 “Cases” and “controls” for the analysis of linkage to QTL 21

2.1.2 “Cases” and “controls” for the analysis of association with QTL 21

2.1.3 Plants used for expression study 22

2.1.4 Field assessment of late-blight 22

2.2 Cultivation of *P. infestans* 22

2.2.1 Rye agar media preparation 22

2.2.2 *P. infestans* propagation (when starting the infection from mycelium on rye agar medium) 23
2.2.3 *P. infestans* inoculum preparation 23
2.2.4 Detached leaf assay 24
2.2.5 Whole plant infection assay 24
2.2.6 Tuber slices infection 25
2.2.7 Pathotype specificity assay (Black's differential test) 25
2.3 Frequently used buffers and medium 26
2.4 Molecular biology methods 26
2.4.1 BAC plasmid isolation and purification (Qiagen plasmid purification protocol modified) 26
2.4.2 Isolation of total genomic DNA 27
2.4.3 Southern gel blot analysis 29
2.4.3.1 Genomic DNA and BAC enzymatic digestion 29
2.4.3.2 Agarose gel treatment and DNA transfer to the membrane 29
2.4.3.3 Membrane hybridization and washing 30
2.4.3.4 Radioactive labeling and purification of DNA probe 30
2.4.4 Preparation of MethaPhor agarose gel 30
2.4.5 Determination of DNA and RNA concentration 31
2.4.6 Enzymatic digestion of amplified DNA- cleaved amplified polymorphic sequences (CAPS) 31
2.4.7 Identification of Single Strand Conformation Polymorphism (SSCP) 31
2.4.8 Total RNA isolation 32
2.4.9 First strand cDNA synthesis 33
2.4.10 RT-PCR (reverse-transcriptase polymerase chain reaction) 33
2.4.10.1 Scanning agarose gels from RT-PCR experiment 33
2.4.10.2 Parameters used for scanning the ethidium bromide stained gels 33
2.4.11 Standard PCR 34
2.5 Statistical methods 34
2.6 Set parameters for the DaX software 35
2.7 Databases and Softwares used 35

Chapter 3: Results **37**
3.1 QTL-Analysis for quantitative late blight resistance 37
3.1.1 Selecting the most resistant "cases" and the most susceptible "control" plants in SaKa-Ragis and BNA populations 37
3.1.2 Tagging QTL for *P. infestans* in SaKa-Ragis and BNA populations using SNP, SSCP, SCAR and CAPS markers 39
3.1.2.1 QTL analysis in BNA population 39
3.1.2.2 QTL analysis in SaKa-Ragis population 43
3.1.2.3 Extended marker study on chromosome X in the SaKa-Ragis population 44
3.1.2.3.1 Analyzing CP105 marker in GDE diploid population 45
3.1.2.3.2 Analyzing CP105 in the K31 diploid population 46
3.1.2.4 Haplotype marker CP105 on chromosome X in SaKa-Ragis population 47
3.1.2.5 Analyzing CP105 marker in the whole SaKa-Ragis population 48
3.2 Analysis of association with QTL on chromosome V based on "cases" and "controls" 50
3.2.1 Selecting "cases" and "controls" based on their original phenotypic passport data 50
3.2.2 SNP analysis of genomic markers in "Cases/ controls" association study on chromosome V 51
3.2.3 St*Pto* on chromosome V 55
3.2.4 Presence of a certain genotypic class in only one phenotypic group 56
3.2.5 Tight linkage between SNPs within a marker in the QTL gradient experiment 58
3.2.6 Statistic analysis with "cases" and "controls" genotypes including genotypic data from previously scored PCR based CAPS markers 59
3.3 Expression study on putative candidate genes on chromosome V 60
3.3.1 Phenotypic difference between I88 and G87 after infection with *P.infestans* 60
3.3.2 Monitoring the presence of *Phytophthora infestans* using specific primers for the ribosomal DNA 61
3.3.3 *PR*-1b specific band amplification in infected I88 and G87 plants 63

3.3.4 Differences of infection symptoms on the I88 and G87 parents in independent experiments. 64
3.3.5 Selecting the candidate genes 64
3.3.6 EST search in the TIGR, NCBI and TAIR databases, ORFs start and end position........ 66
3.3.7 Putative function assignment of candidate genes 70
3.3.8 *R*1-specific primers C76-2 71
3.3.9 Equalization of the cDNA using β-Tubulin primers........ 72
3.3.10 Classification of the candidate genes into groups........ 72
3.3.11 Differentially expressed genes........ 74
3.3.11.1 α-amylase gene (ORF45)........ 75
3.3.11.2 EST-unknown function (ORF46) 76
3.3.11.3 Protein kinase (ORF47) 77
3.3.11.4 EST unknown function (ORF 48)........ 78
3.3.12 Southern gel blot analysis for two expressed genes on chromosome V........ 79
Chapter 4: Discussion........ 81
4.1 QTL analysis in two tetraploid breeding populations........ 81
4.1.1 Candidate gene approach 81
4.1.2 QTL analysis in the SaKa-Ragis population........ 81
4.1.3 QTL analysis in the BNA population 82
4.1.4 "Alien" segregation........ 83
4.1.5 Distorted segregation in GDE and K31 populations........ 84
4.2 QTL gradient experiment........ 85
4.3 Expression study on chromosome V........ 88
4.3.1 Summary results of RT-PCR experiments for 25 PCG (putative candidate genes). 88
4.3.2 Leaf position and age reflect resistance to *P.infestans* 89
4.3.3 The role of anthocyanin in plant defense........ 89
4.3.4 Hypersensitive response (HR) in the initial biotrophic phase of the oomycete in the QR parent G87 after *P.infestans* infection. 90
4.3.5 Differentially expressed genes........ 91

4.3.5.1 *PR*1-b gene 91
4.3.5.2 Amylase (ORF 45) 91
4.3.5.3 Role of Protein kinase genes in plants (ORF47) 92
4.3.5.4 EST (ORF 46) and EST (ORF48) - unknown function. 92
4.3.6 Analyzing *in silico* the data from microarray experiments in the TIGR database 93
4.3.7 Comparisons with previous expression studies upon *P.infestans* infection. ... 94
Summary **96**
Zusammenfassung **97**
References **100**
Appendix **113**
Table A: List of primer pairs used for the QTL analysis. 113
Table B: List of primer pairs used for the expression study on chromosome V. 115
Table C: List of primer pairs used for the QTL gradient experiment on chromosome V. 117
Table D: Digestion pattern of alleles descending from SR1, SR2, NK5 and NK6 for the SSCP markers 118
Table E: Varieties included in the QTL gradient experiment. 119
SNP position for the markers included in the QTL gradient experiment on chromosome V. 121
Domain structure of the predicted ORFs 124
ERKLÄRUNG **134**
Acknowledgments **135**
Lebenslauf **136**

Abbreviations:

ANOVA	analysis of variance
APS	ammoniumperoxodisulfate
ASO	allele specific oligonucleotide
***Avr* gene**	avirulence gene
BAC	bacterial artificial chromosome
BNA	BNA population
bp	base pairs
CAPS	cleaved amplified polymorphic sequence
“cases”	the highly resistant genotypes
CC	coiled coil
“controls”	the highly susceptible genotypes
CTAB	cetyl trimethyl ammonium bromide
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguanidinetriphosphate
dNTP	deoxynucleotidetriphosphate
dTTP	deoxythymidinetriphosphate
EDTA	ethylene diamine tetra acetic acid
Fig.	figure
hpi	hours post inoculation
HR	hypersensitive response
JA	jasmonate acid
KIN	serine-threonine kinase
LB	Luria Bertani
LRR	leucine rich repeat
MAS	marker assisted selection
MPIZ	Max-Planck Institute for Plant Breeding Research

NB	nucleotide binding site
ORF	open reading frame
PCG	putative candidate genes
PCR	polymerase chain reaction
PEG600	polyethyleneglycol (molecular weight=600)
***PR*1**	pathogenesis related gene 1
QR	quantitatively resistant
QRL	quantitatively resistant locus
QS	quantitatively susceptible
QTL	quantitative trait locus/loci
rAUDPC	relative area under disease progress curve
RFLP	restriction fragment length polymorphism
rpm	rounds per minute
RT	room temperature
SA	salicylic acid
SAR	systemic acquired resistance
SARA	SaKa-Ragis population
SCAR	sequence characterized amplified region
SDS	sodium dodecil sulfate
SNP	single nucleotide polymorphism
SSCP	single strand conformational polymorphism
Ta	annealing temperature
TAE	tris acetate EDTA
TBE	tris borate EDTA
TEMED	tetramethyl-ethylenediamine
TIR	toll interleukin receptor
v/v	volume per volume
w/v	weight per volume

Chapter1: Introduction

1.1 Importance and origin of the potato

The potato is the fourth most important crop worldwide, after maize, rice and wheat (online FAOSTAT data 2004). Because of its importance, the potato became an object for extensive studying its genetics of resistance to pathogens, as well as improving cooking and nutrition qualities like chips quality and starch content.

The greatest potato production countries are Russia, followed by China, Poland, India and the western countries, thus the world potato annual production amounts to about 300 million tons per year (Hawkes 1990, online FAOSTAT data 2004).

The introduction of potato to Europe and its arrival details remain and probably will remain an unelucidated historical event. However, there is agreement for two main introductions of potato in Europe, the first introduction of tetraploid *andigena* forms into Spain in about 1570 probably from Colombia and the second introduction into England between 1588 and 1593. There are still some disputes concerning the place of potato origin. In general it is accepted that the potato originates from the Andes of Peru and Bolivia. It is being hypothesed that there are two areas of domestication: a first area in the central Andes and a second independent area in Chile (Hawkes 1990). Juzepczuk and Bukasov (1926) believed that the potato came from Chile because Chilean potatoes, which are adapted to form tubers under long day conditions of southern latitudes similar to the day length in Europe, would adapt immediately to the European climatic conditions. Salaman (1946) contested this hypothesis, as when the first journey, which has been made from Chile through the Strait of Magellan, the potato has already been known in Europe in 1579. According to Salaman it would more plausible, when the potato was sent to Europe from Peru or the north coast of Colombia, particularly from the port at Cartagena. In support to this hypothesis, the first potatoes grown in Europe were "short-day adapted" and tuberized under 12-hours day length or less in the milder regions of Spain, Italy, Southern France, for example. Moreover the earliest herbarium specimen of the potato made by Caspar Bauhin around 1620 that is known to us is obvious by a specimen of subspecies *andigena* from the Andes and not of subspecies *tuberosum* from Chile. The genus *Solanum* contains seven cultivated and 228 wild potato species. The

potato genome occurs in a range of chromosome numbers, from 2n=24 (diploid) in for example *S. stenotomum*, *S. phureja* and *S. ajanhuiri*, 3n=36 (triploid) in *S. chaucha* and *S. juzepczukii*, 4n=48 (tetraploid) in *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *andigena*, 5n=60 (pentaploid) in *S. curtilobum* to 6n=72 (hexaploid) in *S. demissum* (Hawkes 1990).

The cultivated potato was named by Linnaeus (Carl von Linné) from a specimen grown in Europe and the name *Solanum tuberosum* is presently used to include domesticated potatoes from South America, Europe and the USA and derivatives of them in the rest of the world (Ingram and Williams 1991).

The species has been divided into groups by Dodds, as *S. tuberosum* Group *tuberosum* for all the European and North American tetraploid cultivars, and *S. tuberosum* Group *andigena* for all the tetraploid cultivated potatoes in the Andes from Venezuela to Northern Argentina.

Wild potato has a wide geographical distribution showing wide range and climatic adaptation to diverse ecological diversity such as wide range of temperatures and humidity. Some species, such as an *S. acaule* can survive sub-zero temperatures, others such as *S. berthaultii* and *S. gracilifrons* are adapted to hot, dry, semi-desert conditions.

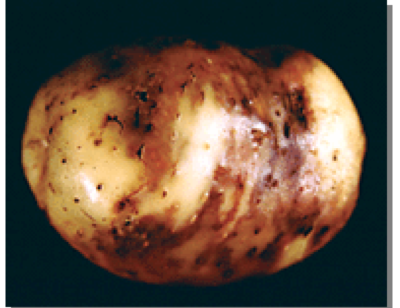
1.2 The late blight pathogen *P. infestans*

1.2.1 The disease symptoms

Potato late blight is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary. The pathogen was the causal agent of the Irish famine in 1845 when millions of people died from starvation, and many were prompted to immigrate to other countries.

Late blight disease symptoms appear on the foliage, stems and tubers of potato (Fig.1.1) The disease can easily destroy a whole potato field within two weeks when the climate conditions favor its development. The disease can be easily transmitted to the next potato generation as the oomycete can over winter in the tubers. When planted the next year in the field, the symptoms develop progressively with the plant growth. The first disease symptoms appear as brownish specks followed by water-soaked lesions on the leave

surface or stems. Later on, the infected areas are covered with cotton- like, fluffy mycelium of *P. infestans*.



(Garelik G. 2002) www.cra.wallonie.be

Fig.1.1. Disease symptoms on potato caused by the oomycete *P. infestans*: all parts of the potato plant are targets for infection. Infected potato leaves are shown on the left, tubers- in the middle and whole plants in the field- on the right.

1.2.2 The life cycle, biology and genome of *Phytophthora infestans*

The oomycete grows at high humidity of almost 100 percent and at temperatures between 15 and 25°C. Over 30°C the oomycete sporulates and waits for favorable conditions to start its life cycle when coming in contact with potato plants.

The infection cycle of *P.infestans* begins when sporangia land on the surface of plant tissue (Fig.1.2) At the above mentioned temperatures, the sporangium releases biflagellate zoospores (3 to 8 per sporangium), which then produce a germ tube on the infected plant tissue. Direct germination of the zoospores is also possible when the temperature is higher then 16°C. After germ tube formation it penetrates through the cuticle or stomata and the haustoria (biotrophic feeding structure of the pathogen) are formed in the plant invaded cell. The invaded host cells eventually dies. *P. infestans* is considered as a hemibiotroph, as in the first infection stage the pathogen requires biotrophic contact with the host plant and in the later phase it proceeds to develop its structures on the killed plant tissue as a necrotrophic pathogen.

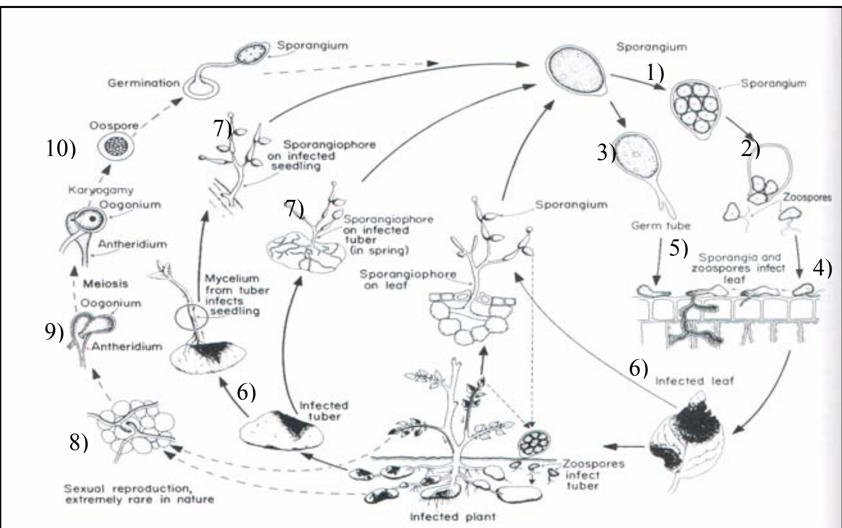


Fig.1.2. Life cycle of *P. infestans* (picture adopted from Agrios 1997)

Asexual reproduction
1) Sporangium (3-8 zoospores); 2) release of the zoospores from the sporangium; 3) direct sporangium germination; 4) zoospore landing on the plant tissue; 5) zoospore germination and haustorium development; 6) infested tissues (leave, tubers, stems); 7) sporangium formation on the sporangiophore
Sexual reproduction
8) Hyphen mating between A1 and A2 mating type; 9) oogonia and antheridia generation;
10) diploid 2n oospore

P.infestans is heterothallic (the male and female organs are on different individuals), having A1 and A2 mating types. The two mating types differ in hormone production and response rather than in morphology of the different sexual forms (Judelson et al. 1997). In response to the hormones, the two mating forms-male (antheridia) and female (oogonia) form gametangia within the mating zone where meiosis undergoes and asexual sporulation is inhibited. Haploid nuclei, one from the antheridia and the second from the oogonia, fuse in the gametangia and generate one diploid, viable nucleus. In progeny of hybrid gametangia (A1A2), only A1 or A2 types develop from the germinated oospore (Judelson et al. 1997, Ingram and Williams 1991).

1.2.3 Migration and genome of *P. infestans*

The A1 mating type most likely migrated to Europe in 1840 where it caused the famous potato famine in 1846. The second migration, namely the appearance of A2 mating type in Europe, is assumed to have occurred in the late 1970. There are four hypotheses for the

occurrence of the A2 mating type in Europe: a) it was always in present but was not discovered; b) it was introduced by migration; c) it arose by mutation or mitotic recombination; d) arose by mating type change. The most supported hypothesis to date is the occurrence of the A2 mating type due to migration from Mexico (Goodwin et al. 1994, 1997).

For a long time, *P. infestans* has been grouped with true fungi. Phylogeny studies based on rRNA (Förster et al. 1999) and mitochondrial gene sequences (Chesnick et al. 1996) confirmed the closer relationship of the pathogen with diatoms and brown algae rather then true fungi. Based on the last phylogeny data, *P. infestans* belongs to the Order Pythiales and the Phylum Oomycota (Fig.1.3) (Judelson 1997, Judelson and Blanco 2005).

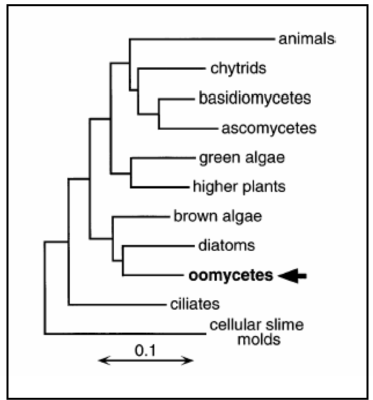


Fig.1.3. Phylogenetic tree based on small rRNA sequence similarities showing the close relationship of the oomycetes to diatoms and brown algae and not to the true fungi (Judelson 1997).

Due to the destructive power of *P. infestans* on potato cultivars and millions of dollars losses every year, the genome of the pathogen is being studied extensively resulting in a recently sequenced and database released mitochondrial genome (www.tigr.org. Genbank Accession NC_002387). Research partners in the *Phytophthora infestans* genome sequencing project have the goal to finish sequencing the *P. infestans* genome till the end of 2005 (www.oardc.ohio-state.edu). The genome size is estimated about 237Mb.

1.3 Breeding for resistance to *Phytophthora infestans*

P. infestans is the causal pathogen of the Irish famine in 1845 on potato cultivars. Since that disaster a lot of effort was devoted to improve resistance of potato cultivars to the pathogen. Expeditions to Argentina, Chile, Bolivia, Ecuador, Mexico and Peru were organized collecting wild potato species. Most of the participating scientists were from England, Germany, Russia, The Netherlands and USA. The first great expedition is considered to be the Russian expedition in 1925 to Mexico when 15 new cultivated species were found. The second, probably the largest collecting expedition occurred in 1939 when more then 1400 potato samples, mostly cultivated species, were brought to Europe. Later, many more small expeditions have been organized enriching the potato germplasm with new wild species (Hawkes 1990).

Important potato germplasm collections are held in IPK Genbank Außenstelle Nord, (Groß Lüsewitz, Germany) supported by the German and the Dutch government. This collection represents the material of H. Ross and his colleagues from Latin America collected in the German botanical-agricultural expedition to the Andes in 1959 (Hawkes1990). To this we owe nowadays many valuable wild potato species with increased resistance to *P. infestans* due to bringing together the efforts of all people participating in the expeditions to Latin America in search of new germplasm. Surprisingly, Hawkes (1990) summarized that only 13 of 228 wild species had contributed to the resistance germplasm in the European cultivars. The need, for new resistant potato sources arose, because of the appearance of the second A2 mating type in Europe in 1970. The A1 mating type was probably imported around 1840 when shortly after in 1845 the Irish potato famine occurred. The A1 and A2 types can mate and give rise to recombinant and more virulent *P. infestans* strains able to overcome defense apparatus in the plant.

The Mexican hexaploid species *S. demissum*, from which 11 *R* genes have been introgressed into cultivated potato is one of the major sources used to improve resistance in Europe. Wild species such as *S. bulbocastanum* (Song et al. 2003, van der Vossen et al. 2003, Park et al. 2005), *S. berthaultii* (Ewing et al. 2000), *S. pinnatisectum* (Kuhl et al. 2001), *S. phureja* (Costanzo et al. 2005), *S. microdontum* (Sandbrink et al. 2000), *S.*

commersonii (Iovene et al. 2004)*, S. mochiquiense* (Smilde et al. 2005) have been in breeding programs (Hawkes1990).

The first appearance of resistance to the phenyl amide fungicides was reported in 1979 in Ireland, Switzerland and The Netherlands resulting from the migration of the A2 mating type from Mexico to Europe (Fry et al. 1993, Gisi et al. 1996). An urgent task to potato breeders is the finding of new resistant sources among potato germplasm and to introgress it into improved varieties. All 11 *R*-genes introgressed into the cultivated potato from *S. demissum* have race-specific resistance to *P. infestans* (resistance to only one race). *RB* gene and its allelic gene *Rbi-blb*1 introgressed from *S. bulbocastanum* and cloned on chromosome X have broad-spectrum resistance (resistance to all known races to *P. infestans* (Song et al. 2003, van der Vossen et al. 2003, 2005)). The gene might be race specific as all the others *R* genes, when a new virulent strain appears. Resistance conferred by introgression of wild species into cultivated results in qualitative (Kuhl et al. 2001) or quantitative resistance-when several genes are introgressed into cultivated potato (Sandbrink et al. 2000, Ewing et al. 2000, Iovene et al. 2004, and Costanzo et al. 2005).

When trying to cross resistant wild species to cultivated, problems often arise from genotype differences as well as ploidy level, embryo balance number (EBN) (Johnston and Hanneman 1982) or post-zygotic barriers. To overcome such cytological problems several procedures have been successfully applied based on bridge crossing (Carputo et al. 2003), doubling chromosome numbers, using 2n gametes and somatic hybridization (Szczerbakowa et al. 2003, Zimnoch-Guzowska et al. 2000).

1.4 Prospects for durable resistance

Several strategies have been described to achieve durable resistance in plants (McDowell and Woffenden 2003). One way to engineer broad-spectrum resistance in plants is to coordinate the expression of an *R*-gene with the corresponding *Avr* transgene from the pathogen where the transgene is controlled by a pathogen-inducible plant promoter. This strategy enables the synthesis of the *Avr* product upon pathogen attack and eventual by the interaction between *R* and *Avr R-Avr* which will trigger a resistance response in the

plant. This strategy unfortunately can not be applied extensively at the moment for potato and *P. infestans* because of missing knowledge of cloned and characterized *Avr* genes from the pathogen. For example, just very recently the first *Avr* gene *Avr*3 from *P. infestans* was cloned and characterized by Armstrong and colleagues (2005). Other transgenic method is to express antimicrobial proteins in the plants. Alternative to the transgenic approaches is the application of naturally occurring defense-inducing compounds onto the plant. For example the application of salicylic acid as a foliar spray induces systemic acquired resistance. The disadvantage of the above mentioned strategies is that they induce a hypersensitive response (HR) around the infection site, which is an effective mechanism against biotrophic pathogens but not against necrotrophs, which colonize mainly killed plant tissue (McDowell and Woffenden 2003).

The most used and robust strategy in breeding programs is the alternative to single-gene deployment; multiple *R*-genes (pyramids) can be bred into individual plant lines. Pyramiding several *R*-genes into a cultivar provides more durable resistance since mutations in several *Avr* genes of the pathogen would be required to escape detection, which is not so likely and fast occurring in terms off pathogen virulence gene evolution. Normally, the *Avr* gene is recognized by the corresponding *R*-gene in the plant and triggers the defense responses. When a mutation occurs in the *Avr*-gene it can not be recognized anymore by the corresponding *R*-gene in the plant and becomes virulent, thus the plant becomes susceptible to the pathogen.

The development of marker assisted selection (MAS) whereby breeders select for molecular markers linked to *R*-genes, enables pyramiding of more than one effective *R* gene in the individual lines. This process creates new *R*-genes combinations for which pathogen may not be ready to evolve matching virulence genes (Pink, 2002). The recently cloned *RB* gene from *S. bulbocastanum* on potato chromosome VIII (Naess et al. 2000, Song et al. 2003, and van der Vossen et al. 2003) confers broad-spectrum resistance to late-blight in potato. The gene was found in a cluster with other three *R*-gene analogs (RGA) derived from the RB haplotype of *S. bulbocastanum*, where only one of the RGA`s, namely RGA2-RB, confers full resistance to *P. infestans*. Findings of such importance will certainly provide a valuable resource for using such potato species for

developing late-blight resistant potato cultivars with broad spectrum resistance to *P. infestans*.

Wild potato species exhibit different levels of resistance ranging from immunity to susceptibility. Vleeshouwers et al. (2000a) show the differences in response to *P. infestans* in a collection of wild species where the different species react rather in a quantitative manner with major differences in severity and timing of developing HR (hypersensitive response). The wild potato species *S. demissum* from which 11 resistance genes (*R*-genes) have been introduced into cultivated potato provides race-specific hypersensitive resistance in the field, which is rapidly overcome by new virulent pathotypes of *P. infestans* (Staples 2004).

Creating pyramids of *R*-genes in single cultivated potato lines, combined with finding novel *R* genes especially without race specificity such as the *RB* gene will facilitate the breeding process. Moreover, there are already achievements in combining of *S. tuberosum* with *S. bulbocastanum* via somatic hybridization followed by backcross procedures. Progeny of this cross showed resistance to *P. infestans* that was comparable to those in *S. bulbocastanum* over four year-trial test (Helgeson 1998).

1.5 Plant Disease Resistance Genes

1.5.1 Major classes, Structure and Function

Plant diseases can dramatically reduce crop yields and have disastrous impact especially in developing countries. Pesticides and chemical control can provide effective protection against plant diseases, but have also environmental adverse effects. Therefore much effort is invested towards understanding innate resistance mechanisms in plants (McDowell and Woffenden 2003).

In the past ten years over 40 *R* genes have been cloned conferring resistances to bacteria, fungi, oomycetes, nematodes, viruses and insects (Martin et al. 2003). The vast majority of the cloned genes belong to the nucleotide-binding –leucine rich repeat (NB-LRR), extracelular LRR (eLRR), or LRR-Kinase super families. The plant disease resistance genes are divided into five major classes according to their structural domains (Dangl and Jones 2001, Mc Dowell and Woffenden 2003, Martin et al. 2003). There are several *R*

genes that do not belong to any of the five classes like *Mlo* of barley (Buschges et al. 1997), *Hm1* (Johal et al. 1992) of maize and *Ve* (Kawchuk et al. 2001) proteins in tomato. Thus, the first major class of resistance genes belongs to the NB-LRR proteins with the N-terminus having a coiled-coil domain (CC) or Toll and Interleukin-1 receptor like domain (TIR). Interestingly, analysis of plant EST databases revealed no obvious TIR-NBS-LRR-like protein in monocots. This fact might suggest some fundamental differences in resistance mechanisms between dicots and monocots. The second class of proteins has only an extracelular LRR (eLRR) and transmembrane domain. The third class LRR-kinase superfamily consists of an eLRR fused to a cystoplasmic serine-threonine kinase domain (KIN). The fourth class of genes has only Ser-Thr kinase domain. The fifth class of *R*-genes is membrane anchored and contains a putative coiled-coil domain (Fig.1.4).

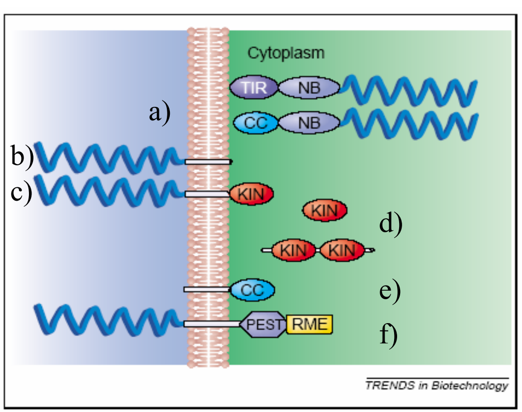


Fig.1.4. Major Families of *R* proteins (McDowell and Woffenden 2003):

a) NB-LRR proteins- nucleotide binding site attached to a leucine-rich repeat and a CC or TIR domain at the N-terminus (*RB* gene in potato, Song et al. 2003, van der Vossen et al. 2003, *P* gene in flax Dodds et al. 2001);
b) eLRR proteins family-consists of extracytoplasmic leucine-rich repeats anchored to a transmembrane domain *e.g*. all *Cf*-genes in tomato (Jones et al.1994);
c) LRR-kinase super family consists of an eLRR fused to a cytoplasmic serine-threonine kinase domain (KIN) (*Xa*21 gene in rice Song et al. 1995);
d) Ser-Thr kinase without LRRs (*Pto* gene in tomato (Tang et al. 1996), *Rpg*1 gene in maize (Brueggeman et al.2002));
e) Membrane anchored, fused to a putative coil-coiled domain (only one gene *RPW*8 in Arabidopsis (Xiao et al. 2003));
f) Proteins containing putative extracellular LRRs, domain for protein degradation (PEST) and short protein motif for targeting the protein for receptor-mediated endocytosis (RME) (*Ve*2 gene in tomato (Kawchuk et al. 2001)).

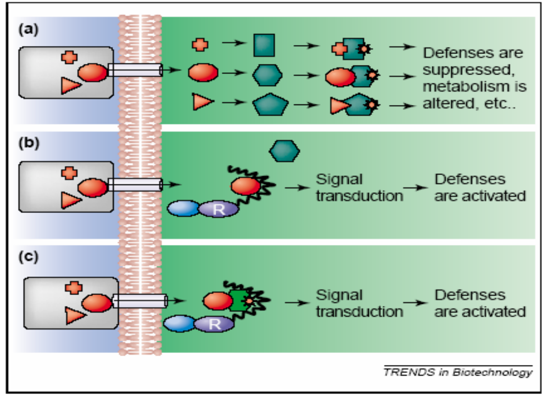


Fig.1.5. Interaction between Avr proteins and R proteins, the red figures depict the virulence proteins from the pathogen (McDowell and Woffenden 2003):

a) the plant is not expressing the *R* gene product resulting in a compatible interaction;
b) the product of the *R*-gene from the plant interacts directly with the product of the avirulence gene from the pathogen resulting in an incompatible interaction: Floor's Gene-for-gene hypothesis (Flor 1956);
c) Guard hypothesis- the pathogen (red) is recognized by guard protein, which interacts with the guardee *R*-gene product.

R-genes encode putative receptors in the plant that respond to the products of avirulence genes (*Avr*) expressed by the pathogen during infection resulting in activation of the defense response, hence resistance in the plant.

It has been proposed and assumed for a long time that the product of the *R* genes interacts directly with the corresponding *Avr* protein from the pathogen. The theory which is built on this assumption was called the gene-for-gene theory (Flor1956). It was shown only in two cases that direct interactions of the *Avr* gene and the *R*-gene occurred *in planta*: the first case *avr*Pto and the tomato *Pto* gene (Tang et al. 1996) and the second case *avr* Pi-ta and *Pi-ta* gene (Jia et al. 2000). Hence, the lack of more demonstrable direct *R*-*Avr* interactions the gene-for-gene theory was formulated to the "guard hypothesis" by Van der Biezen and Jones in (1998). This model predicts that *R* proteins activate resistance when they activate another plant protein (a guardee) that is targeted and modified by the pathogen (Fig.1.5).

1.6 Qualitative versus quantitative resistance traits

Two kinds of resistance exist in nature: qualitative and quantitative. The qualitative resistance is controlled by one gene (resistant gene *R*-gene); it is characterized by two discrete phenotypic classes (resistant and susceptible) and follows Mendelian inheritance. In contrary to the qualitative resistance, the quantitative resistance is governed by many/ unknown number of genes responsible for the resistance phenotype. Genetic variation in nature more often displays the features of a quantitative continuous distribution of the observed phenotype which falls into many phenotypic classes.

A quantitative trait locus (QTL) is a locus in the genome having one or more genetic factors (genes), which contributes to the observed quantitative phenotype in the population under investigation. The genetic variation underlying quantitative phenotypes is a result of multiple segregating QTLs each explaining a portion from the total quantitative variation, and whose expression is modified by interaction with genes in other QTLs "called- epistasis" and a large proportion of the quantitative effect is explained by the impact of the environmental factors (Paran and Zamir 2003, Mackay 2001, Gebhardt and Valkonen 2001, Koornneef et al. 2004).

Despite extensive efforts in studying the quantitative traits of inheritance only ten QTL genes have been cloned so far, and 12 more genes accounting for natural variation were identified in *Arabidopsis thaliana* (Koornneef et. al 2004, Paran and Zamir 2003). The allelic variation of a gene giving rise to the quantitative effect has a huge causal variability on the altered protein function, loss of function, changed expression level, truncated protein, amino acid substitution or even deleted gene. Allelic variation can occur not only in the coding but also in the non-coding regions causing altered regulation and stability of the gene (Paran and Zamir 2003, Koornneef et al. 2004).

1.6.1 Overview of QTLs to *P. infestans* in potato

QTLs for *P. infestans* in potato have been found on almost every potato chromosome. About 20 QTLs for resistance to the oomycete have been identified in diploid and tetraploid potato populations (Fig. 1.6).

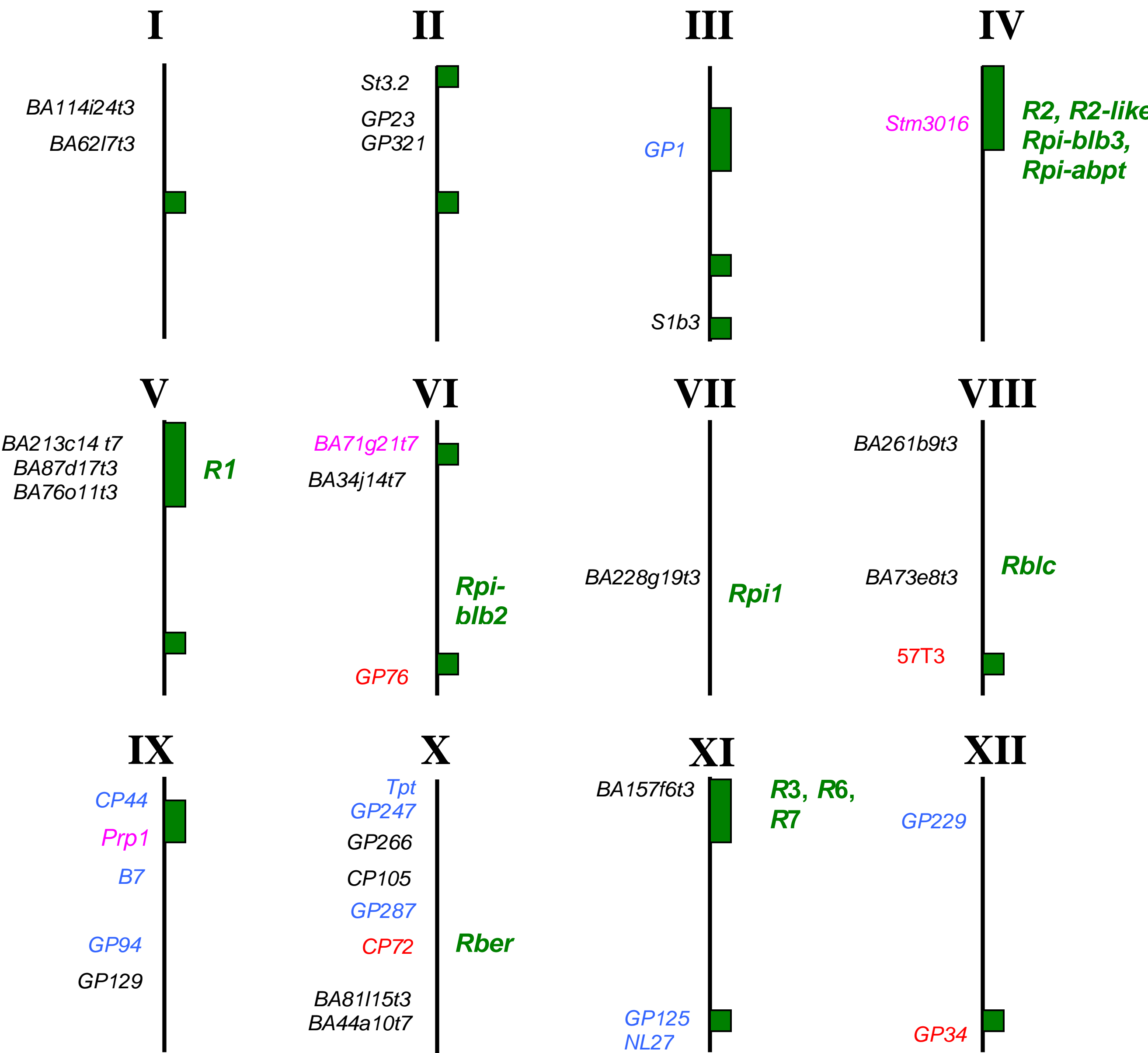


Fig.1.6. Schematic overview of known QTL for resistance to *P. infestans* on the 12 potato chromosomes. Major resistant genes for *P. infestans* are shown (green bold). SNP markers tested in the current study are shown (black), SSCP markers (blue), CAPS markers (red), SCAR marker (pink).

The strongest and most reproducible QTL explaining up to 60% of the phenotypic variation remains on chromosome V. This QTL effect was found in almost every mapping experiment (Bormann et al. 2004, Bradshaw et al. 2004, Ewing et al. 2000,

Visker et al. 2003a, 2004, Collins et al. 1999, Oberhagemann et al. 1999, Leonards-Schippers et al. 1994, Ghislain et.al. 2001), as well in association study (Gebhardt et al. 2004).

Four other reproducible QTL were detected on chromosome III (Leonards-Schippers et al. 1994, Collins et al. 1999, Oberhagemann et al. 1999, Ghislain et al. 2001, Visker et al. 2003a, Ewing et al. 2000), on chromosome IV (Leonards-Schippers et al. 1994, Oberhagemann et al. 1999, Sandbrink et al. 2000, Bradshaw et al. 2004, Collins et al 1999), chromosome XI (Leonards-Schippers et al. 1994, Oberhagemann et al. 1999, Ewing et al. 2000, Collins et al. 1999, Bormann et al. 2004, Ghislain et al. 2001) and chromosome XII (Ghislain et al. 2001, Bormann et al. 2004, Collins et al. 1999, Oberhagemann et al. 1999, Leonards-Schippers et al. 1994).

Several minor QTLs were identified with lower impact in explaining the phenotypic variance and less reproducible in different studies and in different year's tests: on chromosome I (Leonards-Schippers et al. 1994, Collins et al. 1999), on chromosome II (Oberhagemann et al. 1999, Collins et al. 1999), on chromosome VI (Collins et al.1999, Oberhagemann et al. 1999), on chromosome VII (Ghislain et al. 2001), chromosome VIII (Ghislain et al. 2001, Bormann et al. 2004, Oberhagemann et al. 1999, Meyer et al. 1998, Ewing et al. 2000, Collins et al. 1999), chromosome IX (Collins et al.1999, Oberhagemann et al. 1999, Bormann et al 2004, Leonards-Schippers et al. 1994), chromosome X (Sandbrink et al. 2000, Ewing et al. 2000, Collins et al. 1999), chromosome XI. The minor QTL effects should not be underestimated as the alleles might interact with alleles from major QTLs or loci showing no effect in single marker tests but displaying epistatic effects. Recent studies started to concern epistasis and show significant interaction while the markers studied separately did not show linkage to the QTL (Bormann et al. 2004, Ewing et al. 2000) or the QTLs show additive positive epistatic effect (Visker et al. 2003a).

It is interesting to note, that in most of the tested populations, QTL for plant maturity co-localize with QTL for late-blight resistance. In only few studies QTLs for maturity were separated from the QTLs for late-blight resistance (Ewing et al. 2000, Bormann et al. 2004, Bradshaw et al. 2004, and Visker et al. 2003a).

The traits ’resistance to *P. infestans*’ and ’late maturity’ seem to be linked. The reason for this phenomenon is probably the fact that the potatoes were introduced from South America where normally potatoes are grown under short day conditions at high altitude. When the crop was transferred to Europe with longer days, potatoes tuberise and mature later. Late maturity is a negative trait for potato breeders who wish to have resistant and early potatoes and to be able to harvest the crop earlier. It is still unknown whether these two traits are a causal effect of one gene with pleotropic effects, or of closely linked genes (Visker et al. 2003a). It is therefore interesting to find QTLs with effects only for resistance or only for maturity or QTLs for resistance to *P. infestans* and early maturity. Finding such QTLs will enable designing potato crosses that will generate progeny with the desired QTLs for resistance to *P. infestans* and early maturity.

1.6.2 Major *R*-gene clusters, mapped and cloned *R*-genes in potato for resistance to *P. infestans*

R-genes tend to cluster in the genome resulting probably from tandem gene duplication and recombination (reviewed in Gebhardt and Valkonen 2001, Leister 2004, Hulbert et al. 2001). A major cluster or hot spot for resistance was found on chromosome V where at least three genes for resistance to oomycete and viruses have been localized and two major QTLs *Gpa* (Kreike et al. 1994) and *Grp*1 (Rouppe van der Voort et al. 1998) conferring resistance to the potato cyst nematode *Globodera pallida* and *Globodera rostochiensis.* The first gene *R*1 gene (Ballvora et al. 2002) conferring resistance to *P. infestans*, genes *Nb* (Marano et al. 2002) and *Rx*2 (Ritter et al. 1991) both conferring resistance to potato virus X have been cloned. A second major cluster of resistant genes is located on potato chromosome IV, where four genes have been localized: *R*2 (Li et al. 1998), the recent localized *R*2 gene- like (Park et al. 2005b), Rbi-*blb*3 (Park et al. 2005a) and *Rpi-abpt* (Park et al. 2005c).

To the distal upper part of chromosome XI three genes were mapped *R*3, *R*6, *R*7 (El-Kharbotly et al. 1994, 1996), of which one *R*-gene *R*3a (Huang et al. 2005) was cloned.

Apart from resistance gene clusters, four *R*-genes conferring resistance to *P. infestans* were localized on chromosome VII (*Rpi*1 Kuhl et al. 2001), and on chromosome X R_{ber}

(Naess et al. 2000), of which two cloned on chromosome VIII (*Rbi-blb*3 van der Vossen et al. 2003, Song et al. 2003) and on chromosome VI (*Rbi*-blb-2 van der Vossen et al. 2005).

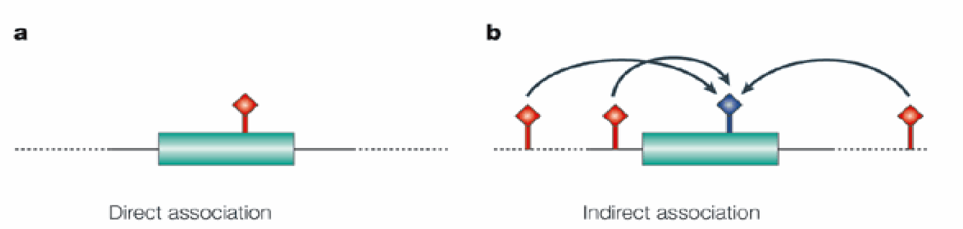
1.7 Single nucleotide polymorphism markers (SNPs)

Since Sanger's discovery in 1987 of DNA sequencing technique the quantitative genetics entered a new era, namely using the smallest DNA polymorphisms -single nucleotide polymorphisms. The SNPs account for the most informative and powerful polymorphisms looking directly into the disease predisposing alleles. SNPs are the most frequent, non-ambiguous, with possibilities for high-throughput detection and account for 90% of the polymorphisms in the human genome (Brookes et al. 1999). The high-throughput detection techniques develop with enormous speed, giving the hope to increase the quality and decrease the costs of the SNPs detection methods (Syvänen 2005). Due to the fast developing SNP technology already 9 million SNPs are documented in the human genome dbSNP database (Hirschhorn and Daly, 2005) and 56 670 single-nucleotide polymorphisms in Arabidopsis (Borevitz and Chory 2004). Applying the knowledge from the available SNP data high-density maps can be designed particularly important for associating SNPs with a phenotype of interest.
SNPs occur in the coding, non-coding and regulatory regions of a gene causing amino acids substitutions, transitions or frame-shifts (Tabor et al. 2002).
SNPs can be exploited for finding linkage disequilibrium (LD) between the tested marker and eventually the gene causing the disease. The SNP can be located directly in the gene or in the vicinity of gene (Fig.1.7).

1.8 The concept of linkage disequilibrium in plants

LD refers to a non-random inheritance of genetic variation in a population of individuals related by descent. In the last years LD has been extensively studied, especially in human genetics where recent findings show that genetic variants are transmitted in haplotype blocks resulting from high LD and low recombination frequencies in the particular part of

the genome under investigation (Phillips et al. 2003). Thus, it will be possible in the future with lower numbers of SNPs to tag larger chromosome fragments, as several SNPs are in a haplotype block and are transferred to the next generation together in a block. Theoretically, few SNPs in the LD region will be enough to study the chromosomal region where the haplotype block exists.



(*Adopted* from Hirschhorn and Daly 2005)
Fig.1.7. Linkage disequilibrium (LD): Direct a) and b) indirect association of SNP to the causal gene

Thus studying SNP haplotypes is a more informative approach in associating an allele with a trait rather then studying single SNPs (Rafalski 2002). The question arises how far does LD extend in the genome? LD depends very much on the plant population, it extent largely depending on several factors: recombination rate, inbreeding, genetic isolation between lines, mutation rate (Gupta et al. 2005). In self-compatible (inbreeding) organisms such as *Arabidopsis thaliana* (Nordborg et al. 2002, reviewed in Gupta et al. 2005) LD can be extensive because of already reached homozygosity in the studied region. An exception to this rule is studies which showed that LD in an inbreeding plant like soybean-*Glycine max* can decay in a very short interval (Zhu et al. 2003). In inbreeding species, the finding of large haplotype blocks is a straightforward process because the homozygous regions resulted form repeating selfing events.

In potato the finding of haplotype blocks is still an adventure because of its high heterozygocity. Potato is an autotetraploid organism with tetrasomic inheritance (Gebhardt and Valkonen 2001, Simko 2004). Despite the complexity of the potato genome, there are few studies, which show that LD in potato may be between 0.3-1cM (Gebhardt et al. 2004, Simko 2004).

The status in studying the haplotype distributions in the potato genome is the cloning of PCR products, which is very expensive, elaborative and time consuming. Another possibility is to use a lower ploidy level potato. The last is possible as using di-haploid *S. tuberosum* (2n=2x=24) by anther or pollen culture or using of intraspecific hybridization of tetraploid *S. tuberosum* with diploid *S. phureja* – cultivated mostly in South America (2n=2x=24). *S. phureja* chromosomes are eliminating at the early stage of embryo development resulting in producing di-haploid *S.tuberosum* (Simko 2004).

1.9 Candidate gene approach versus Genome-Wide analysis

Candidate gene approach had been pioneered in 1990 by Trudy F. C. Mackay in *Drosophila melanogaster* when study the *achaete-scute* region for association with variation in bristle number (Mackay et al. 1990). Candidate genes are often genes which are known they are involved in the certain biological pathway. Candidate genes may be causal for a QTL or genetically linked it (Pflieger et al. 2001, Tabor et al. 2002, Leister et al. 1996 and Rickert et al. 2003). The candidate gene approach is particularly helpful when studying a biological pathway in outcrossing species where LD rapidly decays, therefore requiring a huge number of SNP markers to perform genome-wide analysis (Neale and Savolainen 2004, Gupta at al. 2005, Rafalski 2002). Contrary to the candidate gene approach the genome wide analysis refers to studying the whole genome with evenly markers distributed. The approach is powerful for identifying common disease predisposing alleles in a population (Hirschhorn and Daly 2005, Carson et al. 2004). The disadvantages of the approach are: very laborious, expensive and time consuming. The most profound genome-wide study reported so far in plants may be in *Arabidopsis thaliana* where 824 DNA fragments have been analyzed in 94 accessions (Nordborg et.al. 2005).

In potato, 10 000-300 000 markers have been purposed or evenly distributed markers at each 10 cM from the genome in order to perform a genome-wide analysis (Rickert et al. 2003, Bormann et al. 2004). To perform such genome-wide analysis will be only feasible in a long term project and will rely on improving high-throughput methods for SNP genotyping and lowering the cost of the methods (Hirschhorn and Daly 2005). Because of

the requirement of high marker numbers in the whole-genome approach, the candidate gene approach is more feasible, especially when the candidate genes are selected on the basis of above mentioned criteria and the possible role of the candidate genes in the biological process is known.

1.10 Expression studies in potato

A comprehensive analysis of differentially expressed genes could contribute of better understanding of the molecular processes involved in plant-pathogens interactions in quantitative resistance mechanisms of the plant. A powerful method, suppression subtractive hybridization (SSH) has been used to constructed cDNA libraries enriched for genes that were up-regulated in the compatible or incompatible interactions between potato and *P. infestans* (Wang et al. 2005, Ros et al 2004, Beyer et al. 2001). A recent large scale gene expression study was reported where more then 7000 cDNA clones were studied in compatible interaction between *P. infestans* and potato (Restepo et al. 2005). Alternatively, to the large scale gene expression study, such can be performed with candidate genes localized in a QTL region of subject and gain insight into the molecular basis of moderately compatible and non compatible interactions between potato and *P. infestans*.

1.11 Goal of the thesis

The goal of the PhD work was to genotype two new tetraploid breeding populations with DNA markers and to search for linkage to some of the previously described QTLs for resistance to *P. infestans* in experimental diploid potato populations. The goal was to transfer the knowledge from diploid to tetraploid breeding material. CAPS, SSR, SSCP and mainly SNP markers were to be used to genotype the two unrelated tetraploid potato breeding populations. The DNA markers were chosen in close vicinity to previously described QTLs for *P. infestans* or close to already mapped *R*-genes such *Rber* on chromosome X.

The second goal was to find genes located in a QTL region on chromosome V differentially expressed upon *P. infestans* infection using RT-PCR technique. The genomic region where the cloned *R*1 gene is localized was sequenced to identify positional candidate genes besides the *R1* gene family. The goal was to find other genes for resistance to *P. infestans* in region of about 400 kb, besides *R*1 gene. Previous study showed that parents of a diploid population segregating for QTL for resistance to *P. infestans* on chromosome V lack the *R*1 gene, despite the very strong QTL effect.

The third and the last goal of the PhD thesis was to search the QTL region on chromosome V for a QTL gradient and to narrow down genetically the region with candidate genes for quantitative resistance to *P. infestans*. For this purpose six single copy genomic markers spanning a genetic distance of about 8-10 cM were chosen. The markers were amplified, sequenced and scored for SNPs. For this experiment 32 late maturing and late blight resistant plants and 33 early mature and susceptible plants were selected. The two groups of plants represented the most extreme groups of plants from a whole population of 610 tetraploid genotypes.

Chapter 2: Materials and Methods

2.1 Plant material

2.1.1 “Cases” and “controls” for the analysis – of linkage to QTL

The “cases” and “controls” study were 23 highly resistant (“cases”) and 23 highly susceptible (“controls”) plants of two tetraploid populations. The individuals have been selected from a total of 270 F1 individuals for the BNA population and 196 F1 individuals for the SaKa-Ragis population. The parents of the BNA population were: NK5 (resistant) and NK6 (susceptible). The parents of the SaKa-Ragis population were SR1 (resistant) and SR2 (susceptible) parent. The plant material was provided by BNA and SaKa-Ragis.

2.1.2 “Cases” and “controls” for the analysis of association with QTL

As “cases” and “controls” for the association study 32 most late maturing and most resistant plants (cases) and 33 early maturing and highly susceptible plants (controls) were selected from a collection of 610 tetraploid *S. tuberosum* genotypes preserved in the IPK Genbank (Groß Lüsewitz, Germany).

This population is preserved in IPK Genbank (Groß Lüsewitz, Germany). The plant material originates from North and South America, North, South, Middle and Eastern European countries and represents 143 years of breeding history.

The plants of this population are not directly related between each other. They are separated by up to six meiotic generations (associated individuals), (Gebhardt et al. 2004).

The original passport data of the individuals included in this study is reported in Table E, Appendix.

2.1.3 Plants used for expression study

The parents I88 and G87 of the GDE diploid population (Oberhagemann et al. 1999) were used for the RT-PCR experiment. The I88 is moderately susceptible, whereas G87 moderately resistant to late blight. A strong QTL Pin5A for resistance to *P. infestans* on chromosome V in GDE population has been detected although both parents lack the major *R*1 gene for resistance to *P. infestans*.

2.1.4 Field assessment of late-blight

The two tetraploid populations were provided by "BNA" (Böhm- Nordkartoffel Agrarproduktion OHG, Ebstorf, Germany) and "SaKa-Ragis" (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany). The field assessments for the two populations were performed in the years 2001 and 2002. Both populations were inoculated with a *P. infestans* complex race R1-R11. The complex race was able to infect finally both tetraploid populations. None of the known major resistance genes were detected in the parents of the two independent tetraploid populations. Field experiments, phenotypic data and frieze dried plant material were obtained by the two Potato Breeding Companies. The field experiments were carried out in the field of the breeding station at Ebstorf (BNA) and Windeby (SaKa-Ragis).

2.2 Cultivation of *P. infestans*

2.2.1 Rye agar media preparation

(personal communication W.Giffers, MPIZ, Cologne)

200 g Rye grains were added to 800 ml ddH_2O and autoclaved. The rye grains extract was sieved and the grains were discarded. ddH_2O was added to the rye grain extract and adjusted to 1L. 39g/L Potato Dextrose Agar (Becton, Dickinson and Company, Le Pont de Claix, France) was added to the solution and autoclaved again. The medium was mixed in order to get homogeneous mixture and then poured into "Petri" dishes.

2.2.2 *P. infestans* propagation (when starting the infection from mycelium on rye agar medium)

(personal communication W.Giffers, MPIZ, Cologne)

A rye agar piece with *P. infestans* mycelium was cut from four to six weeks old cultures and transferred into a new "Petri" dish with freshly prepared rye agar. For propagation on leaves, one rye agar piece (d=5mm) with *P.infestans* mycelium was excised and put on the abdominal site of the leaf. 50-100 µl sterile water were added exactly on top of the rye agar piece for providing 100 % humidity required for zoospore mobility. The infected leaves were kept in a transparent plastic box (2.2.4).

2.2.3 *P. infestans* inoculum preparation

The agar piece from the primary infection was lifted up and discarded. 1 to 2 infected detached potato leaves were soaked in 5-10 ml ddH_2O and carefully vortexed in order to release the sporangia from the sporangiophore. The sporangia were counted using a hemocytometer camera (Neubauer, 0.1 mm depth, 1/400 mm^2). For the whole plant infection experiment 40 sporangia/ µl were used. The inoculum was incubated for 2 h at 4-8 °C (in the fridge) followed by transfer it to room temperature for 20 min and then used for infection. Under the microscope the typical "lemon-like" shape *P. infestans* sporangia was observed (Fig.2.1). The picture with the sporangia was taken using Microscope Zeiss Axioplan 2 imaging (Carl Zeiss, Göttingen, Germany) and software Diskus 4.50 FireWire (Carl Hilgers, Königswinter, Germany).

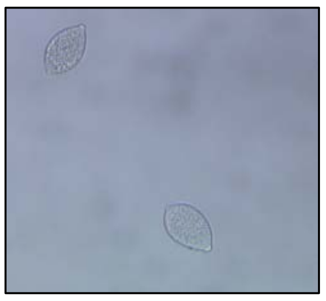


Fig.2.1.Typical “Lemon-like” shape *P. infestans* sporangia

Fig.2.2. Detached leave test performed in a plastic box

Fig.2.3.Cultivating *P. infestans* on tuber slices

2.2.4 Detached leaf assay

Detached potato leaves from the susceptible genotypes Desirée and I88 were cut with a sterile razor blade and placed on a prewet Whatman paper on a metal grid in a plastic box (20 x 20 x 6 cm). The bottom of the plastic box was filled in with ddH_2O to keep high humidity. The Razor blade was changed each time when another plant had to be cut to prevent transmission of infections. Each detached leaf was inoculated with two 50 µl drops (2.2.3). The lid of the box was sealed with autoclave tape in order to maintain high humidity, a very essential parameter to enable growth of the oomycete. The box was kept in a light chamber for 16-24 h at 10 °C, 13 h light and 11 h dark and then transferred to a light chamber- 16 °C, 14 h light and 10 h dark. This step facilitates the release of the zoospores from the sporangia (Fig.2.2).

2.2.5 Whole plant infection assay

4 weeks old plants grown under green-house conditions were infected with the complex *P. infestans* race R1-11. Every leaflet was sprayed twice with 10ml-spraying bottle (cat. №10007245, neoLab, Heidelberg, Germany) and marked to prevent collecting non treated leaves. A leaflet is a part of the complex potato leaf which usually consists of five

leaflets. Plants were infected and kept in a growth chamber at 80 % humidity, 17 ºC , 16 h light and 8 h dark, light 100 %. The whole plant was covered with an absolutely transparent autoclave bag, tightened with elastic band. Plants were infected always at the second, third and fourth complex leave (middle part), counted from the lowest part of the plant to prevent leaf age difference effects. The leaf samples for RT-PCR experiment were taken at 24, 48 and 72h post-infection, transferred immediately to a falcon tube with liquid nitrogen and kept at -70 ºC until use. The samples were taken in the late afternoon (3-8 PM), depending on the starting point of infection. The infected plants were kept in the growth chamber until the infection symptoms with *P. infestans* were observed and then autoclaved.

2.2.6 Tuber slices infection

Tubers of cultivar Granola, provided by SaKa-Ragis Pflanzenzucht GbR (Windeby, Germany) were washed thoroughly using detergent. The whole tuber was sterilized by soaking in 75% ethanol and flaming. The tubers were cut in 1 cm thick slices and placed in a plastic box on dry Whatman paper without H_2O for 24h in order to get a dry surface. Each tuber slice was inoculated with two drops each of 50 µL *P. infestans* inoculum or with one rye agar piece *P. infestans* mycelium (d=5mm), (Fig.2.3). 24h post-inoculation the tuber slice was turned up-side down. The plastic boxes were incubated under the same conditions as described in (2.2.4). *P. infestans* mycelium from 4-5 tuber slices was collected using a paint brush and dipped in 5 ml dH_2O. The inoculum was placed at 4 °C for 2 h and then kept at RT for at least 30 min to release the zoospores from the sporangia (Fig.2.3).

2.2.7 Pathotype specificity assay (Black's differential test)

The detached leaf (2.2.4) assay was carried out with eleven potato genotypes each carrying a single known resistance (*R*) gene. The plants are named "Scharnhorst *R*1 to *R*11". They were obtained from the former MPIZ breeding station (Scharnhorst, Germany) and are now maintained at MPIZ-Cologne. The detached leaves were infected

with *P. infestans* to determine pathotypes specificity. The specificity of the strain was determined as absence or presence of infection symptoms on the plant carrying a single known *R* gene.

2.3 Frequently used buffers and medium

Denaturation buffer:
1.5 M NaCl
0.5 M NaOH

Neutralization buffer:
1 M Tris
1.5 M NaCl
pH 7, 4 (HCl)

Stripping buffer:
0.1% (w/v) SDS
(sodium dodecylsulfat)

Hybridization buffer:
7 % (w/v) SDS
200 mM Na_2HPO_4 x
$2H_2O$ pH 7.0
(phosphoric acid)

Washing buffer:
2 x SSPE
0.1% (w/v) SDS

SSPE buffer:
3 M NaCl
200 mM NaH_2PO_4
20 mM EDTA
pH7.0 (phosphoric acid)

10 x TBE buffer:
890 mM Tris
890 mM boric acid
20 mM EDTA, pH 8.0

1 xTAEbuffer
40 mM Tris
2 mM EDTA pH 8.0
0.01 % (v/v) acetic acid

Sephadex-G50 (medium) buffer
10 mM Tris pH8
0.5 M EDTA
4 M NaCl
10 % (w/v) SDS
2-4 % (w/v) Sephadex
G-50(medium)

CTAB-lysis buffer:
100 mM Tris pH9.5
1.4 M NaCl
20 mM EDTA
2 % (w/v) CTAB
0.5 % PEG 600

LB medium, pH 7.0 (1l):
10 g bacto-trypton
5 g bacto-yeast extract
10 g NaCl
15g agar

2.4 Molecular biology methods

2.4.1 BAC plasmid isolation and purification (Qiagen plasmid purification protocol modified)

The BAC library has been constructed from *Hind*III partially digested high-molecular-weight genomic DNA of the potato genotype P6/210 in the binary vector pCLD0454.

E.coli strain DH10B (Invitrogen, CA, USA) has been transformed with the binary vector (Jones et al. 1992, Ballvora et al. 2002). Bacterial cells were plated on LB medium (+13 mg/l tetracycline) and incubated at 37 °C for overnight. A single colony was picked, transferred to 5 ml LB liquid medium (+ tetracycline) and cultured with shaking at 37 °C overnight. 100 µl from the pre-culture were inoculated in 100 ml selective LB medium and incubated at 37 °C for 14 hours with vigorous shaking (250-300rpm). The culture was centrifuged at 4000 rpm for 30 min. The bacterial pellet was resuspended in 10 ml P1 buffer (Qiagen Plasmid Purification Handbook, Hilden, Germany) containing 100 µg/ml RNase A. 10 ml P2 buffer (Qiagen Plasmid Purification Handbook) were added to each sample, mixed by inverting 4-6 times and incubated for 5 min at room temperature. 10 ml chilled P3 (Qiagen Plasmid Purification Handbook) buffer was added to each tube, immediately mixed and incubated on ice for 15 min. The lysed cells were centrifuged at 4000 rpm for 30 min at 15 °C and the supernatant was discarded. The lysate was centrifuged again at 4000 rpm for 15 min at 15°C and purified on Qiagen-tip 100 column (Qiagen, Hilden, Germany). The column was equilibrated by adding 4 ml QBT buffer (Qiagen Plasmid Purification Handbook) to the column and let it dry by gravity flow. The lysate was applied to the column. Qiagen-tip 100 column was then washed using 3 x 10 ml QC buffer (Qiagen, Hilden, Germany). BAC plasmid DNA was eluted using 5 x 1 ml pre-warmed 50°C QF buffer (Qiagen, Hilden, Germany), precipitated with 3.5 ml isopropanol at RT and centrifuged immediately at 10000 rpm for 15 min at 4°C. The DNA pellet was washed with 70 % (v/v) ethanol and centrifuged again at 10000 rpm for 10 min at 4°C. The BAC pellet was air-dried, dissolved in ddH_2O and the concentration was measured using a Smart Spec ^{TM}3000 (Bio-Rad, München, Germany).

2.4.2 Isolation of total genomic DNA

CTAB-Method with purification on Qiagen Tip 100 columns (Qiagen, Hilden, Germany)
0.3-0.4 g freeze dried leaf material was powderized by vortexing with two to tree ceramic balls (d=5-6mm) per falcon tube and extracted with 20 ml pre-warmed (74 °C) CTAB-lysis buffer containing 0.25 % (v/v) β-mercaptoethanol. The suspension was very well vortexed and incubated at 74 °C for 20 min with temporary mixing. After the suspension

was cooled down to RT, one volume of chloroform/isoamylalkohol (24:1) was added to the sample, vortexed very well and centrifuged at 4000 rpm for 15 min at 10 °C. The upper aqueous phase was transferred to a new falcon tube, treated with 20 µl RNase A (10mg/ml) (Qiagen, Hilden, Germany) and incubated at 37 °C for 30 min. Then 20 ml ddH_2O were added to the sample, the pH was adjusted to 7.0 with 1N HCl and controlled with pH indicator strips (Merck, Darmstadt, Germany). The sample was vortexed and applied to a Qiagen-tip 100 column (Qiagen, Hilden, Germany). Equilibration, washing and elution steps were the same as in (2.4.1) with exception of the amount of QC buffer added to the column- 2 x 7.5 ml per sample. 0.7 volume isopropanol was added to the sample, exposed at 4 °C for overnight precipitation and centrifuged at 10000 rpm at 4 °C for 15 min. The pellet was washed using 2 ml 70 % (v/v) ethanol and again centrifuged at the same conditions for 1 min. The sample was air dried, dissolved in 100 µl dH_2O and the concentration was measured using Smart Spec ^{TM}3000 (Bio-Rad, München, Germany).

Qiagen DNeasy Plant Mini Kit (modified)

Total genomic DNA was isolated after a modified protocol using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). 20 mg freeze dried leaf material was ground to fine powder with two metal spheres (d=4mm). The plant material was ground with a Qiagen Mixer Mill Type MM 300 (Qiagen, Hilden, Germany) set for 30 sec at 30 beats per sec. The DNA was extracted using 600 µl AP1 (Qiagen, Hilden, Germany) heated to 65 °C buffer and incubated at 65 °C for 10 min. The suspension was mixed with 195 µl AP2 buffer (Qiagen, Hilden, Germany), cooled on ice for 15 min, frozen for 30 min at -70 °C, thawed and centrifuged at 6200 rpm for 5 min at RT. The upper phase (450 µl) was mixed with one volume isopropanol, inverted up-side down for 10 min and centrifuged at 6200 rpm for 5 min at RT. The DNA pellet was washed with 100 µl 70 % (v/v) ethanol and centrifuged again at the same conditions. Pellets were dried under vacuum and resuspended in 100 µl dH_2O.

2.4.3 Southern gel blot analysis

2.4.3.1 Genomic DNA and BAC enzymatic digestion

5µg genomic DNA or 3ng BAC DNA were digested with 0.5U/ µl restriction enzyme not cutting within the gene sequence. The restriction reaction was performed in 20 µl total volume and incubated in a PCR machine overnight at the temperature appropriate for the enzyme. The whole digestion reaction or 3 µl for the BAC positive control was loaded on agarose gel and run for 18h at 2 V/cm in 1x TAE buffer. The gel was stained in 1 x TAE buffer including 0.5 µg /ml (w/v) ethidium bromide.

2.4.3.2 Agarose gel treatment and DNA transfer to the membrane

The gel was treated with 0.2 N HCl for 10 minutes, rinsed with dH_2O and incubated for 45 min in denaturation buffer. The gel was again rinsed with dH_2O, incubated for 30 min in neutralization buffer and marked at the upper left corner. Whatman paper was cut with exactly the same size as the gel, prewetted in 2 x SSPS buffer and placed on the gel. Bridge Whatman paper prewet in 2 x SSPS buffer was put on a glass plate and in direct contact with 10 x SSPE transfer buffer. The gel was turned up- side down and placed on the bridge Whatman paper in direct contact with the transfer buffer. Biodyne B membrane (Paal Corporation, Dreieich, Germany) was cut exactly the same size as the gel, placed on the top of the gel and marked at the upper right corner. Four Watman paper sheets prewetted in 2 x SSPC buffer were placed on top of the Biodyne membrane. The sandwich assembly was finished by stacking about 9 cm of tissue paper on top. Weight of c.a. 500 g was placed on top of a glass plate on top of the tissue paper stack. Parafilm was put around the edges of the gel to prevent contact of the tissue paper with the bridge paper. The sandwich assembly was left for overnight transfer. DNA was covalently cross-linked to the membrane using a UV Strata linker 2400 (Stratagene, La Jolla, CA, USA) followed by drying it at 80 º C for 1hour.

2.4.3.3 Membrane hybridization and washing

The membrane was pre-hybridized with 20 ml hybridization buffer containing 0.1 mg/ml herring sperm DNA denatured at 95 º C for 5 min. Pre-Hybridization was performed at 65 ºC in a hybridization oven (Bachofer, Reutlingen, Germany) over night (approx. 16 h) in a hybridization glass tube (27 cm x 3 cm). The radioactive labeled probe was added to the fresh hybridization buffer with newly denatured herring sperm DNA and incubated for 16h under the same conditions as the pre-hybridization step. The membrane was washed with 30 ml washing buffer for 15 min in the hybridization tube followed by a second wash (shaking) for one hour at RT in access of buffer. The membrane was wrapped in Saran folio and exposed to Phosphoscreen (Molecular Dynamics) for 16h.
The membrane was stripped in 0.1 % (w/v) SDS for 10 min at 70 º C.

2.4.3.4 Radioactive labeling and purification of DNA probe

50-100 ng PCR product in a total volume of 12 µl was denatured at 95º C for 5 min and put immediately on ice. The Feinberg and Vogelstein (1984) method was used for random primer labeling: 4 µl 5x OLB buffer (0.1 mM each dATP, dCTP, dGTP, dTTP; 1M HEPES pH 6.6; 1mg/ml hexadesoxyribonucleotide pdN6 (Pharmacia); 0.1 M Tris-HCl, pH 8; 3.7µM $MgCl_2$; 0.35% (v/v) β-mercaptoethanol); 3 µl of [α^{32}P] –dCTP (30µCi/µl) and 0.5 U/µl Klenow enzyme (Roche, Mannheim, Germany) were added to the denatured probe and incubated at 37 º C for 1h. A 1ml syringe was clogged with cotton fibers and filled in with Sephadex G50-medium suspension (Amersham Bioscience AB, Uppsala, Sweden). The 20µl radioactive probe was then applied to the column and the second fraction was collected as a radioactive labeled probe. For each fraction 300µl Sephadex buffer were used.

2.4.4 Preparation of MethaPhor agarose gel

MethaPhor Agarose (Cambrex Bio Science Rockland, USA) was soaked before boiling in pre-cooled 1 x TBE buffer. Agarose was added slowly to the buffer to prevent forming

of bulks. The solution was cooled to 50-60 °C and casted slowly to prevent forming of bubble formation in the gel. After polymerization the gel was placed in the fridge for 30 min to obtain optimal resolution of the bands afterward.

2.4.5 Determination of DNA and RNA concentration

The quality and quantity of DNA and RNA were measured on a 1% agarose gel containing 0.5 µg /ml (w/v) ethidium bromide and run at 2V/cm in 1x TAE buffer. The Quantity of the genomic DNA was determined by comparing the band intensity on the gel with a reference λ_{50} (50ng/µl) marker with concentration on the gel 100 ng, 200 ng, 300 ng and 400 ng. Additionally, the quantity of the DNA was measured using a UV spectrometer (Smart Spec 3000, Bio-Rad, München, Germany).

2.4.6 Enzymatic digestion of amplified DNA- cleaved amplified polymorphic sequences (CAPS)

Monomorphic PCR products (5-7 µl) were digested with seven restriction endonucleases, six with a 4 bp recognition site (*Alu*I, *Mse*I, *Rsa*I, *Taq*I, *Dpn*II and *Nla*III, New England Biolabs, Frankfurt am Main, Germany) and one with a 6 bp recognition site-(*Hinf*I). The digestion reaction was performed in 20 µl total volume of buffer recommended by the supplier of the enzyme, 0.1U/µl enzyme and incubated at the temperature required for the enzyme.

-For SSCP analysis, the digestion was carried out in 10 µl total reaction volume.

2.4.7 Identification of Single Strand Conformation Polymorphism (SSCP)

Monomorphic PCR products from parents SR1, SR2, NK5, NK6 were digested in 10 µl total volume using seven restriction endonucleases *Alu*I, *Mse*I, *Rsa*I, *Taq*I, *Dpn*II, *Hinf*I, and *Nla*III (0.1U/µl). 9 µl denaturing solution were added to 4 to 6 µl of the digestion reaction, depending on the amount of the PCR product), denatured at 95 °C for 4 min and put on ice immediately. 6-7 µl from the denatured mix were applied to the SSCP gel.

The enzyme, which resulted in the most defined and informative polymorphic bands was used to check 8 to 10 individuals per population for segregation of the polymorphic bands. Finally, the DNA from the 23 “cases” and 23 “controls” population was digested and applied to the gel. The gel was run at 1.5 V/cm for 16-18h in 0.6 x TBE buffer.

SSCP gel preparation

SSCP gel:
0.6 x TBE buffer
5 % (v/v) glycerol
0.25 x MDE gel solution
(Cambrex, Rockland, USA)
0.062 % (v/v) TEMED
0.05 % (w/v) APS

Denaturing solution:
95 % (v/v) deionized formamide
0.01 M NaOH
0.05 % (v/v) xylen cyanol
0.05 % (v/v) bromphenol blue

SSCP gel staining

The polyacrylamide gel was incubated 3min in fixation solution, immediately transferred in staining solution for 5 min, washed in dH_2O for 2 min, transferred to developing solution for 15-20 min, and again treated in fixing solution for 5min. The gel was thoroughly rinsed with dH_2O to get a clear background and sharp bands on the gel.

SSCP solutions:

Fixation solution:
10 % (v/v) ethanol
0.5 % (v/v) acetic acid

Staining solution:
0. 2 % (w/v) $AgNO_3$
10 % (v/v) ethanol
0.5 % (v/v) acetic acid

Developing solution:
0.75 M NaOH
0.27 % (v/v)
formaldehyde

2.4.8 Total RNA isolation

The whole plant material collected per time point after infection and per genotype was ground to fine powder using liquid N_2 and stored at -80 °C. 100 mg grinded material was used for total RNA isolation using RNAwiz isolation buffer (Ambion, Austin, USA) following the supplier’s protocol. The total RNA was treated with DNA- free^{TM} reagent (Ambion, Austin, USA) following the supplier’s protocol.

2.4.9 First strand cDNA synthesis

First strand cDNA synthesis was carried out using 4 µg total RNA, SuperScript™ II RNase H reverse transcriptase (Invitrogen, Karlsruhe, Germany), primer oligo p $(dT)_{15}$ (Roche, Mannheim, Germany) and RNAseOUT (Invitrogen, Karlsruhe, Germany) as recommended in the protocol for the enzyme and following strictly the supplier's instructions.

2.4.10 RT-PCR (reverse-transcriptase polymerase chain reaction)

RT-PCR reactions were performed with the following conditions: cDNA was amplified in a total volume of 20 µl of 20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.66 mM $MgCl_2$ (Invitrogen, Karlsruhe, Germany); 0.2 mM each of dATP, dCTP, dGTP and dTTP (Carl Roth& Co. KG, Karlsruhe, Germany); 1 µM of each primer and 0.03 U/µl Taq Polymerase (Invitrogen, Karlsruhe, Germany) was used for the RT-PCR. The RT-PCR products were separated on an agarose gel as in (2.4.5).

2.4.10.1 Scanning agarose gels from RT-PCR experiment

1.5 % agarose gels from the RT-PCR analysis were scanned on Typhoon 860 Phosphor Imager (Amersham Bioscience) and quantified using Image Quant Version 5 (Molecular Dynamics, Krefeld, Germany).

2.4.10.2 Parameters used for scanning the ethidium bromide stained gels

The following parameters were set to scan the ethidium bromide stained agarose gels
Acquisition mode: -fluorescence
Emission filter: -Rox 610BP30
Pixel Size: 50 micron
Focal plane: +3mm
PMT: 600V
Laser: Green (532nm)
Sensitivity: Normal

2.4.11 Standard PCR

Standard PCR was carried out using 50 ng genomic DNA template and the same PCR conditions as for the RT-PCR (2.4.10), the only difference being the primer concentration with 0.3 μM for each primer. Standard PCR and RT-PCR were performed in a T3 Thermocycler (Biometra, Göttingen, Germany). PCR reaction conditions for standard and RT-PCRs were: 94°C for 2 minutes, 45 cycles denaturation at 92 ºC for 35s, annealing at T_a as specified in (Tables A, B, C, Appendix) for 35 s, extension at 72 °C according to the rule of amplification of 1kb per 1min and a final extension at 72 °C for 10 minutes.

2.5 Statistical methods

Whole population test

The nonparametric one-Sample Kolmogorov-Smirnov Test was used to check whether the phenotypic data of the whole population were normal distributed. The normal distributed phenotypic data was tested with compare means test and one-way ANOVA test (software SPSS 13 for Windows, SPSS Inc., Chicago, IL, USA).

“Cases” and “controls”

For the purpose of “cases” and “controls” linkage and association study Descriptive statistics was used combined with Cross table and Chi-square test.

Chi-square association test for testing minimum sample size

For the purpose of “cases” and “controls” study the minimum number of individuals required was calculated according to the formula:

$$\chi^2 = \sum_{class} (n_{observed} - n_{expected})^2 / n_{expected} ,$$

$n_{observed}$- the observed frequency of the individuals per genotypic class per phenotypic group
$n_{expected}$- the expected frequency of individuals per genotypic class per phenotypic group

The formula was computed and used only for the significant SNPs in the “cases” and “controls” linkage and association study.

Data scoring

In tetraploid potato five allelic states are possible: homozygous (AAAA), (aaaa) or heterozygous AAAa (triplex/simplex), AAaa (duplex/duplex), Aaaa (simplex/triplex) “A” presenting the presence and “a” the absence of the marker allele. The polymorphic bands of the parents for the SSCP markers were scored as present (1) or absent (0). The genotypic data as (1) and (0) was used for the statistic test. For the SNP markers the five possible allelic states were coded with digits and used for the statistic test.
Example plants having: AAAA-1; AAAT-2; AATT-3; ATTT-4 and TTTT-5

2.6 Set parameters for the DaX software

DaX software for quantification of SNPs (single nucleotide polymorphisms) version 7.2
DaX software version 7.2 (Eindhoven, The Netherlands) was used to quantify SNPs from the trace files. The software uploads automatically the files in Abigene format and gives the segregation ratio of every SNP for every individual. The numeric SNP data such as 2:2, 3:1, 1:3, 4:0 or 0:4 from the DaX software was transferred into letters and used in this format for the Descriptive statistical test. Example: At a SNP position in one genotype the allele dosage was 3:1 (format from the DaX software) and converted for the statistic test in AAAT.

2.7 Databases and Softwares used

Databases

Arabidopsis Information Resource; http://www.arabidopsis.org/index.jsp
Blast Search for TIGR Unique Potato Gene Indices; http://www.tigrblast.tigr.org/tgi/
Blast Search in Sol Genomics Network for Potato Gene Indices;
http://www.sgn.cornell.edu
NCBI Blast analysis; http://www.ncbi.nlm.nih.gov/
Retrieval of potato sequences, snp, mapping data; http://www.gabi.rzpd.de/
Potato Pedigree Database; http://www.dpw.wau.nl/pv
Potato Microarray Clone Batch Search
http://www.tigr.org/tdb/potato/search/potato_search_batch.shtml

Softwares

Converts a DNA sequence into its reverse, complement, or reverse-complement counterpart; Stothard (2000), (software)
http://www.ualberta.ca/~stothard/javascript/rev_comp.html
Translation of a nucleotide sequence to a protein sequence
http://www.expasy.org/tools/dna.html
Multiple sequence alignment (Corpet 1988); http://www.probes.toulose.inra.fr
DaX software for quantification of SNPs; http://www.dax.nl
Statistical software; http://www.spss.org
Multiple sequence alignment editor, analyzer and shading utility for windows; Nicholas KB, Nicholas HB, Deerfield DW (1997): GeneDoc, version 2.6.002 (software) www.psc.edu/biomed/genedoc
Primer pairs design and search for restriction sites of restriction endonucleases in a nucleotide sequence; Hasting Software, Inc. (1994); Gene Runner, version 3.05 (software) www.generunner.com
Gene structure prediction program;
http://www.opal.biology.gatech.edu/GeneMark/eukhmm.cgi

Chapter 3: Results

3.1 QTL-Analysis for quantitative late blight resistance

3.1.1 Selecting the most resistant "cases" and the most susceptible "control" plants in SaKa-Ragis and BNA populations

The 23 "cases" and 23 "controls" were selected from the whole SaKa-Ragis population consisting of 196 genotypes. All genotypes from the population are represented on the (Fig.3.1). The selection of the "cases" and "controls" genotypes from the whole population and Fig.3.1 have been performed by Dr. Jens Lübeck (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany). Each dot represents one genotype from the whole population. In orange are the selected highly susceptible "controls", above the regression curve (green); in yellow are the selected highly resistant "cases", below the regression curve. The results on the Fig.3.1 had been obtained as an average phenotypic data for maturity for years 2001 and 2002 and average rAUDPC from the replica experiments in year 2002. The data for year 2001 for SaKa-Ragis population has not been concerned because normal distribution of the phenotypic data has not been observed (personal communication Dr. Jens Lübeck). Each genotype was evaluated in the field for the quantitative traits maturity and resistance to *P. infestans*. The resistance score for each genotype is explained by the AUDPC (area under progress curve) value. The AUDPC value is calculated in the following way: The disease symptoms were evaluated up to 15 times, where the period between two evaluation days was between several days up to one week. The evaluation corresponds to scores between 1 and 9, where score 1-highly resistant and score 9-highly susceptible. With the results a saturation curve is obtained. The area under this curve is calculated resulting in an AUDPC value for each genotype. For susceptible plants the AUDPC have higher values in comparison to the AUDPC values for resistant plants. The disease symptoms for susceptible genotypes go up to score 9, which will result in a steep slope of the curve resulting in high AUDPC value. Disease symptoms on resistant genotypes develop slower and the scores may be much less then 9, obtaining a curve with a flat slope and hence smaller AUDPC value. Each AUDPC value is divided by the mean AUDPC from the whole year, resulting in the

“relative” AUDPC value- rAUDPC. This calculation is done to prevent differences from the environmental factors during the different years of field assessment. rAUDPC data were plotted against maturity scores and a regression curve was calculated. “Cases” and “controls” with maturity corrected resistance were selected, that had the largest distance between the actual score for each individual (dot on the graph) to the regression curve (in green). Such calculations for “maturity corrected resistance” are needed, because resistance to *P. infestans* is correlated with late maturity and is an undesirable combination of traits in potato cultivars. In order to brake down this correlation, the genotypes are fixed for the maturity trait and further selection is based only on the resistance to *P. infestans*. For BNA population, the extremely resistant “cases” and extremely susceptible “controls” have been selected from the whole population without considering “maturity corrected resistance”.

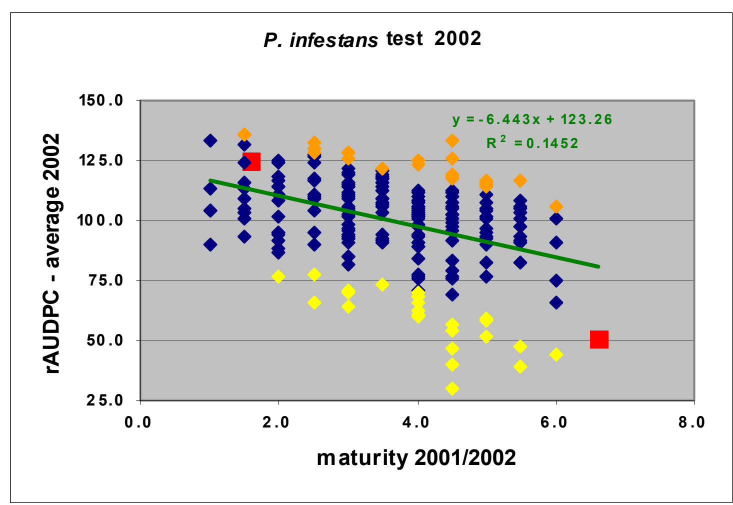


Fig. 3.1. Regression curve of maturity versus rAUDPC values of the SARA population (calculation were performed by Dr. Jens Lübeck-SaKa-Ragis Pflanzenzüchtung, GbR). The highly resistant “cases” are shown as yellow dots; highly susceptible “controls” as orange dots. Upper left rectangle shows the susceptible SR2 parent (red); lower right rectangle resistant SR1 parent (red).

3.1.2 Tagging QTL for *P. infestans* in SaKa-Ragis and BNA populations using SNP, SSCP, SCAR and CAPS markers

QTL analysis was performed on 23 highly resistant "cases" and 23 highly susceptible "controls" plants selected from the whole population. First, the SR1 and SR2 parents of the SaKa-Ragis and the NK5 and NK6 parents of the BNA populations were analyzed for SNPs. In case of gut quality trace files, amplicons of the whole population (46 plants) were sequenced and analyzed for SNPs. The SNPs markers tested in both populations were chosen based on the information available in the PoMaMo database for the polymorphisms and positions of SNPs in the corresponding marker. Markers with low quality sequence in the parents were not considered further. Only the successfully scored polymorphic SNP, SSCP, CAPS and SCAR markers are reported. The schematic potato map with the chromosomal location of all analyzed markers is shown in (Fig. 1.6), (Introduction). Table 3.1 shows the summary results from the SNPs evaluated markers in SaKa-Ragis and BNA populations. A threshold of p value ≤ 0.01 was accepted for the SNP, SSCP, SCAR and CAPS markers, due to small size of the analyzed populations.

3.1.2.1 QTL analysis in BNA population

The BNA population was genotyped for SNPs in amplicons of 16 marker loci, for two SCAR, two CAPS and eight SSCP markers (Tables 3.1, 3.3, and 3.4). The 28 markers were distributed on the twelve potato chromosomes and tagged QTLs for *P. infestans* known from previous studies. SNP evaluation in the BNA population resulted in 170 SNPs in a total region of 5690 bp considering only the well readable sequence (Table 3.2.). Four SNP markers were significantly linked to quantitative resistance to *P. infestans*: Marker GP 23, (snp 183) located on chromosome II; marker S1b3, (snp 195 and snp 257) on chromosome III; markers BA76o11T3, (snp 336) and marker BA87d17-(snp109) on chromosome V (Table 3.5). None of the tested SSCP, CAPS and SCAR markers were significantly linked to QTL for resistance to *P. infestans* (Table 3.3. and 3.4).

Table 3.1. **Results of the SNP analysis of the SaKa-Ragis and BNA populations evaluated for quantitative resistance to *P. infestans*.**

LG [1] group	Marker	№ SNPs evaluated in SARA population	№ SNPs evaluated in BNA population	№ InDel evaluated	SARA № significant SNPs (P≤ 0.01)	BNA № significant SNPs (P≤ 0.01)	Amplicon size, bp
I	BA114i24t3	7	0	0	0	n.a.[2]	220
	BA62l7t3	4	4	0	0	0	480
II	St3.2	4	0	0	0	n.a.[2]	350
	GP23	0	7	0	n.a.[2]	2	100
	GP321	8	7	0	0	0	580
III	S1b3	21	20	0	0	1	280
V	BA213c14t7	26	6	0	0	0	510
	BA87d17t3	13	10	0	0	1	510
	BA76o11t3	2	2	1	0	1	70
VI	BA34j14t7	0	21	0	n.a.[2]	0	360
VII	BA228g19t3	7	6	0	0	0	480
VIII	BA73e8t3	9	10	0	0	0	180
	BA261b9t7	13	13	0	0	0	260
IX	GP129	7	11	0	0	0	270
X	CP105	20	15	0	5	0	370
	GP266	13	20	0	1	0	330
	BA81l15t3	4	2	0	0	0	450
	BA44a10t7	11	0	0	0	n.a.[2]	330
XI	BA157f6t3	16	16	0	0	0	460
		∑=185	∑= 170	∑ = 1	∑ = 6	∑ = 5	∑=5690 (BNA) ∑=5800 (SARA)

[1] LG group= chromosome, [2] n.a. - not analyzed marker

Table 3.2. **Summary of the successfully analyzed SNP markers in the BNA population.**

Number of loci screened	16
Number of significant loci:	4
Total length of amplicons analyzed per individual, bp	5690 bp
Total number SNPs	170
Significant SNPs	5
SNP frequency	1/ 33 bp

Table 3.3. **Results of segregating SSCP alleles from the SSCP markers in the SaKa-Ragis and BNA populations**

LG	Marker	Polymorhic parent(s)		№ of individuals having the polymorphic band		Restriction enzyme	P value	
		Saka-Ragis	BNA	Saka-Ragis	BNA		Saka-Ragis	BNA
III	GP1	n.p.[2]	NK5	n.a.[1]	R20/S23	*Mse*I	n.a.[1]	0.267
IX	CP44[a]	SR1	NK5	R22/S20	R16/S13	*Mse*I	0.182	0.361
	CP44[b]	SR2	NK5	R19/S11	R19/S19	*Mse*I	0.494	0.581
	CP44[c]	SR2	NK5	R11/S11	R15/S18	*Mse*I	0.614	0.563
	CP44[d]	SR1	NK5	R21/S20	R15/S13	*Mse*I	0.182	0.361
	CP44[e]	SR2	NK6	R19/S20	R17/S19	*Mse*I	0.494	0.437
	CP44[f]	SR2	NK5	R10/S11	R18/S18	*Mse*I	0.490	0.565
IX	B7[a]	SR2	NK5	R11/S10	R13/S12	*Hinf*I	0.5	0.5
	B7[b]	SR2	NK6	R13/S14	R13/S20	*Hinf*I	0.5	0.03
	B7[c]	SR2	-	R18/S18	-	*Hinf*I	0.63	-
IX	GP94[a]	SR1	NK6	R14/S13	R9/S10	*Hinf*I/*Dpn*I[3]	0.5	0.5
	GP94[b]	+	-	R8/S9	-	*Hinf*I	0.5	-
X	TPT[a]	SR1	n.p.[2]	R11/S12	n.p.[2]	*Nla*III	0.5	n.p.[2]
	TPT[b]	+	n.p.[2]	R15/S8	n.p.[2]	*Nla*III	0.041	n.p.[2]
X	GP247	SR2	NK5	R33/ S15	R15/S33	*Mse*I	0.5	0.282
X	GP287[a]	SR1	n.p.[2]	R12/S10	n.a[1]	*Nla*III	0.386	n.a.[1]
	GP287[b]	SR2	n.p.[2]	R14/S11	n.a[1]	*Nla*III	0.282	n.a.[1]
	GP287[c]	SR2	n.p.[2]	R13/S15	n.a[1]	*Nla*III	0.385	n.a.[1]
	GP287[d]	SR2	n.p.[2]	R12/S13	n.a[1]	*Nla*III	0.500	n.a.[1]
XI	NL27[a]	SR1	NK5	R16/S9	R14/S17	*Mse*I	0.041	0.38
	NL27[b]	SR2	NK5	R12/S15	R12/S9	MseI	0.281	0.281
	NL27[c]	SR2	-	R5/S9	-	*Mse*I	0.171	-
	NL27[d]	SR1	-	R17/S8	-	*Mse*I	0.01	-
XI	GP125[a]	n.p[2]	NK6	n.a.[1]	R11/S15	*Rsa*I	n.a.[1]	0.193
	GP125[b]	n.p.[2]	NK5 and NK 6	n.a.[1]	R21/S20	*Rsa*I	n.a.[1]	0.500
XII	GP229	n.p.[2]	NK5	n.a.[1]	R19/S15	*Rsa*I	n.a.[1]	0.171

R-resistant- "cases" and S-susceptible- "controls"
[1]n. a.-not analyzed; [2]n. p. - not polymorphic; + alien segregating allele; alleles assigned as **a**, **b**, **c**, **d** in the parents of both populations display different polymorphic bands
LG- linkage group (chromosome); - not segregating allele; [3]-*Hinf*I-restriction endonuclease was used for SARA population; *Dpn*I-restriction endonuclease for BNA population

Table 3.4. **Results of the segregating alleles from the CAPS and SCAR markers in SaKa-Ragis and BNA populations**

LG	Marker	Polymorphic parent		№ of individuals having the polymorphic band		Restriction enzyme	P value	
		SARA	BNA	SARA	BNA		SARA	BNA
IV	STM3016	SR1	NK5	R12/S15	R12/S13	SCAR	0.281	0.5
VI	BA71g21T7[a] BA71g21T7[b]	SR2	-/-	R13/S13 R21/S18	R19/S13	SCAR	0.488 0.231	0.06
VIII	57T3	SR1	NK5	R14/S9	R23/S23	*Rsa*I	0.193	0.5
IX	Prp1	SR1	n.p.[1]	R19/S21	n.p.[1]	SCAR	0.333	n.a.[2]
X	CP72	SR1	NK5	R10/S13	R18/S21	*Dpn*II	0.5	0.5
XII	GP34	SR1	n.a.[2]	R17/S18	n.p.[1]	*Alu*I	0.5	n.a.[2]
XII	GP76	SR1	n.a.[2]	R15/S19	n.p.[1]	*Rsa*I	0.171	n.a.[2]

R-resistant to *P. infestans*- "cases"; S-susceptible to *P.infestans*- "controls"
[1]n.p.- not polymorphic marker; [2]n.a.-not analyzed marker; -/- the allele segregated neither from NK5 nor from NK6 parents; **a** and **b**- segregating alleles

Analyzing the SSCP, CAPS and SCAR markers resulted in 15 alleles segregating from the NK5 (resistant) parent and 5 segregating alleles from the NK6 (susceptible) parent in the F1 progeny of these parents (Tables 3.3 and 3.4). The minimum number of genotypes required for the SNP analysis was calculated for all significant SNP positions in accordance to the observed frequency of individuals per genotypic class per phenotypic group. The threshold for the chi-square test of linkage was set to P value < 0.01, (Table 3.5). Four of the five significant SNPs fulfilled the criterion with exception of snp 257 in marker Slb3 on chromosome III, where more genotypes were required. For Snp109 in marker BA87d17t3 on chromosome V the minimum number required individuals were not calculated because 12 genotypic classes were observed with very less genotypes per genotypic class. In SCAR marker, BA71g21T7 on chromosome VI (Table 3.5.), allele neither from parent NK5 nor from parent NK6 segregated in 33 genotypes from the BNA population. Probably, there was cross-pollination in the population or NK5 and NK6 are not the parents of the BNA population, despite none of the analyzed SNP, CAPS and SSCP showed alien allele segregating in the population. The segregating alleles from the NK5 and NK6 parents for the SSCP markers are shown in (Table D), appendix.

Table 3.5. **Results of the significant SNPs in SARA and BNA populations. Numbers shown in red are the SNPs for which the numbers of the genotypes in the assay was not sufficient.**

LG	Marker	Population	SNP	Sequence	Individuals in the assay	Required individuals	P value
II	GP23	BNA	snp183	GTC(C/A)ACT	48	33	0.007
III	S1b3	BNA	snp195	TCT(C/T)TGC	48	46	0.017
			snp257	CAT(A/T)ACT	48	69	0.017
V	BA87d17t3	BNA	snp109	ATG(T/G/C)AC	-	-	0.004
	BA76o11t3	BNA	snp336	ACAT(C/G)GAT	46	32	0.001
X	CP105	SARA	snp54	TC(C/G)TCG	48	42	0.009
			snp58	TCG(T/C)TGC	48	42	0.009
			snp143	ACC(C/T)GAA	48	42	0.009
			snp191	ATG(T/C)ACT	48	39	0.004
			snp329	ATT(T/G)TTA	48	42	0.009
X	GP266	SARA	snp130	CCT(G/A)AC	46	57	0.009

-not analyzed locus for minimum number required individuals

3.1.2.2 QTL analysis in Saka-Ragis population

The SaKa-Ragis population was genotyped with 17 loci with 185 SNPs, three SCAR, four CAPS and seven SSCP markers in the F1 progeny of "cases" and "controls" (Tables 3.1, 3.3 and 3.4). The 31 polymorphic markers were distributed on all 12 potato chromosomes. The two significant markers in the SaKa-Ragis population, CP105 and GP266, are localized on chromosome X. Five SNPs in marker CP 105-(snp 54, snp 58, snp 143, snp 191 and snp 329) and one SNP in marker GP 266- (snp130) were significantly linked to QTL for resistance to *P. infestans*. 185 segregating SNPs in SaKa-Ragis population were tested in 5800 bp total, considering only the well readable sequences. Thus, the SNP frequency resulted in one SNP per 31 bp, (Table 3.6.) The significant SNPs were tested with the chi-square test for linkage to QTL. For all significant SNPs in marker CP105, the numbers of the plant genotypes were enough in order to state the significant value of the markers. In case of marker GP266-(snp130), the

numbers of individuals in the study were not enough. Analyzing the SSCP, SCAR and CAPS markers resulted in 13 alleles segregating from SR1 (resistant) and 14 alleles from SR2 (susceptible) parent in the F1 progeny of these parents (Tables 3.3 and 3.4) . None of the tested SSCP, CAPS and SCAR markers resulted in significantly linked to the quantitative trait resistance to *P. infestans* with exception of marker NL27 on chromosome XI which was slightly significant (Table 3.3). The segregating alleles from the SR1 and SR2 parents in the SSCP analysis are shown in (Table D, Appendix).

Table 3.6. **Summary of the analyzed SNP markers in SaKa-Ragis population.**

Number of loci screened	17
Number of significant loci	2
Total length of amplicon analyzed per individual, bp	5800 bp
Total number SNPs	185
Significant SNPs	6
SNP frequency	1/ 31 bp

Alien allele segregated in 23 of 46 in total genotypes in the SaKa-Ragis population in marker TPT (Table 3.3). An aberrant allele segregated in 17 of the 46 in total genotypes in marker GP94 (Table 3.3).

3.1.2.3 Extended marker study on chromosome X in the Saka-Ragis population

Four loci were analyzed for SNP on chromosome X, where CP105 was the most significant marker (Table 3.1). In order to test how large the QTL effect on chromosome X for this population extends; four SSCP markers were selected and analyzed (Fig.1.6, Introduction, chromosome X). Analyzing the SSCP markers GP247, CP72, GP287 and TPT resulted in non-significant p values. CP105 marker was sequenced in two additional diploid populations GDE and K31 population. These two diploid populations have been tested in a previous study and segregated for QTL for resistance to *P. infestans* (Oberhagemann et al.1999) but not tested for markers in the genomic region where CP105 marker is localized.

3.1.2.3.1 Analyzing CP105 marker in GDE diploid population

The GDE diploid population was chosen in order to test the marker CP105 in a genetic background different from the previously analyzed tetraploid populations. This population has been evaluated for *P. infestans* resistance in the field in years 1996 and 1997 and segregated for QTL for *P. infestans*.

The CP105 marker was sequenced in GDE population consisting of 86 genotypes and resulted in 14 polymorphic segregating SNPs. Exact position and segregation ratio of the 14 SNP analyzed are summarized in Table 3.7. The phenotypic data for GDE population were tested with Kolmogorov-Smirnov test and resulted in normal distribution of the phenotypic data. The segregating SNPs were tested with One – way ANOVA test. In all 14 segregating SNPs in GDE population the observed SNP segregation was distorted Table 3.7. None of the SNPs was significantly linked to the QTL for resistance to *P. infestans* on chromosome X.

Table 3.7. **SNP position, expected and observed segregation ratio of the 14 SNPs analyzed in GDE population is shown.**

SNP position	I88	G87	Expected segregation ratio	Observed segregation ratio
snp63	GA	GA	1 GG : 2 GA : 1AA	1 GG : 27 GA : 0 AA
snp 68	CA	CA	1 CC : 2 CA : 1 AA	1 CC : 2 CA : 0 AA
snp 81	CT	TT	1 TT : 1 CT	1 TT : 24 CT
snp 133	CA	CA	1 CC : 2 CA : 1 AA	1 CC : 5 CA : 0 AA
snp 143	CC	CT	1 CC : 1 CT	1 CC : 2 CT
snp144	GG	AG	1 GG :1 GA	2 GG : 1 GA
snp 149	CT	CT	1 CC : 2 CT : 1 TT	1 CC : 2 CT : 0 TT
snp 157	TT	AT	1 TT : 1 AT	2 TT : 1 AT
snp 158	TT	CT	1 TT : 1 TC	2 TT : 1 TC
snp 181	AT	AT	1 AA : 2 AT : 1 TT	1 AA : 42 AT :1 TT
snp 237	CT	CC	1 CC : 1 TC	1 CC : 42 TC
snp 281	AG	GG	1 GG : 1 GA	1 GG : 6 AG
snp 326	AG	AG	1 AA : 2 AG :1 GG	0 AA : 85 AG :1 GG
snp 371	CT	TT	1 TT : 1 CT	1 TT : 42 TC

From the five significant SNPs analyzed in cases and controls, only snp143 was heterozygous in the GDE population (Tables 3.5 and 3.7). Snp 68, 149, 157 and 158 in the GDE population were not analyzed in the CP105 marker in the “cases” and “controls” study.

3.1.2.3.2 Analyzing CP105 in the K31 diploid population

Similarly, to the GDE population the Kolmogorov-Smirnov test was performed, to test whether the phenotypic data of the population are normal distributed. The normally distributed data were tested with One-way ANOVA test. Sequencing the CP105 marker in the K31 population in 90 genotypes revealed 12 segregating SNPs. Exact position, segregation ratio and parental SNP of the segregating SNPs in K31 population are reported in Table 3.8. Similarly as in the GDE population, in the K31 population all segregating SNPs had distorted segregation ratio. None of the 12 analyzed SNPs were significantly linked to the QTL for resistance to *P. infestans*.

Table 3.8. **SNP position, expected and observed segregation ratio of the 12 SNPs analyzed in K31 population is shown.**

SNP position	P3	P38	Expected segregation ratio	Observed segregation ratio
snp 63	GA	GA	1 GG : 2 GA : 1 AA	1 GG : 36 GA : 7 AA
snp 65	AT	AT	1 AA : 2 AT : 1 TT	1 AA : 36 AT : 7 TT
snp 66	AG	AG	1 AA : 2 AG : 1GG	3 AA : 1 AG : 0 GG
snp 81	CT	CT	1 CC : 2 CT : 1 TT	1 CC : 39 CT : 5 TT
snp 131	CT	CT	1 CC : 2 CT : 1 TT	0 CC : 4 CT :1 TT
snp133	CA	CA	1 CC : 2 CA : 1 AA	1 CC : 4 CA : 0 AA
snp181	AT	AT	1 AA : 2 AT : 1 TT	1 AA : 4 AT : 0 TT
snp203	AA	AG	1 AA : 1 AG	11 AA : 2 GG : 1 AG
snp237	CT	CT	1 CC : 2 CT : 1 TT	16 CC : 4 CT : 0 TT
snp281	AG	AG	1 AA : 2 GA : 1 GG	1 AA : 4 AG : 0 GG
snp326	AG	AG	1 AA : 2 AG : 1 GG	1 AA : 4 AG : 0 GG
snp371	CT	CT	1 CC : 2 CT : 1 TT	1 CC : 4 CT : 0 TT

From the five significant SNPs analyzed in cases and controls, none was heterozygous in the K31 population (Tables 3.5 and 3.8). Snp 65 and 66 in the K31 population were not analyzed in the CP105 marker in the "cases" and "controls" study.

3.1.2.4 Haplotype marker CP105 on chromosome X in Saka-Ragis population

Five significant SNPs in a PCR product of 350bp could be resolved in two distinct haplotypes **a** and **b** in the SR1 (resistant) and in SR2 (susceptible) parents. The three haplotypes were composed in *silico* and their segregation was followed in the F1 progeny of the SaKa-Ragis population. The haplotypes **a** and **b** from the SR1 and SR2 parents were followed in the progeny. All five SNPs from the haplotype **b** were linked and transferred as a haplotype in the progeny (Table 3.9). In the susceptible tetraploid SR2 parent the four homologous chromosomes were haplotype-**a**, identical to the haplotype **a** for two of the chromosomes from the resistant tetraploid SR1 parent. SR1 parent carries other distinct haplotype **b** which was absent in the susceptible parent SR2 (Table 3.9). The significant SNPs comprising the two distinct haplotypes were not polymorphic in the BNA population.

Table 3.9. **Haplotype composition of parents SR1 and SR2 based on 5 SNPs in marker CP105 on chromosome X**.

parent	Homologous chromosomes	haplotype	snp54	snp58	snp143	snp191	snp329
SR1- resistant parent	1	a	C	T	C	T	T
	2	a	C	T	C	T	T
	3	b	G	C	T	C	G
	4	a	C	T	C	T	T
SR2- susceptible parent	1	a	C	T	C	T	T
	2	a	C	T	C	T	T
	3	a	C	T	C	T	T
	4	a	C	T	C	T	T

The genotypes of the "cases" and "controls" fell into two distinct genotypic classes according to the two haplotypes inherited from the parents SR1 and SR2. A Descriptive

test (Cross tab) was performed for the two segregating haplotypes and resulted in p value 0.004. The haplotype **b** segregating from the resistant SR1 parent was present in 15 of the 24 genotypes in total in the “cases”, where in 5 of the 24 genotypes from the “controls” this haplotype was observed (Fig.3.2).

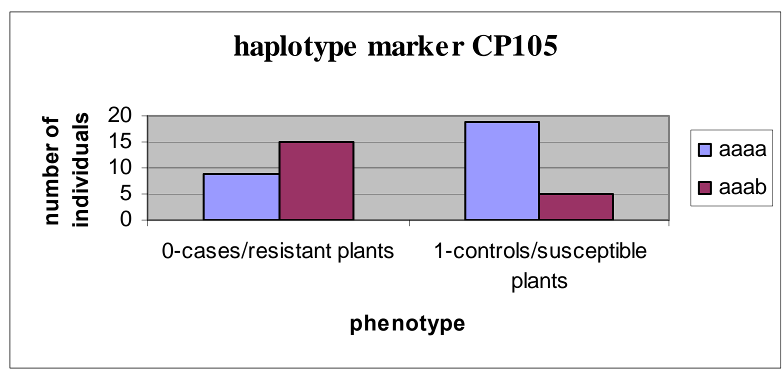


Fig. 3.2. Distribution of haplotypes of marker CP105 on chromosome X in the “cases” and “controls” in the SaKa-Ragis population .

3.1.2.5 Analyzing CP105 marker in the whole SaKa-Ragis population

CP 105 marker was sequenced in the whole population consisting of 192 individuals, where for 21 genotypes not well sequence could be obtained. Nine of 20 SNPs analyzed in the “cases” and “controls“ segregated in the whole population (Table 3.10). Six SNPs in the beginning of the CP105 sequence tested in the “cases” and “controls” were not analyzed in the whole population, because of not well readable sequences. One significant SNP from the “cases” and “controls” *e.g.* snp 191 was significant in the whole population, p value 0.000. Not fitting to the segregation pattern expected from the parental genotypes SNPs were observed in the whole population (Table 3.10). One-Way ANOVA test was performed only with the 20 individuals left after selecting the genotypes with SNPs segregating according to the parental genotypes and the 46 “cases” and “controls”, resulting in 66 genotypes in total. Snp260 which was not polymorphic in the parents and not observed in the “cases” and “controls” segregated in the whole

population. Deviations from the segregating SNPs from the two parents were not observed in any of the markers tested in the "cases" and "controls" study.

Table 3.10. **Results from the ten analyzed segregating SNPs in the whole SaKa-Ragis population. SNP in the two parents SR1 and SR2 are shown and the deviating SNPs observed in the population.**

SNP position	SR 1 parent	SR2 parent	SNPs deviating from the expected SNP in SR1 and SR2 parents		
snp110	TTTT	TTTC	TTCC (16)[1]	CCCC (4)[1]	
snp131	TTCC	TTTT	TCCC (10)[1]	CCCC (12)[1]	
snp133	CCCA	CCCC	CCAA (44)[1]	AAAA (12)[1]	
snp181	AATT	AAAA	TTTT (1)[1]		
snp191	TTTC	TTTT	TCCC(2)[1]	TTCC (13)[1]	
snp203	AAAG	AGGG	-	-	
snp237	CCCT	CCCC	CCTT (17)[1]	TTTT (1)[1]	
snp260	CCCC	CCCC	CCTT (18)[1]	CTTT (5)[1]	TTTT (11)[1]
snp281	AAAG	AAAA	AAGG (7)[1]	GGGG (4)[1]	
snp371	CCCT	CCCC	CTTT (8)[1]	TTTT (14)[1]	CCTT (61)[1]

[1]Numbers in parenthesis show the number observed individuals per genotypic class not fitting to the segregation pattern expected from the parental genotypes
- SNP segregated according to the genetic model

Genotypes having CCCC at snp position 110, for example were not expected (Table3.10), because parent SR2 is simplex for C, e.g. TTTC. Possible, explanation is that out-crossing has occurred `alien` pollinators, due to not perfectly isolated conditions were the crossings have been performed (personal communication Dr. Jens Lübeck).

3.2 Analysis of association with QTL on chromosome V based on "cases" and "controls"

3.2.1 Selecting "cases" and "controls" based on their original phenotypic passport data

A SNP study was performed on 65 genotypes selected from a collection of 610 tetraploid potato cultivars. This population has been assessed for two quantitative traits: the foliage and tuber resistance to *P.infestans* and the maturity. The genotypes were selected based on their passport data and classified in two groups: the first group was highly resistant to *P. infestans* and late maturing ("cases"), the second group was highly susceptible to *P. infestans* and early maturing ("controls"). This combination of phenotypic traits was selected, because it is more feasible to find resistant, late maturing and susceptible, early maturing potato genotypes. These two phenotypic traits are correlated and it was therefore easy to select a sufficient number of individuals per phenotypic group in order to perform a QTL gradient experiment. For the maturity trait, the genotypes of the two groups "cases" and "controls" fell into two distinct phenotypic groups with clear difference in the phenotypic scores (Fig.3.3). For resistance to *P. infestans*, individuals belonging to classes 1, 2, 3 and 4 were selected for the "control" group with exception of one genotype being of class 5; individuals belonging to resistant classes 6, 7, 8 and 9 were assigned to the group "cases" with exception of two genotypes one belonging to classes 4 and one genotype to class 5, but being highly late mature, therefore considered as belonging to the resistant class (Fig. 3.4). The selected "cases" and "controls" and the passport data for each genotype is summarized in (Table E, Appendix).

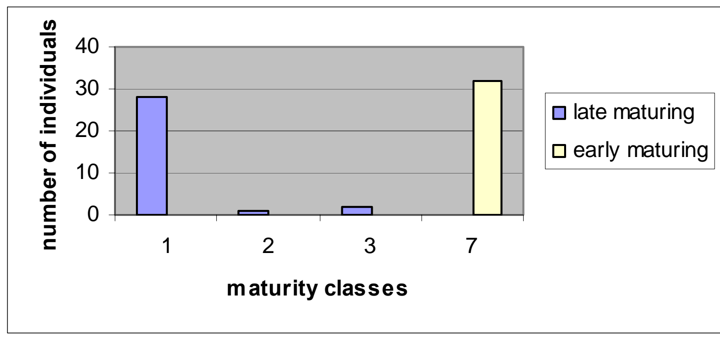


Fig.3.3. Distribution of the genotypes in the "cases" and "controls" study in maturity classes derived from the passport data.

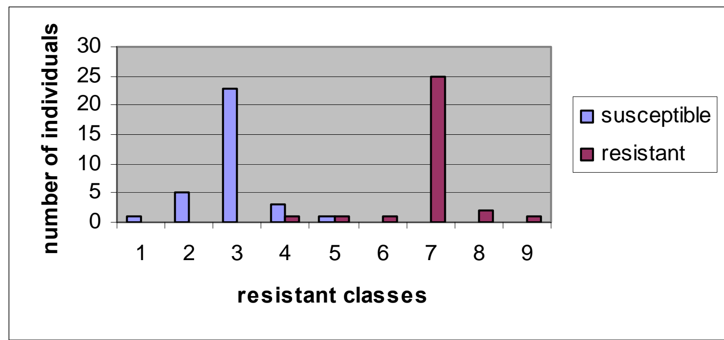


Fig. 3.4. Distribution of the individuals in the "cases" and "controls" study in resistance classes according to the passport data for quantitative resistance to *P. infestans*. Grade scale: resistance to *P. infestans* increases gradually from 1 to 9, where "1" are the most susceptible and "9" are the most resistant plants.

3.2.2 SNP analysis of genomic markers in "Cases/ controls" association study on chromosome V

The purpose of the experiment was to narrow down genetically the genomic region on chromosome V in which the gene/ genes for resistance to *P. infestans* and maturity are localized. The experiment was performed by sequencing and analyzing for SNPs four putative candidate genes ORF №3 (EST no homology) localized on BA47f2, ORF №24 (ATPase protein family-AAA type) in BA122p13, ORF №36 (acid phosphatase) on BA 213c14 and ORF №47 (protein kinase) on BA151m8, the most distal BAC in the physical contig (Fig.3.5). The annotation of the genes comes from the blast search of the predicted potato ORFs in TIGR database from *S. tuberosum* and in TAIR database for the *A. thaliana* homologues genes (3.3.3).

Amplicons for ORF 3, ORF 24, StPto and ORF 47 were sequenced with the forward, while OFR 36 and GP21 with the reverse primer Tables B and C, Appendix.
84 SNP were analyzed in the four markers and did not resulted in finding of QTL gradient in the physical contig on chromosomeV (Table 3.12). The four markers showed very strong association to resistance to *P. infestans* and maturity. The 11 of the 84 analyzed SNPs were significant of p value < 0.005 (Table 3.11).
In order to test whether the association effect declines at marker loci outside the physical contig the single copy RFLP markers, GP21, Uptg1, GP186 and StPto were selected. Markers GP21, Uptg1 and GP186 have been mapped to the distal (telomere) site of the physical contig; while StPto on the proximal (centromere) site. Lp*Pto*- Genbank accession number-U02271 is a protein kinase gene and was cloned in *Lycopersicon pimpinellifolium* (Martin et al. 1993, 1994). The sequence from Lp*Pto* and the corresponding potato EST TC128018 in TIGR database were aligned, resulting in 91% identity at the nucleotide level. Primers were designed for the potato EST. The Lp*Pto* and St*Pto* are homologous genes of potato and tomato both localized on chromosome V in potato and tomato, respectively. Marker GP186 could not be analyzed, as the sequence trace files for this marker did not result in gut quality sequence suitable for SNPs evaluation. A single band PCR product could not be obtained for marker Uptg1, despite several attempts to design primers for different regions of the gene.
129 SNPs were analyzed in six markers: GP21, ORF3 on BAC47f2, ORF24 on BAC122p13, ORF36 on BAC213c14, ORF47 on BAC151m8 and StPto from which 20 significantly liked to resistance to *P. infestans* and maturity (Table3.11). The location of the ORF in the corresponding BACs is illustrated in Fig.3.14. All 20 significant SNPs were tested for minimum number of genotypes required at a threshold p value < 0.01, in order to test whether numbers of individuals included in the association study were enough to perform the statistical descriptive chi-square test. The minimum number of genotypes required for each significant SNP is summarized in Table 3.11. From the 20 significant SNPs, 15 SNPs fulfilled the criteria for threshold p value < 0.01. For 5 of the significant SNPs the number of the genotypes was slightly smaller than the required. The minimum number of individuals required for the different SNPs within the same marker

differed depending on the observed frequency of individuals per genotypic class per phenotypic class.

Table 3.11. **Significant SNPs detected in amplicons of 6 marker loci on chromosome V.**

Marker/ ORF №	BAC	Significant SNP	Sequence[1]	Required[2] individuals	Individuals in the assay	P value
GP21	-	snp1	CCA(C/T)GTA	30	53	0.000
		snp5	TTA(T/C)ACT	45	53	0.002
		snp6	TTT(A/G)CTA	33	53	0.000
		snp11	GAG**(T/C)**ATC(T/A)CT	32	53	0.000
		snp12	GTT**(T/C)**AC(G/A)TT	32	53	0.000
		snp13	GTT(T/C)AC**(G/A)**TT	34	53	0.000
ORF3	BA47f2	snp7	AGA(G/A)GTA	51	65	0.002
		snp12	CA**(C/T)**(T/A)GTA	61	65	0.005
		snp25	CTT**(C/T)**(G/A)TGC	22	65	0.000
		snp33	GTC(T/C)ATC	62	65	0.006
		snp35	CCA**(C/T)**(G/A)CCTT	50	65	0.001
		snp36	CCA(C/T)**(G/A)**CCTT	48	65	0.000
ORF24	BA122p13	snp5	TACA(T/C)**(T/C)**GTCA	68	64	0.008
		snp8	CAG(C/T)ATT	72	64	0.005
		snp17	TTCA(A/G)TTT	70	64	0.008
ORF36	BA213c14	snp1	GGA(A/C)TAT	44	62	0.001
ORF47	BA151m8	snp6	GCC(T/G)ATG	65	64	0.005
St*Pto*	-	snp14	GCA(T/C)TA**(T/C)**CTT	59	59	0.004
		snp21	AAT(G/T)ACTG	57	59	0.003
		snp29	CCT(T/G)GAC	39	59	0.000
Total number of significant SNPs						20

[1]In the sequence where two SNPs are shown, the SNP in red and bold is the significantly associated with resistance/maturity.
[2]Numbers shown in red are the SNPs for which the individuals number was less then the required.
- markers not localized in BAC clone

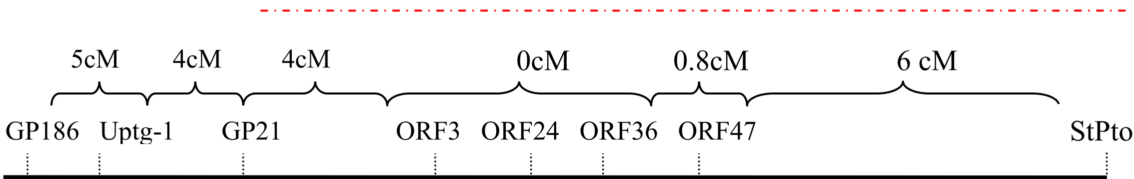


Fig. 3.5. Schematic overview of the genetic distance between the markers analyzed on the potato chromosome V for the purpose of the QTL gradient experiment. Red dashed line shows the region in which the six markers for SNPs were analyzed. The genetic distance is taken from www.gabi.rzpd.de database.

Of 129 analyzed SNPs, 101 SNPs were localized in putative coding and 28 SNPs in the putative non-coding region. The SNP analysis was conducted in a region of 3932 bp referring only the gut readable sequence. The SNP frequency in the coding region on chromosome V was 1/27 bp and 1/35 bp in the non-coding region Table 3.12. The intron sequence in the marker is shown in red in Appendix.

Table 3.12. **SNP frequency in putative coding and non-coding regions of the genes/ markers tested on chromosome V**.

Marker/ORF №	SNPs scored		SNPs frequency, bp	
	Coding region	Non-coding region	Coding region	Non-coding region
GP21	-	12	-	1/25
3	27	9	1/16	1/17
24	22	-	1/24	-
36	13	5	1/26	1/29
47	7	2	1/66	1/61
St*Pto*	32	-	1/17	-
Total number SNPs scored	101	28	1/27	1/35
Total length of coding versus non-coding, bp	2739	1193		

-the sequence contains only coding or non-coding region

The full “cases” and “control” population was analyzed only with ORF № 3. For ORFs № 24, 36, 47, markers GP21 and St*Pto* the exact number of individuals tested per

marker/ ORF is summarized in Table 3.13. The difference in the number of individuals tested per marker/ ORF was due to unreadable sequence trace files for some of the 65 analyzed genotypes.

Table 3.13. **Number of genotypes and number of SNPs analyzed for all six markers on potato chromosome V.**

ORF №/ marker	Putative assignment/ marker	BAC	№ of individuals tested [1]	Significant SNPs	Scored SNPs
GP21	-	+	R25/ S28	6	12
ORF3	No homology	47f2	R32/ S33	6	36
ORF24	ATPase protein family	122p13	R32/ S32	3	22
ORF36	Acid phosphatase	213c14	R30/ S32	1	18
ORF47	Protein kinase	151m8	R32/ S32	1	9
St*Pto*	Protein kinase	+	R27/ S32	4	32
Total number of SNPs scored					129

[1]R-resistant to *P.infestans* / late mature; S- susceptible to *P.infestans* / early mature
+ marker not localized in BAC clone; - no putative assignment

3.2.3 St*Pto* on chromosome V

In order to test whether the primers amplify the St*Pto* gene mapped to the potato chromosome V, St*Pto* amplicons of the parents P18 and P40 of the diploid mapping population F1840 were sequenced. The parents were polymorphic for the St*Pto* locus but homozygous, which would not result in informative segregating polymorphisms in the progeny of these parents. The parents I88 and G87 of the GDE diploid population were chosen to test further whether the amplified St*Pto* PCR product is the St*Pto* previously mapped on chromosomeV. In a previous study (Oberhagemann et al. 1999), the GDE population has been tested with RFLP markers GP179, GP21 and St*Pto* and PCR based markers *SPUD237* and *CP113* on chromosome V. The segregation data of the markers for the whole F1 population were available. Using this information, the data of two alternatively segregating St*Pto* alleles from the susceptible parent I88 were compared to

the segregation data of two GP179 alleles. The two polymorphic St*Pto* alleles from the susceptible I88 parent were obtained after digestion of the St*Pto* PCR product with *Mse*I restriction endonuclease (Fig.3.6). Alleles 2 of St*Pto* were linked with the GP179_1 allele Fig.3.6. Five of 80 individuals in total in the GDE population were recombinant, resulting in a genetic distance between GP179 and St*Pto* markers of about 6 cM.

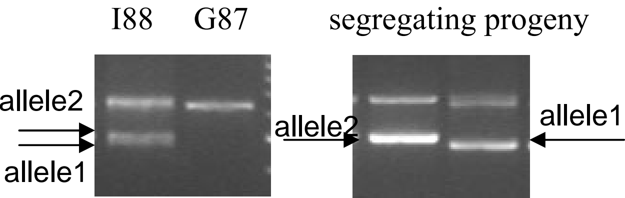


Fig. 3.6. Digestion patterns of the alternative alleles descending from the susceptible I88 parent for the St*Pto* locus on chromosome V. St*Pto* PCR product was digested with *Mse*I restriction endonuclease.

This result confirmed that the primers for the potato EST St*Pto* amplicon amplify a single copy St*Pto* localized on chromosome V.

3.2.4 Presence of a certain genotypic class in only one phenotypic group

The difference between the resistant/ late maturing and susceptible/ early maturing groups in the majority of the SNPs scored was due to a different frequency of genotypes per phenotypic class rather an absence or presence of a genotypic class in only one of the phenotypic groups (Fig.3.7). However, for 12 SNPs of all 129 SNPs tested in the 8-10 cM region presence of a certain genotypic class was observed in only one phenotypic group. The 12 SNPs were significantly associated with resistance to *P. infestans* and maturity. All differences in terms of presence of a genotypic class in one of the phenotypic classes and numbers of individuals per genotypic class are summarized in Table 3.14.

For example, the genotypic class TTTT of snp11 of marker GP21 was present only in the resistant/ late maturing group, whereas the genotypic class CCCC of the same marker was present only in the susceptible/ early mature group (Fig.3.8).

For the remaining 117 SNPs every genotypic class was present in both phenotypic groups the only difference being the frequency of individuals per genotypic class per phenotypic group (Fig. 3.7).

Table 3.14. **Presence of a certain SNP genotypic class and number of individuals observed per genotypic class in the resistant/ late maturing group**

Marker ORF№	SNP №	genotype	Number individuals observed only in the resistant/ late maturing class
GP21	snp1	CCCC	9
	snp6	AAGG and AGGG	6/5
	snp11	TTTT	10
	snp12	TTTT	10
	snp13	GAAA and GGAA	2/8
ORF3	snp25	CTTT	5
	snp36	GGGG	7
ORF24	snp8	CCTT	4
ORF47	snp6	TTTT	4
St*Pto*	snp23	CCCA	4

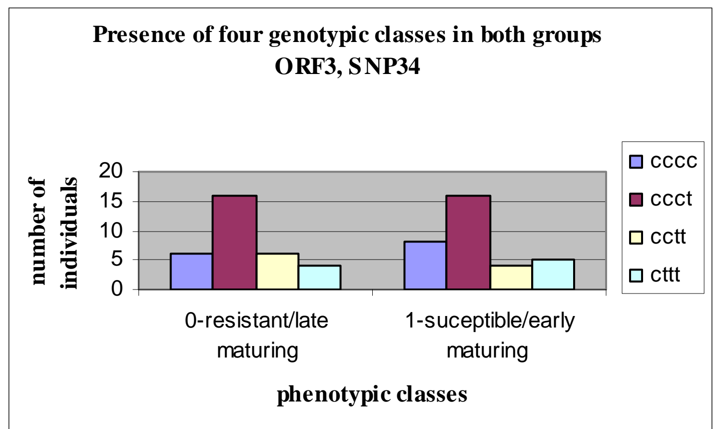


Fig. 3.7. Presence of four genotypic classes in both groups of selected individuals: resistant to *P. infestans* and late maturing and susceptible to *P. infestans* and early maturing

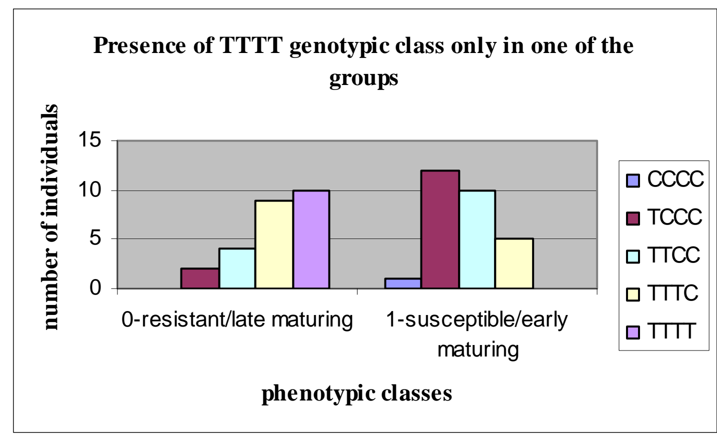


Fig.3.8. Presence of genotypic class TTTT at SNP11 in marker GP21 in only the resistant/ late maturing. Presence of genotypic class CCCC in only the susceptible/early mature group.

3.2.5 Tight linkage between SNPs within a marker in the QTL gradient experiment

The most pronounced difference between the two phenotypic groups was found in marker GP21 where six significant SNPs were scored. Two SNP combinations within the GP21 marker clearly distinguishable between the two phenotypic classes were observed. The SNP combination CCCC-TTTT-TTTT was present only in the resistant/ late maturing class and missing completely in the susceptible/ early maturing class. The second combination CTTT-TCCC-TCCC was present in 11 susceptible/ early maturing and in only one resistant/ late maturing individual (Table 3.15).

Table 3.15. **SNP combinations and their frequency in the resistant/ late maturing (R) and susceptible/ early maturing (S) individuals in GP21 marker on chromosome V.**

snp1	snp11	snp12	№ of individuals having the SNP correlation	snp5	snp6	snp13	№ of individuals having the SNP correlation
CCCC	TTTT	TTTT	R9/ S0	TTTC	AAAG	GGGA	R10/ S9
CTTT	TCCC	TCCC	R1/ S11	TTTT	AAAA	GGGG	R4/ S15

At least three distinct homozygous haplotypes spanning the whole St*Pto* sequence were found. Haplotype 3 was found in 11 susceptible/ early mature and in only one resistant/ late mature genotype (Table 3.16).

Table 3.16. **Three distinct homozygous haplotypes in the St*Pto* sequence**.

haplotype	Haplotype sequence[1]	№ of individuals having the haplotype[2]
1	A T T G G T G G T A G G T T T T T A G G G T C G G T C A T T G G	R5/ S4
2	G T T G C T G C C C C G T C T T T A G G T T A T G T C A T T C A	R7/ S1
3	A T T G G T G G T A G G T T T T T A G G G T C T G T C A G T C G	R1/ S11

[1]SNP numbers are shown in sequence St*Pto* ,(Appendix). The SNP numbers start from 1 and finish with 32. Each haplotype is consisted of 32 SNPs.
[2]R- resistant to *P. infestans* / late maturing group; S-susceptible to *P. infestans* / early maturing group; Nucleotides shown in are the different SNPs between the three haplotypes.

3.2.6 Statistic analysis with "cases" and "controls" genotypes including genotypic data from previously scored PCR based CAPS markers

The descriptive test (Cross tabs) was performed with the selected 65 individuals and genotypic data from the previously scored five PCR-based and CAPS markers in the population consisting of 610 genotypes (Gebhardt et al. 2004). The statistic test reconfirmed the significant p-value for CosA, *R*1 1400 bp and BAC 47f2 650bp markers as it was shown in the whole population test. This test was performed in order to confirm if the previously tested significant markers have also significant p-value with only the selected 65 individuals "cases" and "controls", meaning that the difference between the two groups is of significant value Table 3.17.

Table 3.17. **P values for the "cases" and "controls" in the QTL gradient experiment on chromosome V for the PCR based and CAPS markers scored in a previous study.**

marker	GP179 570bp	CosA 210bp	*R*1 1400bp	*R*1 1800bp	BAC47f2 650bp
P value	0.214	0.007	0.03	0.144	0.014

3.3 Expression study on putative candidate genes on chromosome V

I88 and G87 were chosen as they were the parents of a population having a very strong QTL for resistance to *P. infestans* on chromosome V. Both genotypes do not have the major *R*1 resistance gene, but in the progeny of these two parents a very strong QTL for resistance to *P. infestans* was detected on chromosome V (Oberhagemann et al. 1999). As a consequence of this genetic background I88 and G87 were used to perform the RT-PCR experiments. G87 will be referred to further in the text as the quantitative resistant parent (QR), and I88 as the quantitative susceptible parent (QS, as they both did not show a qualitative resistant or susceptible phenotype in the field (Oberhagemann et.al 1999).

3.3.1 Phenotypic difference between I88 and G87 after infection with *P. infestans*

The resistant (QR) G87 parent reacted with necrotic leasons 48h after infection with *P. infestans* (Fig.3.9 B), while the susceptible (QS) parent I88 showed mycelium growth (Fig.3.A). During the time course of infection, the QS parent I88 developed the infection symptoms much faster then the QR G87 parent. Both parents were infected after two weeks post-inoculation with *P. infestans*, but with large difference in the severity and delay of the symptoms. The QR parent reacted with having necrotic leaves at the infection site and developing very occasional lesions with *P. infestans.* The lesions with *P. infestans* were observed at least 14 days after infection; where the susceptible parent

was well covered with fluffy whitish mycelium and looked as if not developing any delayed defense response to prevent the growth of the oomycete (Fig.3.9).

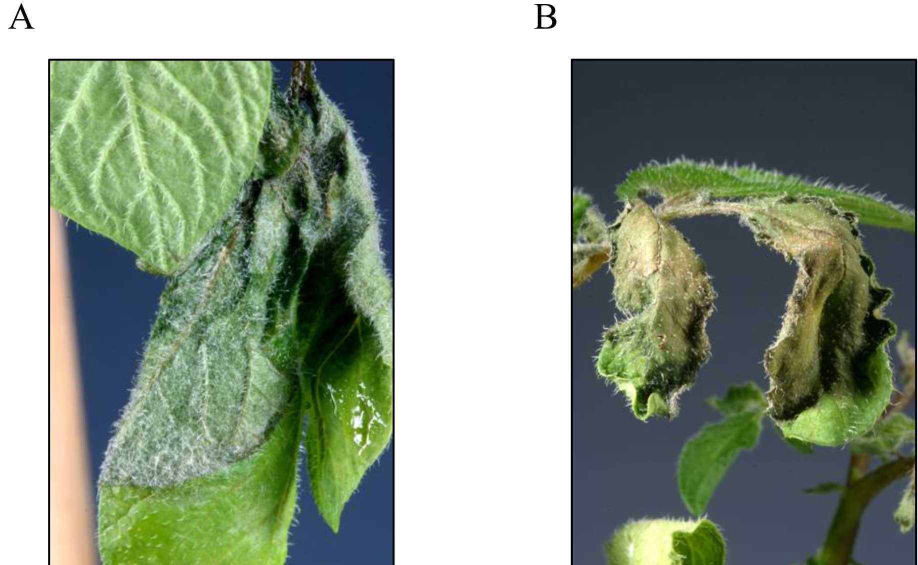


Fig. 3.9. Symptoms on the QS parent I88 are shown on the left and QR parent G87 on the right after *P. infestans* infection

3.3.2 Monitoring the presence of *Phytophthora infestans* using specific primers for the ribosomal DNA

P. infestans specific primers were used to monitor the presence of the oomycete on the infected potato leaves. Genomic DNA was extracted from the same plant samples used for the expression study with the 25 putative candidate genes (PCG). The sequences of *P. infestans* specific primers have been described by Judelson et. al (1997, Table B, Appendix). Primers amplified specifically a 258bp PCR fragment only in infected but not in control plants. Genomic DNA from *P. infestans* (provided by Dr. E. Schmeltzer at MPIZ) was used as a positive control for the standard PCR. As a negative control, genomic *S. tuberosum* DNA was used (Fig.3.10 and Fig. 3.11.)

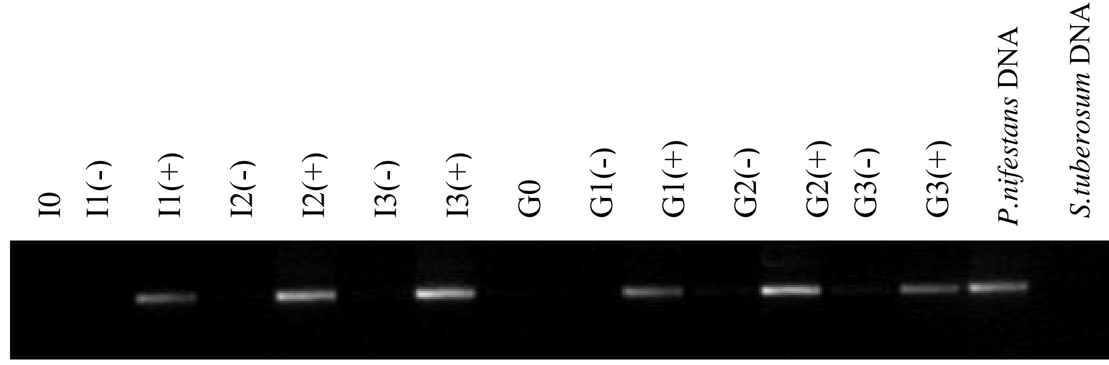


Fig. 3.10. Detection of the *P. infestans* 258bp specific band in infected leaf tissue using primers 08-3 and 08-4.
Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant

The ethidium bromide stained gels were scanned using a Typhoon scanner and quantified using Image Quant Software. The calculations for Fig.3.11 were performed with the mean value from three independent standard PCR reactions. The result showed that in the QS I88 parent the intensity of the PCR product slowly decreased with time after infection, while in the QR G87 parent the PCR band intensity was lower and did not change during infection time course (Fig.3.10 and Fig.3.11).

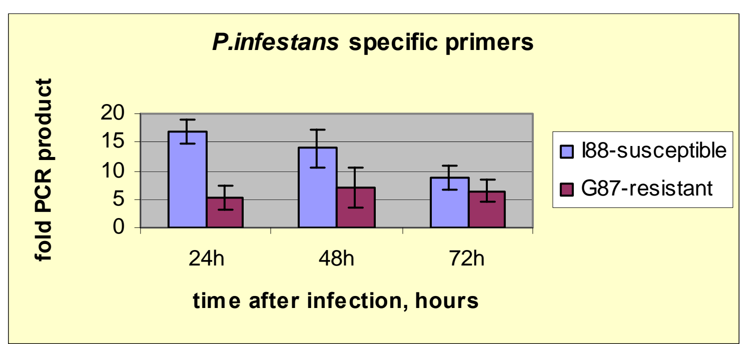


Fig. 3.11. Quantification of the PCR product obtained with *P. infestans* specific primers on infected I88 and G87 plants.

3.3.3 *PR*-1b specific band amplification in infected I88 and G87 plants

Specific primers for the *PR*1-b gene (Catherine 1999) were used in order to check whether the defense reaction of the potato plants infected with *P. infestans* took place. Primers for the *PR*1-b coding sequence were designed based on the cDNA sequence of a *PR*1-b clone (provided by Dr. Sabine Rosahl) Fig.3.12 and 3.13.

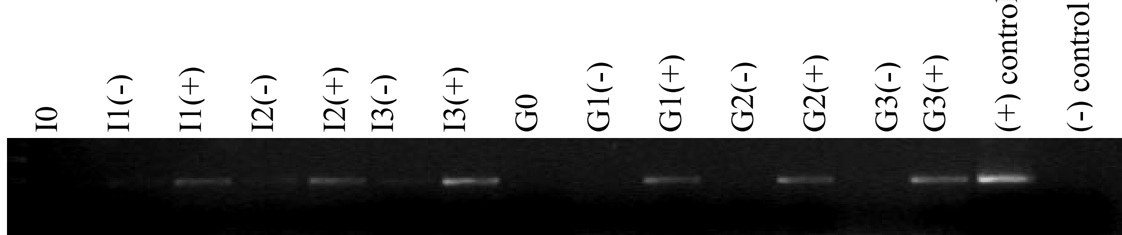


Fig. 3.12. The *PR*1-b 500bp specific band is detected only in the infected plants and not in the uninfected.
Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant

As a positive control for the RT-PCR experiment genomic DNA from I88 or G87 was used (this control applies for all RT-PCR). In the QS I88 parent, the transcript level of *PR*1-b gene increased with the time post-infection, while in the QR G87 parent the *PR*1-b transcript level was lower and remained at similar level during the whole time course of infection.

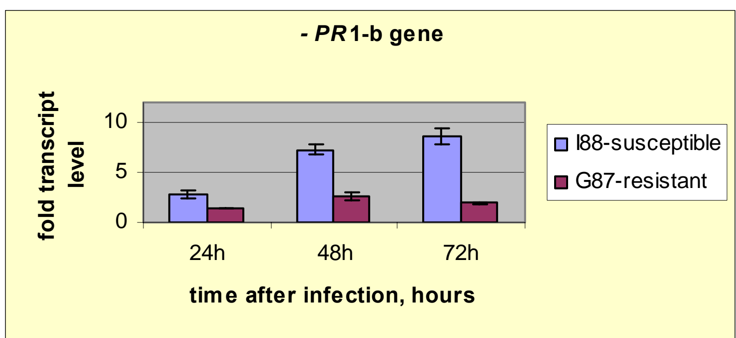


Fig.3.13. Induction of *PR*-1b transcript in I88 and G87 infected plants.

3.3.4 Differences of infection symptoms on the I88 and G87 parents in independent experiments.

The results for the differentially expressed genes were obtained based on one biological replica with two technical repetitions.
The first infection test was performed in the second week of August 2004. Infected plants were observed after 14 days. The RT-PCR for the differentially expressed genes was repeated with material of the second infection experiment performed in May 2005. Plants from the second experiment showed extremely delayed infection symptoms. Primers for the ribosomal *P. infestans* DNA amplified specifically a 258bp PCR fragment only in infected but not in control plants *P. infestans*, showing that there was a successful interaction between the oomycete and the infected plants picture looked identical to (Fig.3.10). The expression of the *PR*1-b gene was delayed. Both I88 and G 87 did not express the gene at the first time point after infection, which is an indication that the successful penetration of the pathogen was delayed. In addition, the *PR*1-b gene was not as strongly expressed as from the successful infection in August 2004, identical picture to (Fig.3.12), but missing PCR fragment at 24h post-inoculation. In a third experiment I88 parent reacted with synthesizing the pigment anthocyanin in the leaves and the plants were not used further for infections with *P. infestans*. The plants were already stressed by too high light intensity in the growth chamber, where the plants were maintained. In order to confirm the results from the differentially expressed genes an additional independent biological replica will be performed.

3.3.5 Selecting the candidate genes

Two BAC physical contigs have been assembled (Ballvora et al. in preparation) one for the “resistant” *R*1 chromosome introgressed from the wild species *S. demissum* Fig.3.14 (upper chromosome) and the second for the “susceptible” *r*1 chromosome from *S. tuberosum* Fig.3.14 (lower chromosome). The major *R*1 gene conferring resistance to *P. infestans* has been localized in the first physical contig-*R*1 and has been cloned (Ballvora et al. 2003).

The sequence from the physical contig was subjected to the Gene prediction software-Gene Mark setting as a blast plant organism *Arabidopsis thaliana* or *Oryza sativa*. The outcome from the prediction software resulted in at least 49 ORFs on both chromosomes. From the 49 ORFs 10 retroelements, 6 truncated and allelic variants of the *R*1 family, two RNA-polymerase genes, one transposase gene and one reverse transcriptase gene were excluded. As candidate genes five ORFs without homology to known genes were considered, as they have an unknown function. The *R*1 gene family was excluded because their analysis is a subject of study in an ongoing experiment in the laboratory. The other above mentioned candidate genes were excluded in this study as not being strong candidate genes playing major role in resistance. Excluding all above mentioned genes, 25 of 49 predicted ORFs were considered as candidate genes in total in the physical contig on chromosome V (Fig.3.14, Table 3.18).

The sequences of the 25 candidate genes were blasted against the TIGR database and searched for the presence of best fit EST sequences. For most of the genes there was a potato EST similar or identical to a certain part of the whole coding region of the gene or to the complete gene or very short sequences with even not thus high nucleotide homology to the gene. Only one gene putative, α-amylase gene had a corresponding EST in the Solanaceae Genome Network (SGN database). For all other 24 ORFs a corresponding EST was found in the TIGR database (Table 3.18). The position of the ORFs on the “resistant” (upper) and “susceptible” (lower) chromosome is shown in Fig. 3.14.

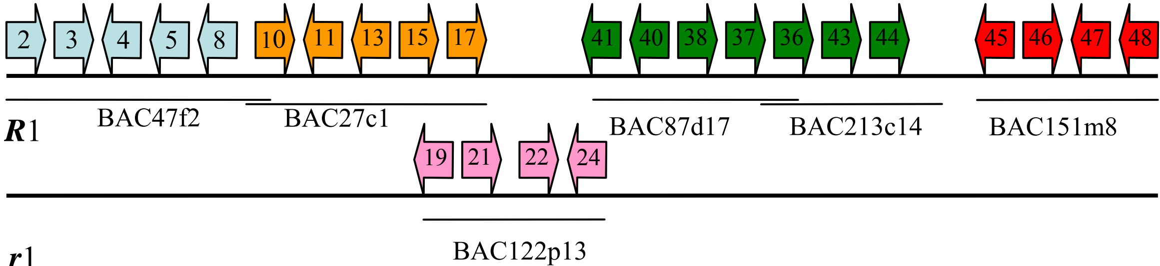


Fig. 3.14. Schematic overview of candidate gene order in the sequenced BACs on potato chromosome V: the upper chromosome carries the *R*1 gene introgressed from *S. demissum*; the lower chromosome carries “susceptible” *r* 1 genes.

BAC47f2 is located towards the telomere (distal), BAC151m8 towards the centromere (proximal) of chromosome V.

3.3.6 EST search in the TIGR, NCBI and TAIR databases, ORFs start and end position

Primers for the 25 ORFs were designed based on comparison of the gene sequences from the annotated BACs with GenBank accession numbers. Primers were designed mainly for the 3`end of the putative coding cDNA sequence and staying away from the putative exon -intron boundaries. Of 25 ORFs, primers for ORFs 5, 11 and 43 could not be designed due to technical reasons The PCR products obtained from the I88 and G87 parents of the 22 putative candidate genes (PCG) were sequenced and resulted in readable genomic sequence for 20 of the PCG (Table 3.19). The correct exon-intron boundaries for each gene were derived from the comparison of the existing EST sequences in the Solanaceae TIGR database with BAC sequences in the NCBI database. The ORF start and the end positions of the 25 candidate genes according to the information in the NCBI database are summarized in Table 3.18. The coding sequence of the candidate genes was blasted against the TIGR database and compared with corresponding ESTs in database. The EST number and percent identity of the candidate genes to the corresponding potato EST are summarized in Table 3.18. For ORFs 5, 8, 10, 11, 15, 40, 43, 44, the blast resulted in a very low percentage of identity (c.a. 70%) as the corresponding EST spanned very short fragment of the gene. The E value a measure for the degree of sequence similarity was recorded, for the 25 candidate genes with the corresponding most closely related gene from *A. thaliana*. For all candidate genes the similarities were much higher at the amino acid than at the nucleotide level, because of presence of the corresponding domains in the gene (Table 3.20).

Table3.18. **"Start" and "End" positions of the putative candidate genes based on comparisons with the NCBI database.**

ORF №	Annotation assignment of the candidate genes	Accession number	BAC assignment	ORF start position	ORF end position
2	ZF-HD homeobox protein	AY730338	BA47f2	68138	69744
3	EST(*A.thaliana*) unknown function	AC146506	PGEC989P08	92985	96911
4	NAM (no apical meristem) gene	AY730335	BA151m8	37304	44271
5	No homology	AY730338	BA47f2	31971	3501
8	No homology	AY730338	BA47f2	13971	15040
10	F-box protein family	AC149288	PGEC446J10	39557	40669
11	No homology	AC149288	PGEC446J10	37292	39324
13	No homology	AC149288	PGEC446J10	23326	24944
15	F-box protein family	AY730337	BA27c1	38118	40400
17	No homology	AC149287	PGEC568D21	31764	33915
19	CAAX amino-terminal protease family	AY730334	BA122p13	46122	51029
21	Methyltransferase	AC151802	PGEC46	172	6010
22	Phytochrome kinase substrate	AY730334	BA122p13	30670	32079
24	ATPase protein family, AAA-type	AY730334	BA122p13	16691	18274
36	Acid phosphatase	AC151815	PGEC472P22	52659	55842
37	Origin recognition complex chain 6	AY730340	BA87d17	39262	41979
38	EST (*A.thaliana*), unknown function	AC149290	PGEC093P17	4363	11430
40	EST (*A.thaliana*), unknown function	AY730340	BA87d17	15346	19378
41	Transcription factor, TCP family	AY730340	BA87d17	2915	3925
43	HVA22 family protein	AC151815	PGEC472P22	87555	89473
44	Zinc-finger protein	AC151815-	PGEC472P22	91685	99406
45	α-amylase	AY730335	BA151m8	1	2365
46	EST (*A.thaliana*) Unknown function	AY730335	BA151m8	3892	11857
47	Protein kinase	AY730335	BA151m8	13173	28378
48	EST (*A.thaliana*) Unknown function	AY730335	BA151m8	30564	37436

Table3.19. **Readable genomic sequence obtained after sequencing amplicons of the I88 and G87 parents with the forward or the reverse primers.**

ORF №	I88, sequencing primer F/R	G87, sequencing primer F/R
2	+/F	+/F
3	+/F	+/F
4	-/F	+/F
10	+/F	+/F
13	+/F	+/F
15	+/R	+/R
19	+F	+F
21	-/F	+/F
22	+/F	+/F
24	+/F	+/F
36	+/F	+/R
37	+/F	+/F
38	+F	+F
40	+/F	+/F
41	+F	+F
44	+/F	+/F
45	+F	+F
46	+/F	+/R
47	+/F	+/F
48	+F	+F

Table 3.20. **Annotation of *S. tuberosum* candidate genes based on sequence comparisons.**

ORF №	Annotation	The nearest *A. thaliana* gene	E value, genomic [1]	E value, protein [2]	Potato EST Accession №	Identity %[3]
2	ZF_HD homeobox protein	At5g15210.1	3e-11	1e-52	TC119476	96
3	EST (*A. thaliana*), unknown function	At5g39785.2	-	3e-59	CK719827 BF053872	94 93
4	NAM (no apical meristem) like	At1g26870.1	6e-16	4e-70	BM110077	100
5	No homology	-	-	-	BQ509476	65
8	No homology	-	-	-	TC113689	54
10	F-box protein family	At3g23880.1	-	8e-12	TC116285	75
11	No homology	-	-	-	TC126661 TC120883	58 54
13	No homology	-	-	-	TC131709	71

(continuation)

Table 3.20 (continuation). **Annotation of *S. tuberosum* candidate genes based on sequence comparisons.**

ORF	Annotation	The closest *A. thaliana* gene	E value, genomic[1]	E value, protein[2]	Potato EST	Identity %[3]
15	F-box protein family	At3g22650.1	-	4e-09	TC116285 TC131047 TC123219	75 67 64
17	No homology	-	-	-	TC124058	65
19	CAAX amino-terminal protease family	At1g14270.1	6e-07	1e-100	TC115259 BE920788	80 95
21	Methyltransferase	At1g26850.1	2e-38	0.0	TC127830	98
22	Phytochrome kinase substrate	At2g02950.1	-	1e-39	TC122676 EST624974	96 96
24	ATPase protein family, AAA-type	At5g40010.1	2e-14	1e-145	TC113578	98
36	Acid phosphatase	At1g69640.1	3e-36	1e-115	TC114785 TC129899	97 96
37	Origin recognition complex, chain 6	At1g26840.1	5e-07	2e-99	U238994	97
38	EST(*A. thaliana*), unknown function	At1g14300.1	7e-15	3e-35	TC119976	98
40	EST(*A. thaliana*), unknown function	At2g02910.1	1e-28	1e-107	TC124262	72
41	Transcription factor, TCP protein	At1g58100.1	4e-23	5e-26	TC115706	92
43	HVA22 family protein	At1g74520.1	-	1e-103	TC112044	71
44	Zinc-finger protein, regulator of chromosome condensation	At1g69710.1	1e-10	0.0	EST514758	68
45	α-amylase glucanhydrolase	At1g69830.1	9e-09	4e-67	SGN-U224541	95
46	EST(*A. thaliana*), unknown function	At1g07040.1	1e-10	1e-119	TC120485	98
47	Serine threonine kinase-gene	At5g15080.1	2e-26	1e-110	TC122442	98
48	EST(*A. thaliana*), unknown function	At3g17900.1	2e-12	1e-101	BF187935	98

[1]E value for the nucleotide sequence comparison between the ORF and the nearest *A. thaliana* homolog.
[2]E value for the protein sequence comparison between the ORF and the nearest *A. thaliana* homolog.
[3]Identity % with potato EST;
- no homology at nucleotide or amino acid sequence

3.3.7 Putative function assignment of candidate genes

The functional assignment of the *S. tuberosum* genes was based on the presence of structural domains in the deduced protein sequences of the candidate genes. The deduced *S. tuberosum* protein sequence from the prediction software was compared to the *A. thaliana* protein sequence database and resulted in the finding of known structural domains in 14 of 20 genes (Table 3.21). For 11 of the PCG no domain was available at the TAIR database. The exact domain structure of the 14 genes is shown as highlighted in red amino acids in Appendix. The domain structure for the fourteen genes was obtained by comparing the deduced *S. tuberosum* protein sequence with the protein sequence of the corresponding *A. thaliana* homolog in TAIR database. For ORFs 45 and 47 the domain could not be found in the database and a literature search was performed instead. The nucleotide sequence of all candidate genes was translated independently from the GeneMark prediction software in order to test for premature stop codons in the amino sequence sequence. All 25 candidate genes translated into correct protein sequences as it was shown by the prediction software.

Table 3.21. **Domain ID number, structure and domain function of the predicted ORFs in comparison to domains present in the corresponding *Arabidopsis thaliana* protein sequences. The Domain function description was found in TAIR database.**

ORF №	BAC	Pfam database ID	Putative assignment	Domain function
2	BA47f2	PF04770	ZF-HD protein dimerisation region	The region is involved in the formation of homo and heterodimers, and forming a zinc finger
4	BA47f2	PF02365	No apical meristem (NAM) protein	Contain DNA-binding domain and pocess ability for homodimerisation
19	BA122p13	PF02517	CAAX amino terminal protease family	CAAX prenyl protease activity
21	BA122p13	PF03141	DUF248	Methyltransferase function
22	BA122p13	PF03128	C x C x C repeat	CXCXC where X can be any amino acid
24	BA122p13	PF00004	AAA ATPase, central region	AAA stands for “A”TPases “A”ssociated with diverse cellular “A”ctivities

(continuation)

Table 3.21 (continuation). **Domain ID number, structure and domain function of the predicted ORFs in comparison to domains present in the corresponding *Arabidopsis thaliana* protein sequences. The Domain function description was at TIGR database.**

ORF №	BAC	Pfam database ID	Putative assignment	Domain function
36	BA87d17	PF01598	Sterol desaturase	Members of these group enzymes are involved in biosynthesis of cholesterol and plant cuticular wax.
37	BA87d17	PF05460	Origin recognition complex subunit 6 (ORC6)	ORC is a six protein complex and functions in DNA replication initiation
40	BA87d17	DUF616	EST	A family of uncharacterized proteins
41	BA87d17	PF03634	TCP family transcription factor	TCP domain is involved in DNA-binding and dimerization
43	BA213c14	PF03134	HVA22, abscisic acid induced	Likely a regulatory protein
44	BA213c14	PF00415	Regulator of chromosome condensation (RCC1)	RCC1 a eukaryotic protein which binds to Ran protein and play a role in regulation of gene expression
45	BA151m8			The domain structure was found in (Yu et al. 2005)
47	BA151m8			The domain structure was found in (Hanks 2005)

3.3.8 *R*1-specific primers C76-2

Standard PCR was performed with I88 and G87 to confirm the absence of the *R*1 gene as it has been assumed based on phenotypic data but has not been checked with *R*1 specific primers. The PCR results confirmed the absence of the *R*1 gene in I88 and G87. The *R*1 specific 1400 bp amplicon was present only in the BA 87d17 positive control where the gene is localized Fig. 3.15.

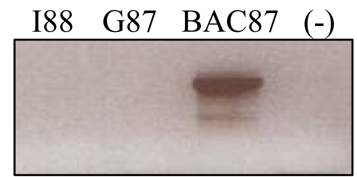


Fig. 3.15. PCR results with *R*1- specific marker C76-2
First lane: QS I88 parent; second lane: QR G87 parent; third lane: BA87d17 (positive control), forth lane: negative control.

3.3.9 Equalization of the cDNA using β -Tubulin primers

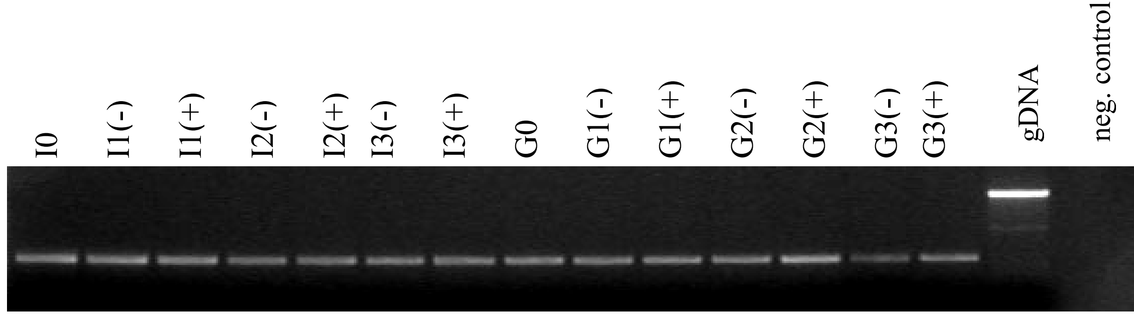


Fig. 3.16. β− Tubulin primers amplify a 525bp PCR product on the DNA level

Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant
gDNA- genomic DNA positive control; neg. control= no template

β-Tubulin primers were used to equalize the cDNA samples for the RT-PCR experiments. The primers were designed using the *A. thaliana* β-Tubulin sequence (At5G44340.1) in the TAIR database and using it to identify a corresponding potato EST (TC126169) in the TIGR database. The primers were designed for the potato EST. They amplified a 525bp fragment with potato cDNA as template and an about 1200 bp fragment with genomic DNA as a template (Fig.3.16).

3.3.10 Classification of the candidate genes into groups

Of the 25 candidate genes analyzed, four were found to be differentially expressed. ORFs 45, 46, 47 and 48 are located in the most proximal BA151m8 in the physical contig on chromosome V. For eight genes, expression was not detected in leaves in leaves (ORFs 2, 4, 8, 10, 15, 17, 40 and 44). Primer design for ORFs 5, 11, 17 and 43 was not successful although several attempts were made locating the primers in different regions of the gene. Among the not differentially expressed genes strongly expressed ORF 21, 24, 36, 37 were distinguished from weakly expressed genes ORFs 3, 13, 19, 22, 38, 41. The results of the expression study on all 25 candidate genes are summarized in Table 3.22. For the not expressed genes the primers were located definitely in exon regions according to the

available EST in the database or based on the prediction exon-intron boundaries according to GeneMark Software.

Table 3.22: **Summary from the expression study conducted on the 25 putative candidate genes.**

ORF №	Putative function assignment based on sequence similarity	Expressed in control G87	Expressed in infected G87	Expressed in control I88	Expressed in infected I88	Differentially expressed Yes/no
2-*R*1	ZF-HD homeobox protein	-	-	-	-	no
3-*R*1	EST(*A. thaliana*), unknown function	+	+	+	+	no
4-*R*1	NAM (no apical meristem)	-	-	-	-	no
5-*R*1	No homology	0	0	0	0	no
8-*R*1	No homology	-	-	-	-	no
10-*R*1	F-box protein family	-	-	-	-	no
11-*R*1	No homology	0	0	0	0	no
13-*R*1	No homology	+	+	+	+	no
15-*R*1	F-box protein family	-	-	-	-	no
17-*R*1/r1	No homology	-	-	-	-	no
19-*R*1/r1	CAAX amino-terminal protease family	+	+	+	+	no
21-r1	Methyltransferase	++	++	++	++	no
22-r1	Phytochrome kinase substrate	+	+	+	+	no
24-r1	ATPase protein family, AAA-type	++	++	++	++	no
36-*R*1	Acid phosphatase	++	++	++	++	no
37-*R*1	Origin recognition complex, chain 6	++	++	++	++	no
38-*R*1	EST (*A. thaliana*), unknown function	+	+	+	+	no

Table 3.22 (continuation): **Summary from the expression study conducted on the 25 putative candidate genes.**

ORF №	Putative function assignment based on sequence similarity	Expressed in control G87	Expressed in infected G87	Expressed in control I88	Expressed in infected I88	Differentially expressed Yes/no
40-*R*1	EST (*A. thaliana*), unknown function	-	-	-	-	no
41-*R*1	Transcription factor, TCP family	+	+	+	+	no
43-*R*1	HVA22 family protein	0	0	0	0	no
44-*R*1	Zinc-finger protein	-	-	-	-	no
45-*R*1	α-amylase	+	+	+	+	yes
46-*R*1	EST (*A. thaliana*), unknown function	+	+	+	+	yes
47-*R*1	Protein kinase	+	+	+	+	yes
48-*R*1	EST (*A. thaliana*), unknown function	+	+	+	+	yes

++ strongly expressed genes**;** +weakly expressed genes; 0- primers not working due to technical reasons; -not expressed genes; *R*1-the genes are located on the “resistant” chromosome or *r*1- on the “susceptible”

3.3.11 Differentially expressed genes

The graphics of the four differentially expressed genes (Fig.3.18, 3.20, 3.22 and 3.24) show the subtraction of the band intensity value of the uninfected (control) plants from the band intensity of infected plants for each time point after infection. For quantifying the PCR product, the tubulin band intensity on the agarose gel was used as an internal standard for each infection time point. For each time point there were two tubulin lanes, one for the non-infected and one for the infected plant. The tubulin band intensity with the lower value for one time point (non-infected and infected plant) was chosen as a standard lane. The relative intensity of the tubulin band for the other lane for the same time point was calculated by the ratio. This ratio was used as a normalization factor by which the band intensity of the ORF for every time point was calculated. The normalization factor for each time after infection *e.g.* 24h, 48h and 72h and for I88 and G87 plants was calculated separately.

The columns in the graphic represent the residual effect of the expression of the gene only due to the *P. infestans* treatment on the potato plant. The sequence of the primers and the cycle parameters of the RT-PCR for all candidate genes are listed in Table: B, (Appendix). RT-PCR conditions for all genes are described in Chapter: Material and Methods.

3.3.11.1 α-amylase gene (ORF45)

Primers for the putative α-amylase gene were located in the first exon, while the reverse primer was in the second exon. The PCR resulted in clearly distinguished size products of 341bp with cDNA template and 970bp with genomic DNA. The α-amylase gene (ORF45) was preferentially expressed in the QS I88 parent. At the first time point after infection, the transcript level of the gene was up regulated. At the second time point, the gene was not up regulated compared to the control plant and at the third time point, the expression of the gene was again up-regulated. In the QR G87 parent the transcript level was almost undetectable at 55 cycles of PCR (Fig.3.17 and Fig. 3.18).

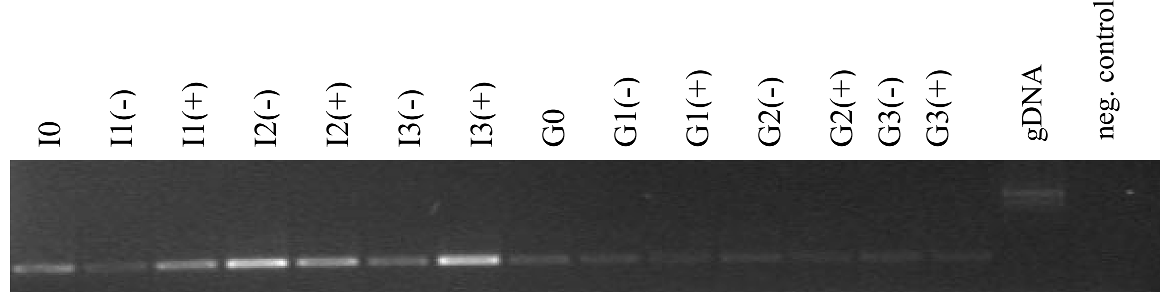


Fig. 3.17. RT-PCR of the α-amylase gene (ORF45) in infected versus non-infected I88 and G87 plants.
Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P . infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant gDNA- genomic DNA positive control; neg. control= no template

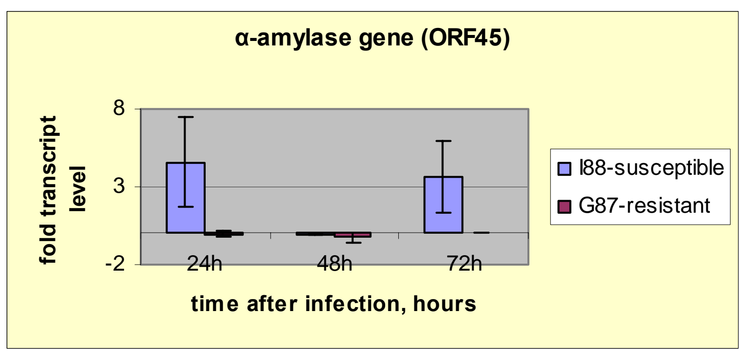


Fig. 3.18. Expression profile of ORF45 (α-amylase)

3.3.11.2 EST-unknown function (ORF46)

The primers for the gene were located in the first and the third exon, resulting in a 386 bp PCR band with cDNA as template and a 1055 bp with genomic DNA template. ORF46 was stronger induced in the QS I88 parent than in the QR G87 parent. I88 transcript level was up-regulated one day after infection, weaker up-regulated at the second time point, and again very strongly up-regulated at the third point after infection. In the QR parent the gene was clearly up-regulated at the second and the third point after infection Fig 3.19 and Fig.3.20.

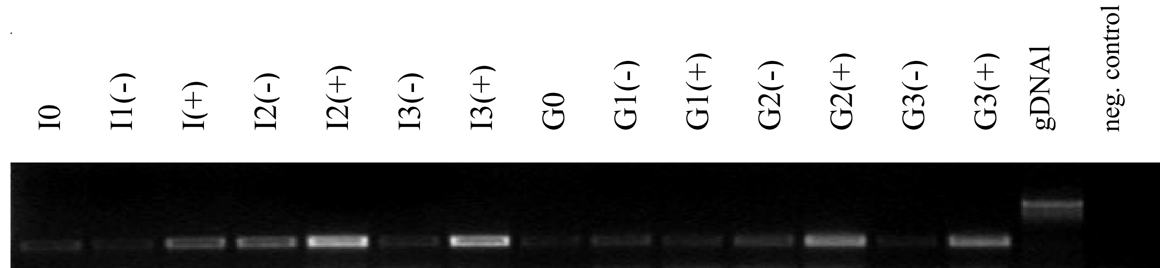


Fig. 3.19. Results from the RT-PCR with EST- unknown function (ORF46)
Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant gDNA- genomic DNA positive control; neg. control= no template

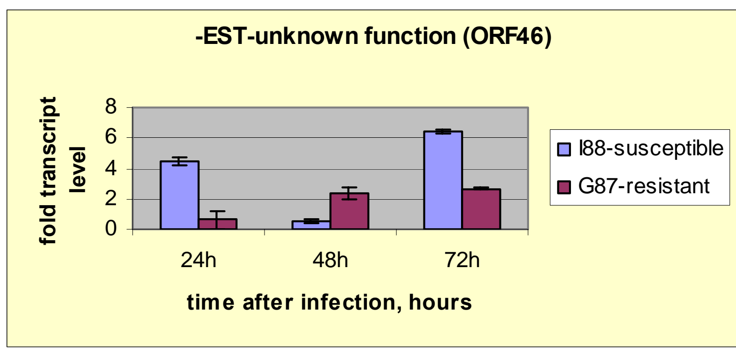


Fig. 3.20. Expression profile of ORF46 (EST-unknown function)

3.3.11.3 Protein kinase (ORF47)

Primers for ORF 47 amplified a 442bp product with cDNA template and a 548 bp product with genomic template. The transcript level of the gene in the QS I88 parent was weakly up-regulated one day after infection, up-regulated two days after infection and near control level three days after infection. In QR G87 the gene was hardly up-regulated at the first time point infection, weakly up-regulated at the second and third time point infection Fig.3.21 and Fig.3.22.

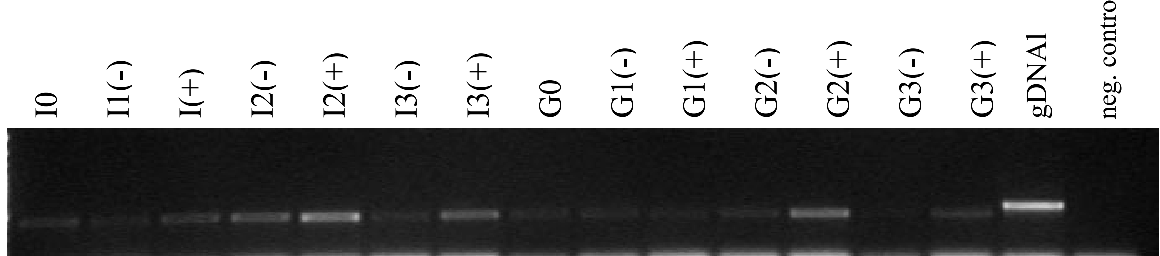


Fig. 3.21. RT-PCR picture with protein kinase gene (ORF47)

Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant gDNA- genomic DNA positive control; neg. control= no template

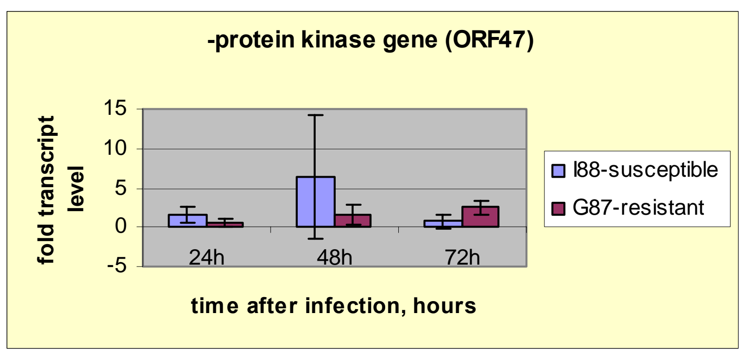


Fig. 3.22. Expression profile of Protein kinase gene (ORF47)

3.3.11.4 EST unknown function (ORF 48)

Primers were located in the sixth and seventh exon of the gene resulting in a PCR band of 491 bp on cDNA template and a band of 617 bp on genomic template. In the QS I88 parent the transcript level of the gene was up-regulated at the first time point after infection, weakly up-regulated at the second time point infection and strongly up-regulated at the last third time point infection. In QR G87 the expression of the gene was weakly up-regulated only at the second time point after (Fig.3.23 and Fig.3.24.)

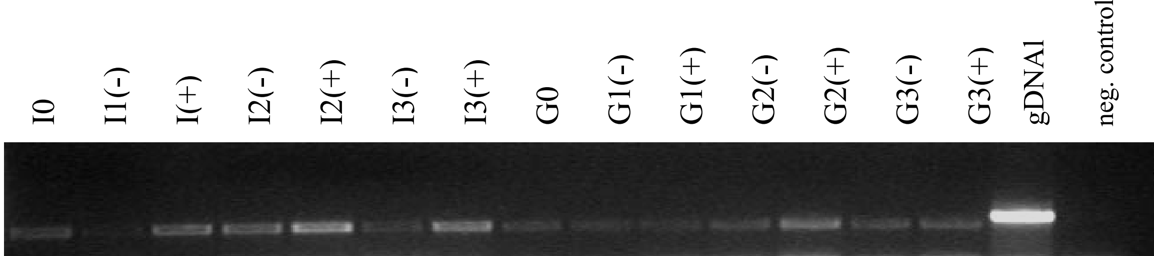


Fig. 3. 23. Results from the RT-PCR with EST-no homology gene (ORF 48)

Abbreviations:
I: I88 parent, G: G87 parent, I0: 0 day non- infected plant; G0: 0 day non-infected plant; I1(-): I 88 first day non-infected control; I1 (+) first day after *P. infestans* infection; 1, 2 and 3 – days after infection; (+): infected with *P. infestans* and (-): non-infected plant gDNA- genomic DNA positive control; neg. control= no template

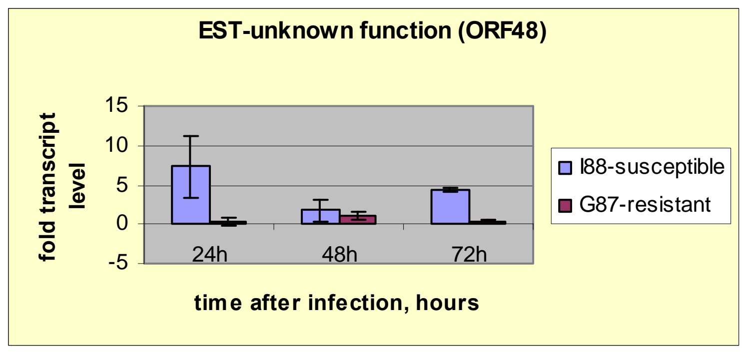


Fig. 3.24. Expression profile of ORF48 (EST-unknown function)

3.3.12 Southern gel blot analysis for two expressed genes on chromosome V

Southern analysis gel blot was performed with two of the 14 expressed genes from the expression study experiment on chromosome V. These two genes were chosen because from the first RT-PCR experiments they seemed differentially expressed. Later, it was found out especially for ORF№ 22 that the forward primer was located in a genomic region with a mismatch base pair, resulting in the difference in detection of the RT-PCR product in I88 parent. The Southern gel blot analysis from the two genes shows that the putative Phytochrome kinase substrate (ORF№ 22) and the ATPase AAAtype (ORF№ 24) genes have low copy numbers in the potato genome. For the phytochrome kinase substrate gene, the copy number is between one or maximum two genes in the potato genome (Fig.3.25). For the ATPase AAA-type protein family the gene copy number may be between two and four genes (Fig.3.26). The Southern gel blots were hybridized with a probe amplified from BAC DNA with the same PCR primers as used for the RT-PCR experiments. As a PCR template BAC122p13 for both genes was used, because both ORF№ 22 and ORF№ 24 were localized in the same BAC from the contig on chromosome V (Fig.3.14). The Southern gel blot analysis shows that both genes are highly polymorphic between I88 and G87 genotypes.

Eco RV Eco RI

I88 G87 P6/210 BA122p13 I88 G87 P6/210 BA122p13

Fig.3.25. Southern gel blot analysis of ORF22 (phytochrome kinase substrate).

Eco RV Eco RI

G87 I88 P6/210 BA122p13 G87 I88 P6/210 BA122p13

Fig.3.26. Southern gel blot analysis of ORF24 (ATPase protein family AAA type).

Chapter 4: Discussion

4.1 QTL analysis in two tetraploid breeding populations

4.1.1 Candidate gene approach

The Candidate gene-approach was used to tag QTLs for resistance to *P. infestans.* A candidate gene is referred to as any gene involved in a metabolic pathway of relevant for a trait of interest and located in close vicinity to a QTL for the same trait (Pflieger et al. 2001). The `BA` markers used in the study for analyzing SNPs, originated from screening a potato BAC library with probes designed for the major class of *R*-genes containing NBS-LRR domains (Rickert et al. 2003, Leister et al. 1996). Primers have been developed for the insertion of BAC clones containing NBS-LRR type genes (Rickert et al. 2003). The BAC ends were mapped on the twelve potato chromosomes. Markers from BAC ends linked to known QTL for resistance to *P. infestans* were selected for SNP analysis. RFLP markers previously shown to be linked to QTLs for resistance to *P. infestans* were also considered based on the assumption that the markers tag previously localized resistant factors but in different genetic backgrounds.

4.1.2 QTL analysis in the SaKa-Ragis population

The SaKa-Ragis population was genotyped using 31 polymorphic SNP, SSCP, CAPS and SCAR markers located on all twelve potato chromosomes (Fig. 1.6). The 31markers, tagged known QTLs for resistance to *P. infestans*, and the knowledge gained from these previous studies was used in two new tetraploid breeding populations. From the 31 polymorphic loci, two were significantly linked to QTL for resistance to *P. infestans*: locus CP105 and GP266 both located on chromosome X (Table 3.5). In total 20 SNPs were analyzed in the amplicon of locus CP105 from which five were significantly linked to QTL for resistance to *P. infestans* SNPs (snp54, snp58, snp143, snp191 and snp329) (Table 3.5). The second locus GP266 was located 1cM proximal to the locus CP105

(Meyer et al. 2005). The genetic distance and the chromosomal location of the analyzed 31 loci can be seen at (www.gabi.rzpd.de) webpage. In total 13 SNPs were analyzed in the amplicon of locus GP266 from which one was significant (snp130), (Table3.5). The two significant loci are localized in close genetic distance to the previously mapped *R* gene (R_{ber}) from the wild species *S. berthaultii* (Ewing et al. 2000) on the distal long arm of the potato chromosome X. For mapping the gene, a RFLP marker TG63 has been used. The results showed that the TG63 marker co-segregated with the allele originating from the resistant parent *S. berthaultii.* 90% of the progeny having a resistant phenotype had the TG63 allele of *S. berthaultii*, while only one susceptible genotype had the TG63 allele linked to resistance. TG63 locus maps c.a. 1cM proximal to the CP105 locus (Gebhardt et al. 1991). The amplicons of the resistant SR1 and the susceptible SR2 parent for the TG 63 marker were sequenced but did not result in SNPs in the parents of the SaKa-Ragis population. Moreover, the dominant gene *Ph*-2 conferring resistance to *P. infestans* from the wild *L. pimpinellifolium* has been mapped on chromosome X in tomato, in a region co-linear with the potato region on chromosome X studied here (Moreau et al. 1998, Tanksley et al. 1992). Giving the position of the R_{ber} in potato and *Ph*-2 in tomato, R_{ber} may be orthologous gene to *Ph*-2 gene in tomato (Ewing et al. 2000). The linkage of the CP105 locus to the QTL for resistance to *P. infestans* could be related to the presence of the previously mapped R_{ber} and *Ph*-2 resistant genes. It has been suggested that co-localization of a major resistance gene with resistance QTL, indicates that major resistance genes can be alleles of a gene or cluster of genes that also control quantitative resistance (Gebhardt and Valkonen 2001, Bormann et al. 2004, Ballvora et al. 2002, Oberhagemann et al. 1999, Leonards-Schippers et al. 1994).

4.1.3 QTL analysis in the BNA population

The BNA population was genotyped using 28 polymorphic SNPs, SSCP, CAPS and SCAR markers. Four loci, GP23 (snp183) on chromosome II, S1b3 (snp195 and snp257) on chromosome III, BA87d17t3 (snp109) and BA76o11t3 (snp336) located on chromosome V (Table 3.5.) were significantly linked to QTLs for resistance to *P. infestans*. The marker GP23 has been identified to tag the QRL (**q**uantitative **r**esistant

locus) *Pin*2A for resistance to *P.infestans* (Bormann et a. 2004, Oberhagemann et al. 1999) and QRL *Eca*2A for resistance to *Erwinia carotovora* ssp. *artroseptica* (Zimnoch-Guzowska et al 2000). Recently, the locus St*PAD*4-1 has been mapped 4cM distal from the GP23 marker on the short arm of chromosome II (Pajerowska et al. 2005). Studies in *A. thaliana* have shown that *PAD*4 is a regulator of defense responses and act upstream from salicylic acid (SA) (Zhou et al. 1998). The two SNPs in marker S1b3 were slightly significant with a P value 0.017 (Table3.5). The marker S1b3 tagged QRL *Pin*3C for resistance to *P. infestans* that was previously detected (Leonards-Schippers et al. 1994, Collins et al. 1999, Oberhagemann et al. 1999). S1b3 is an EST with putative function as respiratory burst oxidase. The oxidative burst has been suggested to be a primary event for triggering the cascade of defense responses against pathogens (Yoshioka et al. 2001). Studies in potato showed that treatment of potato tubers with hyphal wall components from *Phytophthora infestans* induced the respiratory burst oxidases which produce active oxygen species (Yoshioka et al. 2001). The QRL *Pin*5A for resistance to *P. infestans* on chromosome V has been detected in studies on diploid (Bradshaw et al. 2004, Ewing et al. 2000, Visker et al. 2003, 2004, Collins et al. 1999, Oberhagemann et al. 1999, Leonards-Schippers et al. 1994, Ghislain et.al. 2001), one tetraploid (Bormann et al. 2004) F1 potato populations as well in an association study (Gebhardt et al. 2004). This genomic region is a resistance “hot spot” which contains genes for resistance to nematodes, viruses and the oomycete *P. infestans* (Gebhardt and Valkonen 2001). The mapping of cloned plant genes active in pathogen defense close to QRL is supportive to the hypothesis that allelic variants or paralogues of major resistance genes contribute to quantitative disease resistance (Gebhardt and Valkonen 2001, Trognitz et al. 2002, Ballvora et al. 2002, Pajerowska et al. 2005).

4.1.4 “Alien” segregation

CP105 locus on chromosome X in SaKa-Ragis population

The CP105 locus on chromosome X was analyzed for SNPs in the 46 genotypes of the (“cases” and “controls”) study, which resulted in five significant SNPs (Table 3.5). In order to evaluate the significance of the locus in the whole population, the amplicons of 192 genotypes were sequenced and analyzed for SNPs. 10 SNPs were evaluated in the

whole population, nine of which were among the 20 analyzed SNPs in the “cases” and the “controls” study (3.1.2.5). One SNP (snp260) was not polymorphic in the SR1 (resistant) and SR2 (susceptible) parent but segregated in the whole population (Table 3.10). This SNP has been detected in a previous study in which the SR1 and SR2 parents have been evaluated for the presence of SNPs at CP105 locus and snp 260 was present in sequence of the parents (Rickert et al. 2003). None of the 46 genotypes in the “cases” and “controls” segregated for snp 260. The only explanation of this result could be that out-crossing has occurred by `alien` pollinators, due to not well isolated conditions where the plant crosses have been performed (personnel communication Dr. Jens Lübeck- SaKa-Ragis Pflanzenzucht GbR).

In tetraploid potato five allelic states are possible at a SNP locus: homozygous (AAAA) or TTTT and heterozygous AAAT (triplex/simplex), AATT (duplex/duplex), or ATTT (simplex/triplex). For example when both parents of a population are heterozygous duplex/duplex, *e.g.* AATT, the following five genotypic classes in the F1 progeny of the corresponding two parents are expected assuming tetrasomic inheritance: AAAA, ATTT, AATT, AAAT and TTTT. From the SNP allele dosage information of the parents, the expected genotypic classes in the F1 progeny can be deduced. Nine SNPs in locus CP105 did not segregate in the population according to the expected allele dosage information from the parents (Table 3.10). Of 192 genotypes, 20 genotypes had the expected allele dosage at all nine SNPs.

4.1.5 Distorted segregation in GDE and K31 populations

Distorted segregation ratio was observed in the SNPs analyzed at the locus CP105 in the two diploid populations GDE and K31 (Tables 3.7, 3.8). The populations have been analyzed using PCR based and RFLP markers and distorted segregation at other loci was observed (Oberhagemann et al. 1999). The most trivial explanation for this result is that the sequencing of the amplicons is not sensitive enough to differentiate and quantify the presence of all alleles segregating from the parents in the corresponding population.

4.2 QTL gradient experiment

The QTL for resistance to *Phytophthora infestans* on chromosome V is being studied extensively since it has been detected as carrying the major *R*1 resistant gene and being one of the major QTL for resistance to *P. infestans* in the potato genome (Leonards-Schippers et al. 1992, 1994, Oberhagemann et al. 1999). This is the first genomic region in potato for which physical contigs have been assembled. A physical contig has been assembled for three haplotypes of the hexaploid *Solanum demissum* with the largest contig covering 1 Mb (Kuang et al. 2005). Another physical contig of about 400 kb was assembled for *Solanum tuberosum* with introgression from *Solanum demissum* (Ballvora et al. in preparation). The objective of the experiment was to narrow down genetically the genomic region in which the gene(s) for resistance to *P. infestans* are localized. For this purpose, 32 genotypes highly resistant to *P. infestans* and late maturing ("cases") and 33 highly susceptible to *P. infestans* and early maturing genotypes ("controls") were selected. The selection was performed in order to differentiate the alleles responsible for resistance or susceptibility to *P. infestans* and lateness or earliness between the selected two groups. For the "cases" and "controls" approach initially four loci (ORFs) spanning the whole physical contig on chromosome V were chosen (Fig.3.5). The main reason to choose these four particular loci was that the ORFs were expressed genes in leaves according to the RT-PCR experiment, indicating that they might be candidate genes for playing a role in the QTL for resistance to *P. infestans* on chromosome V. Second, the primers for these four genes resulted in amplicons with well readable sequences appropriate for SNP analysis. SNPs in all four loci ORF 3, ORF 24, ORF 36 and ORF 47 were highly significant (Table 3.11). In order to test whether the QTL effect declines outside the physical contig two additional loci were analyzed, GP21 and St*Pto* (Fig.3.5). GP21 is a RFLP marker (Gebhardt et al. 1989); St*Pto* is the ortholog of the *Pto* gene from tomato (Martin et al. 1993), conferring resistance to the bacterial pathogen *Pseudomonas syringae*. The positional coincidence of the two orthologous genes in potato and tomato on chromosome V is in concordance to the high co-linearity found between the tomato and the potato genomes (Grube et al. 2000). Primers for St*Pto* have been previously

designed by Leister et al. (1996), but they bind to a region where the sequences of the two protein kinases *Pto* and *Fen* are identical, with exception of only one mismatch in the forward primer. This primer combination was not used in this study, because we aimed at differentiating the *Pto* from the *Fen* gene in order to analyze the SNP in only one gene. SNPs in St*Pto* and GP21 amplicons were also highly associated with the traits. Analyzing six loci for SNPs resulted in 20 significant SNPs in a genetic region of 8-10 cM (Table 3.11) without any evidence for having a QTL gradient in the analyzed region. To confirm the statistical significant P values, the population was analyzed with two independent statistic tests, a non-parametric descriptive test and Fisher's exact test. All 20 significant SNPs were corroborated in both independent statistic tests. Association of SNPs within the same locus was observed where certain SNP combinations were present or absent in one of the phenotypic group. The clearest association between SNPs was between three SNPs in locus GP21 (snp 1, snp 11 and snp 12), (Table 3.15). This SNP combination was absent in the genotypes resistant to *P. infestans* and late maturing genotypes and was present only in the susceptible, early maturing genotypes. The genotypes selected for the study had clear distinguishable phenotypic data between the two groups concerning the trait maturity. All genotypes fell in two phenotypic classes 1 and 7 according to their passport data, where score 1 means very late maturing and score 7 very early maturing genotypes (Fig.3.3). Exceptions are three individuals, where two genotypes had score 3 for maturity and one genotype had score 2. The evaluation of the trait maturity is more reliable, than the evaluation of resistance to *P. infestans.* The trait maturity can be evaluated precisely in the field and by distinguishing the early from the late maturing genotypes. In contrast, evaluating resistance to *P. infestans* is a more relative measurement depending to some extend on subjective evaluations by humans and environmental factors. The potato cultivars of the germplasm collection originated from different countries, where evaluation for resistance to *P. infestans* is not uniform (Gebhardt et al. 2004). Based on this fundament knowledge it can be stated more surely that the observed difference in the frequency of genotypes per genotypic class is rather between the early and late maturing genotypes. The extended linkage disequilibrium (LD) found along the whole analyzed 8-10 cM genomic region on chromosome V (Fig.3.5) can have several explanations. First, the LD in potato is not yet finely dissected

as not many association studies have been conducted using SNPs to resolve its structure (reviewed in Gupta 2005). In a previous association study (Gebhardt et al. 2004) 610 genotypes have been analyzed with few PCR-based markers where LD extended up to 0.2cM. In this germplasm collection there have been only up to six meiotic recombination events separating the genotypes, in several classes even less. LD may not have broken down and the large LD effect observed in this study could be the result of studying an unrecombined genomic region. Moreover, the substructure of this population has not been assessed which could result in structuring the genotypes in smaller subgroups. In such structured populations false positive associations can be detected between loci and a phenotype even if there is no physical linkage to the locus responsible for the phenotypic variation (Simko 2004, Pritchard et al. 2000). A search in the Potato Pedigree Database (www.dpw.wau.nl) was performed for the ancestors of 21 randomly chosen genotypes from the 65 analyzed genotypes. For example five cultivars, Belchip, Europa, Vindika, Lotos and Leyla have the cultivar Katahdin as common ancestor, which is responsible for almost a quarter of the germ plasm in North American cultivars (Love 1999, Simko 2004). Three other cultivars Elin, Filli and Iris have the cultivar Aquila as common ancestor. This type of observation could be in support that probably the population of 65 genotypes is sub-structured. A large LD is a hallmark for inbreeding plants such as Arabidopsis, maize, soybean but not potato, which is an outcrossing organism and thus is expected to display rather fast declining LD (Nordborg et al. 2002, Thornsberry et al. 2001, and Zhu et al. 2003). However, reports in inbreeding organism such as soybean show that LD also declines remarkably fast, which is in contrast to the general excepted rule for the differences in extend of LD in inbreeding and outcrossing species (Remington et al. 2001, Gupta 2005). The data are being analyzed for LD between SNP loci, as a more extensive study is required in looking into the significant associations between the loci. The possibility can not be excluded that introgression from various wild species occurred in the 65 tested genotypes, thus resulting in introducing different chromosomal segments contributing to resistance or maturity. QTL for resistance to *P. infestans* and maturity on chromosome V have been detected in several studies (Leonards-Schippers et al. 1994, Oberhagemann et al. 1999, Collins et al. 1999, Sandbrink et al. 2000, Visker et al. 2003a and Bormann et al. 2004) where the source of

resistance to *P. infestans* originates from seven different wild *Solanum* species. The possibility of presence of two QTLs in the region on chromosome V cannot be excluded too, but statistical tools and knowledge constraint to resolve this problem in highly polymorphic and tetraploid organisms such as *Solanum tuberosum*. Recent work of Kroymann and Mitchell-Olds (2005) reports the presence of two QTL for growth rate in a region of 210kb in *A. thaliana*. Potato has an almost three times longer live cycle than Arabidopsis and in addition is an outcrossing species. Methods to identify haplotype structure in a tetraploid organism such as *Solanum tuberosum* ssp. *tuberosum* are not well developed. Defining allele composition in each chromosome in a tetraploid organism is a challenging task and thus hampering solving haplotype structures.

4.3 Expression study on chromosome V

4.3.1 Summary results of RT-PCR experiments for 25 PCG (putative candidate genes).

Due to the large number of PCG in the physical contig, not all 49 genes could be included to study their expression upon *P. infestans* infection by RT-PCR.

Of 25 PCG, primers for three of the genes (ORFs 5, 11 and 43) could not be designed due to technical reasons (Table 3.22). The *R*1 gene, its allelic, duplicated and truncated variants were excluded from the RT-PCR experiment, because their analysis is subject of an ongoing experiment in the laboratory. The 10 retroelements were excluded as candidate genes because it has been shown that they function in chromosomal arrangements and epigenetic processes (Wolf-Ekkehardt Lönnig and Saedler 2002). The RNA-directed RNA polymerases were not considered as they function in post-transcriptional gene silencing processes (Maine 2000). The QTL *Pin*5A for resistance to *P. infestans* in the population of which parents I88 and G87 were used for the RT-PCR experiment has been detected by evaluating resistance in tubers and foliage (Oberhagemann et al. 1999). Of 22 PCG analyzed using RT-PCR, 14 genes were expressed in leave tissue, of which four genes were differentially expressed upon *P. infestans* infection (Table 3.22). Eight genes were not expressed in potato leaves, namely ORF 2 (ZF-homeobox protein), ORF 4 (NAM (no apical meristem) gene), ORF 10 and

ORF 15 (F-box genes), ORF 8 and ORF 17 (no homology), ORF 40 (EST *A. thaliana*), unknown function and ORF44 (Zinc-finger protein). This result is consistent with the fact that for all these eight genes there was very weak similarity to any of the existing 158,000 potato ESTs (Ronning et al. 2003) in the TIGR database with exception for ORFs 2 and 4. The EST for the two not expressed genes: ORF2 (ZF-HD homeobox protein) and ORF 4 (NAM (no apical meristem) originates from callus and roots which could be an explanation why the genes were not expressed in RT-PCR experiment where leave tissue was used.

4.3.2 Leaf position and age reflect resistance to *P. infestans*

The material for the expression study was collected from the second and third completely expanded potato leave counting from the oldest leave. The specific conditions in harvesting the plant material were required based on the results of Visker and colleagues (2003b), who demonstrated that plant age influences the resistance to *P. infestans.* The conclusion has been made that the basal leaves are more susceptible than the apex leaves, and young plants are more susceptible (Visker et al. 2003b).

4.3.3 The role of anthocyanin in plant defense

The QS I88 and the QR G87 parents used for the RT-PCR experiments were infected and maintained in a growth cabinet under the conditions required for the growth of the oomycete (2.2.4). The different infection experiments were performed in two growth chambers. In one of the growth cabinets the I88 genotype reacted with obvious change in the color pigment in the leaves. The G87 genotype did not react in changing the leaves pigmentation. The leaves looked completely dark-red, like synthesizing the pigment anthocyanin. The parameters temperature, humidity, day lengths were identical to the previously performed experiment. The only difference was in the light intensity of 260µE instead of 200 µE (microeinsteins) like the conditions are in a green-house with natural day-light. Additionally to the change in the pigment color, the plants had a stunted growth habit which is an indication for too strong light intensity. Anthocyanin pigments

have been shown to possess antioxidant properties (Gould et al. 2002). High irradiance, temperature extremes, UV radiation, mineral imbalance, mechanical injury and pathogen attack have all been shown to enhance the production of anthocyanins in leaves (Chalker-Scott 1999). In an experiment of ectopic expression of anthocyanin 5-o-glucosyltransferase in potato tuber, a two- fold increase in resistance to the bacterium *Erwinia carotovora* subsp. *carotovora* (Lorenc-Kukula et al. 2005) has been observed strengthening the role of the anthocyanins in plant defenses against pathogens. Since, the plants were stressed under the conditions of differences in light intensity, defense responses may have been activated in the host plants and infection with the *P. infestans* did not take place.

4.3.4 Hypersensitive response (HR) in the initial biotrophic phase of the oomycete in the QR parent G87 after *P. infestans* infection.

Typical black speckles (HR symptoms) were observed on the site place of infection on the potato leaves in the quantitave resistant parent G87 but not in the susceptible I88. After a time period, the defense barriers in the QR G87 parent were overcome and disease symptoms developed but much weaker and later than in I88. Some leaves in G87 developed complete necrotic leasons which were not observed at all in the I88 parent (Fig.3.9). HR is an effective defense mechanism in race-specific resistance causative for cell death at the site of infection in the host plant (Hammond-Kosack and Jones 1996). In potato, HR responses upon *P. infestans* infections have been reported to become visible at different time points post-infection depending mainly from the degree of the resistance of the potato cultivars analyzed. Thus, Vleeshouwers and colleagues (2000a) observed HR symptoms in the first 22 h in the biotrophic phase of the oomycete. Recently Wang et al. 2005 identified HR at 48h post-infection in accordance to our observation where HR was observed after 48 hpi in the QR parent G87.

4.3.5 Differentially expressed genes

4.3.5.1 *PR*1-b gene

Upregulation of the transcript level of *PR*1-b at 24hpi, 48hpi and 72hpi gene in the QS parent I88 and almost steady state of upregulation in the QR G87 parent was observed (Fig.3.13). PR proteins are grouped in ten families each sharing amino acid sequence, enzymatic or biological activities (van Loon et al. 1994). The class one PR1 proteins exhibit antifungal activities and are active against *P. infestans*. This class is represented by four PR1 proteins PR1-a, b, c and g. PR1-a, b and c are acidic and PR1-g is a basic protein (Niderman et al. 1995). The PR1-g protein has been shown to have the weakest, PR1-b moderate and PR1-a and c the strongest inhibitory activity on *P. infestans* zoospores germination in tomato and tobacco (Niderman et al. 1995). In the moderately resistant G87 parent, the basal expression level of the *PR*1-b gene was higher and not detectable in the susceptible I88. The higher basal expression level in the G87 parent is in agreement with the results of Vleeshouwers et al. (2000b), where the authors found that there is a correlation between the levels of resistance in various potato cultivars and wild *Solanum* species and the basal expression level of *PR*-1 gene. In contrast, the expression level of the *PR*-1b gene in the susceptible parent I88 was gradually increased with the time post-inoculation and basal expression of *PR*1-b was not observed.

4.3.5.2 Amylase (ORF 45)

The putative α -amylase gene was up-regulated at 24 hpi and 72 hpi in the QS parent I88, but very weakly expressed in the QR parent G87 (Fig.3.18). α –amylase (EC 3.2.1.1) is an endoamylolytic enzyme that hydrolyzes the α-1, 4 glucosidic linkages of starch (Beck et al. 1989, Yu et al. 2005). A direct role of α –amylase in plant defense against pathogens has not been reported. Probably, there is an other mechanism of acting of α –amylases by which the carbon is exported through the triose phosphate/phosphate transporter (Scheidig et al. 2002, Schleucher et al. 1998) that is utilized by the pathogen for its own growth and thus induction of an α -amylase gene in the QS parent I88 benefits the pathogen.

4.3.5.3 Role of Protein kinase genes in plants (ORF47)

ORF 47, a putative protein kinase was up-regulated in the QS I88 parent at 24hpi, 48hpi and 72hpi with the strongest upregulation at 48 hpi (Fig.3.22). In the QR G87 parent the transcription level of the gene was upregulated gradually with the highest expression level at 72 hpi. The putative function assignment as a protein kinase was based on the presence of the ATP-binding domain in the amino-terminal end and the presence of peptide binding and phosphotransfer domains in the carboxy-terminal end (Hanks 2003). The exact position and the domain structure of the gene are shown in appendix. Plant receptor kinases play key roles in the cell-cell recognition process during development, defence against pathogens and self-incompatibly. There are *R*-genes protein kinases shown to be functional protein kinases in plants acting in defence against pathogens. *Pto* and *Fen* genes in tomato conferring resistance to the bacterial pathogen *Pseudomonas syringae* (Loh and Martin 1995), the rice *Xa21* gene, which confers resistance to *Xanthomonas oryzae* (Song et al. 1995). *FLS*2 gene in Arabidopsis confers flagellin insensitivity (Gomez-Gomez and Boller 2000). Thus, ORF47-protein kinase induced after *P. infestans* infection is a plausible candidate gene acting in defence against the oomycete.

4.3.5.4 EST (ORF 46) and EST (ORF48) - unknown function.

ORFs 46 and 48 encoding proteins of- unknown function were differentially expressed (Fig. 3.20 and 3.24). For the two ESTs no domain could be found in the deduced protein sequence, although domains were present in the corresponding *A. thaliana* protein sequence (Table 3.21).

4.3.6 Analyzing *in silico* the data from microarray experiments in the TIGR database

A search for the four differentially expressed genes was performed in the available data from a series of microarray expression studies on potato in the TIGR database- (Solanaceae gene Expression DB). From ORFs 45, 46, 47 and 48 only ORF 46 was represented on the cDNA chip under clone name STMFB74 and STMHZ21 for the corresponding potato EST TC120485. The search for changes in the transcript levels of these ESTs was conducted in the study: "Defence signaling in potato", ID 62. A slight (up to 1.5-fold) up regulation of the transcript level of the STMFB74 probe was observed under treatment with salicylic acid and methyl-jasmonate (MeJA) in compatible interactions in expression study ID64. Similarly, results with EST probe STMHZ21 for the same EST TC120485 indicated up-regulation of the transcript level up to 1.8-fold upon treatment with arahidonic acid on potato leaves. The observed transcript up-regulation upon salicylic acid (SA) treatment in a compatible interaction with *P. infestans* in the microarray expression study is in accordance with the observation of an increased transcript level of the *PR*1-b gene upon *P. infestans* infection in the RT-PCR experiments. *PR*1 is a marker gene for the SA-dependent pathways for resistance to pathogens in plants (Glazebrook 2001) and is typically induced by necrotizing pathogens and HR (Huitema et al. 2003). The first observation on the signaling role of salicylic acid (SA) in plant defense against pathogens has been made by White in (1979). Yu D. and colleagues (1997) concluded that in potato, SA has exclusively a high basal level and does not play a major role in orchestrating the SAR defense responses but is rather involved in the local defense response in potato upon infection with *P. infestans*. Another study in potato points to the potential importance of SA generation at the site of infection, but not systemically (Coquoz et al. 1995). These results suggest the importance of SA rather in the local response then in SAR development in potato. The observations are in agreement with our finding as we studied the local defence response upon *P. infestans* infection and not the SAR response and found elevated transcript levels of *PR*1-b. By contrast, in tobacco and *A. thaliana* (Coquoz et al. 1995, Chen et al. 1995), potato has an up to 100-fold higher constitutive level of SA than tobacco and *Arabidopsis thaliana*.

Since neither this high level of SA nor the application of exogenous SA enhanced resistance against *P. infestans*, potato plants appear to have a poor SA signal perception and/or transduction mechanism (Yu et al. 1997). Similarly to the SA response, upregulation of transcript level of probe STMHZ21 has been observed in the microarray data upon treatment with arachidonic acid (AA). Arachidonic acid is an elicitin released from *P. infestans* during infection and elicits defence responses in potato (Bostock 2005, Tyler 2002, Yoshioka et al. 2001). The slight up to 1.5 fold increase of transcript level of STMFB74 upon MeJA treatment has been recorded in the microarray study ID 64. JA is a signaling molecule acting as plant growth and stress regulator (Glazebrook 2001, Turner et al. 2002). Cross-talk between SA and JA pathways leads the induction of defence gene expression and potentiates each other's activities (Reymond and Farmer 1998). It has been shown in tobacco, that the gene *PR*1-b is synergistically induced by the combination of MeJA and SA, which might be relevant to the up-regulated transcript level of STMFB74 by exogenous application of the two regulator molecules SA and JA on the potato plants in the microarray study ID64 (Xu et al. 1994).

4.3.7 Comparisons with previous expression studies upon *P. infestans* infection.

In none of the published microarray (Wang et at. 2005, Restepo et al. 2005) and macroarray expression studies (Ros et al. 2004, Beyer et al. 2001) the four differentially expressed genes have been reported to be involved in the response to *P. infestans.* A possible explanation is that the four genes were not represented among the cDNA clones, neither in the microarrays studies, nor in the macroarrays, where limited numbers of clones have been studied.

It is possible, that more of the 25 analyzed genes are differentially expressed upon infection with *P. infestans*, but the semi-quantitative RT-PCR technique used in this study is not sensitive enough to differentiate minor differences in gene expression between the QS and QR parents. From the selected 25 ORFs, only eight genes were not expressed in leaves which exclude them as being candidate genes for resistance to *P. infestans.* The ten not differentially expressed genes (Table3.22) are being considered as putative candidate genes as these genes might not have changed transcript levels but may

have different enzymatic properties, or may undergo post-translational modifications, or may have variable protein stability.

Summary

Late blight is the most devastating potato disease worldwide caused by the oomycete *Phytophthora infestans*. Lots of breeding efforts are devoted to improve the field resistance to late blight of potato cultivars. Appearance of new pathotypes of *P. infestans* and the quantitative phenotype of field resistance make the conventional breeding process difficult. The genetics of inheritance of quantitative resistance is not yet fully understood. Marker assisted selection could help to solve the problem to reliably discriminate resistant from susceptible cultivars.

The first part of the thesis was focused on identifying genomic regions responsible for resistance to late blight in two new, independent, tetraploid F1 families 'SaKa-Ragis' and 'BNA'. DNA markers known from previous studies to be linked to resistance loci in the potato genome were tested in the two families, which had been field evaluated in years 2001 and 2002 for late blight resistance and showed normal distribution of the resistance phenotype, indicating polygenic inheritance in both populations. A "cases" and "controls" study was performed. The "cases" were 23 highly resistant and the "controls" were 23 highly susceptible individuals selected from each F1 family. Both populations were genotyped using SNP, CAPS, SSCP and SCAR markers. Markers significantly linked to QTL (quantitative trait locus) for resistance to *P. infestans* were found on chromosomes II, IV and V in the 'BNA' population and on chromosome X in the 'SaKa-Ragis' population. The QTL on chromosome X was further characterized by increasing the marker coverage and SNP haplotype construction for CP105, the most significant marker locus.

The second part of the thesis was focused on the most significant and reproducible known QTL for resistance to *P. infestans* on potato chromosome V. This QTL is part of a hot spot for resistance to pathogens in the potato genome (Gebhardt and Valkonen 2001). A major QTL for plant maturity maps to the same genomic region. A genomic region of about 400 kbp including the *R1* gene for resistance to *P. infestans* has been sequenced to identify positional candidate genes besides the *R1* gene family (Ballvora et al. 2002).

Two contigs have been assembled, one for the “resistant” homologous chromosome with an introgression from the wild potato species *Solanum demissum* and the other from the “susceptible” allele (Ballvora et al., in preparation). The two contigs were subjected to Gene Mark- gene prediction software resulting in the annotation of 49 ORFs (open reading frames). Based on putative function assignment, 24 of 49 ORFs were selected for further characterization. The expression of the 24 selected ORFs was analyzed by RT-PCR in cDNAs from leaves uninfected and infected with *P. infestans,* of two diploid potato genotypes, which were the parents of a population showing a major QTL effect on chromosome V but lacked the *R1*-gene. For 8 ORFs expression was undetectable in leaves. Four genes were up-regulated upon *P. infestans* infection. The remaining 12 genes were equally expressed in both infected and uninfected leaves, in different intensities. Furthermore, a genetic approach was pursued for the same QTL on chromosome V in order to narrow down genetically the genomic region in which the gene/genes for resistance to *P. infestans* and maturity are localized. For this objective, 32 highly resistant and late maturing tetraploid cultivars and 33 highly susceptible and early maturing cultivars were selected. The amplicons at six loci on potato chromosome V were sequenced in the 65 cultivars and analyzed for SNPs. At least one SNP in all six loci were significantly associated with maturity and resistance to *P. infestans*, suggesting extended linkage disequilibrium in a genetic region of about 8 cM on potato chromosome V.

Zusammenfassung

Die Kraut- und Knollenfäule ist die weltweit zerstörerischste Krankheit der Kartoffel und wird durch den Oomyzeten Phytophthora infestans verursacht. Viele Bemühungen in der Züchtung sind darauf gerichtet, die Feldresistenz von Kartoffelsorten gegen die Krautfäule zu verbessern. Das Auftreten neuer *P. infestans* Pathotypen und der quantitative Phänotyp der Resistenz erschweren den konventionellen Züchtungsprozess. Marker-gestützte Züchtung könnte helfen, das Problem der verlässlichen Unterscheidung von resistenten und anfälligen Pflanzen zu lösen.

Der erste Teil der Doktorarbeit konzentrierte sich auf die Identifizierung von genomischen Regionen für Resistenz gegen die Krautfäule in zwei neuen, voneinander unabhängigen, tetraploiden F1 Familien 'SaKa-Ragis' und 'BNA'. In den beiden Familien wurden DNA Marker getestet, die, wie aus vorhergehenden Studien bekannt, mit Resistenzloci im Kartoffelgenom gekoppelt sind. Beide Familien waren in den Jahren 2001 und 2002 im Feld auf Krautfäuleresistenz geprüft worden. Der Resistenzphänotyp war in beiden Populationen normal verteilt und zeigte damit polygene Vererbung. Es wurde eine „case/control“ Studie durchgeführt. „Cases“ bzw. „controls“ waren 23 hoch resistente bzw. 23 hoch anfällige Individuen, die aus jeder F1 Familie selektiert worden waren. Beide Populationen wurden mit SNP, CAPS, SSCP und SCAR Markern genotypisiert. Mit QTL (quantitative trait locus) für Resistenz gegen *P. infestans* signifikant gekoppelte Marker wurden in der ‚BNA' Population auf den Chromosomen II, IV und V gefunden, und in der ‚SaKa-Ragis' Population auf Chromosom X. Das QTL auf Chromosom X wurde weiter charakterisiert durch Analyse weiterer Marker und durch Konstruktion von SNP Haplotypen für CP105, den signifikantesten Marker Locus.

Der zweite Teil der Doktorarbeit konzentrierte sich auf das signifikanteste und reproduzierbarste bekannte QTL für Resistenz gegen *P. infestans* auf Kartoffelchromosom V. Dieses QTL ist Teil eines ‚hot spots' für Pathogenresistenz im Genom der Kartoffel (Gebhardt and Valkonen 2001). Ein Haupt-QTL für die Reifezeit der Pflanzen kartiert in die gleiche genomische Region. Eine Genomabschnitt von 400 kbp einschließlich des *R1* Gens für Resistenz gegen *P. infestans* (Ballvora et al. 2002) ist sequenziert worden, um positionelle Kandidatengene neben der *R1* Genfamilie zu identifizieren. Zwei ‚contigs' wurden konstruiert, eines für das ‚resistente' homologe Chromosom mit einer Introgression aus der Wildkartoffelart *Solanum demissum* und das andere für das ‚anfällige' Allel (Ballvora et al., in preparation). Die Untersuchung beider Contigs mit dem ‚Gene Mark' Gen-Annotationsprogram ergab 49 ORFs (open reading frames). Basierend auf der möglichen Funktions-Annotation wurden 24 der 49 ORFs für eine weitere Charakterisierung ausgewählt. Die Expression der 24 ausgewählten ORFs wurde mittels RT-PCR in cDNAs von nicht-infizierten und von *P. infestans* infizierten Blättern analysiert, und zwar in zwei diploiden Genotypen, den Eltern einer

Nachkommenschaft, die einen Haupt-QTL Effekt auf Chromosom V gezeigt hatte, die aber nicht das *R1* Gen enthielt. Die Expression von 8 ORFs war in Blättern nicht nachweisbar. Vier Gene zeigten eine erhöhte Expression in *P. infestans* infizierten Blättern. Die übrigen 12 Gene waren in infizierten und nicht infizierten Blättern gleich exprimiert, mit unterschiedlicher Intensität. Zusätzlich wurde für das gleiche QTL auf Chromosom V ein genetischer Ansatz verfolgt, um die Genomregion einzugrenzen, in der das Gen/die Gene für Resistenz gegen *P. infestans* und Reifezeit lokalisiert sind. Zu diesem Zweck wurden 32 hoch resistente, spät reifende, tetraploide Sorten und 33 hoch anfällige, früh reifenden Sorten ausgewählt. Die PCR Produkte an sechs Loci auf dem Kartoffelchromosom V wurden in den 65 Sorten sequenziert und auf SNPs analysiert. In allen sechs Loci war mindestens ein SNP signifikant mit Reifezeit und mit Resistenz gegen *P. infestans* assoziiert. Dieses Ergebnis deutet auf ein ausgedehntes Kopplungs-Ungleichgewicht in einer genetischen Region von etwa 8 cM auf dem Kartoffelchromosom V hin.

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Posters from this Dissertation:

Tagging QTL for Late Blight in Tetraploid potato by genotyping highly resistant "Cases" and highly Susceptible "Controls –GABI meeting (Bonn, Germany), February 2004

Functional Characterization of a candidate locus controlling quantitative resistance to the oomycete *Phytophthora infestans*, MPIZ-Cologne, September 2005

Talks:

Tagging QTL for Late Blight in Tetraploid potato by genotyping highly resistant "Cases" and highly Susceptible "Controls" , Plant and Animal Genome XIII Conference, San Diego, California Workshop Molecular Markers, January 2005

Appendix

Table A: **List of primer pairs used for the QTL analysis.**

LG	Marker	Primer	Primer	Ta (°C)	Product size (bp)	Poly-morphism
I	BA114i24t3[1]	fa1	CTT TGG CTT TTG AAC AAA GCA AAC	56	350	SNP
		ra1	CTA ACA CAA TGT ACT TGC AGG TG			
II	BA62l7t3[1]	f	GTG CTA ATA TCG TAA GAA GTG GC	58	500	SNP
		r	ACA GAT TCA GGT GAT GCA CGT G			
II	St3.2[1]	Fa1	AGC AAC TTA GGT CAC AAC CAC AC	56	700	SNP
		Ra1	TAT CTT GAA TTG TTT CCC TGC AGC			
	GP23[1]	Fa1	CAA TAT CTT CAG GAC AAC CAA CC	56	300	SNP
		Ra1	CTT TAA CAG CCT GCA CTA TGG TG			
	GP321[1]	F	GCA CAA AAC AAA CCA ACG CAA	56	850	SNP
		R	TCA CCT TGT TAT CTC CTA TGC			
	S1b3[1]	Fa1	ATG TTT GTC AAC TGT GCT GCG G	56	300	SNP
		Ra1	AAT TAG TAA TTA GTA ATT ACC TCA GAG			
III	GP1[2]	f	GTC TGC TAT CGG TCC GAT C	58	600	SSCP-*Mse*I
		r	TCT ACT CCA TCA AAT TCT CCT G			
IV	STM3016[3]	f	TCA GAA CAC CGA ATG GAA AAC	53	110/150[9]	SCAR
		r	GCT CCA ACT TAC TGG TCA AAT CC			
V	BA213c14t7[1]	A	CAA TTG ATT CAT TTT ATG TAG CGA G	56	600	SNP
		B	TCT TGA CGC AAA CCT CTG CGA G			
V	BA87d17t3[1]	C	GTA GTA CAT CAA CAT ACA TTT TGC GG	58	600	SNP
		D	CTC AGA ATT CAG AGC TTC AAC TGA TG			
V	BA76o11t3[1]	C	CAG GAC ATC AAT ATA AAT ACT GTT GC	58	300	SNP
		D	CGT ACG TAT GAG GAG TCT GTA TC			
V	CP76-2[4]	Sf2	CAC TCG TGA CAT ATC CTC ACT A	55	1400	ASO
		SR	CAA CCC TGG CAT GCC ACG			
VI	BA34j14t7[1]	F	AAA AGT TGA GCC CAT TCG AGT GA	56	600	SNP
		R	GAG TTT CTC ATA CAA ACC TCC TC			
	BA71g21t7[1]	f	CAC AAT CAT CTG ACC ATC GAA AC	56	250/370	SCAR
		r	CTA GGT TGT GTA ATG ATG TAG CG A			
VII	BA228g19t3[1]	f	TCT GTA AGA CAT GCT TGT TGT TGA	58	550	SNP
		r	CAG ACC CAT TAC TTG TTG TTT CG			
VIII	BA261b9t7[1]	f	TCA AAA TTC ACA GGG TGA TTG GC	58	400	SNP
		r	ATG AAG TTA CTC AGG CTA ACA GG			
	BA73e8t3[1]	f	TGG CCT GAA TTT GGA TCA AAT GG	56	350	SNP
		r	AGG GCC TTC TTG GTT AAT ATC AC			
	57T3[4]	for	CAA TCC GTT GGA GTT TAG ACG ATC	58	800	CAPS-*Rsa*I
		rev	GAA GAG GAG AAC CTT GGT GGA TGG			
IX	GP94[1]	f	ATG TAT CAC AAT CAC ATT CTT GCT C	58	400	SSCP-HinfI/DpnI
		r	TGT AAA ACC AAC AAG TAG TGT TGC			
IX	Prp1[5]	#7	GTG ACA TGA GCA CAT AAG TC	55	200/250/600[9]	SCAR
		#8	GCA ACT TCA CTT CTG CCA TC			

Tab. A. (continuation) **List of primer pairs used for QTL analysis.**

LG	Marker	Primer	Primer sequence	Ta (°C)	Product size (bp)	Polymorphism
IX	CP44[2]	f r	TCA CAA CCA CCG AGA CTT TG TTA GTT GAA ACC ACA ACA ACA C	56	500	SSCP-*Mse*I
IX	B7	f r	GGG CCT AGT TCA ATG TAC AGT TGC TAT GGT TGT TCC	53	600	SSCP-*Hinf*I
IX	GP129[1]	f f	GTG GTA GCA AAG TAT TCA TC CGT TAT CTG GAC TCC TTT AG	56	500	SNP
X	CP72[6]	f r	CAA GAA ACC TCA AAT GATCAAG TCA TCA GTA AAG ATT TGG AAC C	60	500	CAPS-*Dpn*II
X	GP247[6]	f r	AGT TGG ATA AGG TCT TGC CC GCA GAA GCA TGG TAG CCA C	60	500	SSCP-*Mse*I
X	TPT[2]	f r	CCT TCT CTC TCA CTG CCA ATG CTC ACC AAG CAA TAT ACC ACC	60	1500	SSCP-*Nla*III
X	TG63[6]	f r	GGT TTC CCA ATG TGT GAC CCG ATT TAC CTA GAG TGC	56	400	n.p.
X	GP266[1]	f r	CCA GCT GTC AAA TTC ATA GAA GG ATC CCA CTT ACA GAC CAA GAA TG	56	554	SNP
X	BA81l15t3[1]	f r	CCA CTT CCT TGT GTT TTC ATG AC ATT GAA TCC TGT CAA GCC AAC AC	58	500	SNP
X	BA44a10t7[1]	f r	ATA TTC CTC CGG AAA CCT TTT CC ATG GGA ATA GGT CTA GTT CGA TG	56	400	SNP
X	CP105[6]	f r	TAA GCT CCC CAA GTC ATT GCA GG GCC GAA CTT GTT TCA CAT AAC ATG	58	350	SNP
X	GP287[6]	f r	TCA TTC CCA AGA CAC TCA TGC ACT CAA CCA CCA GCT CAA GAC	59	600	SSCP-*NlaIII*
XI	BA157f6t3[1]	f r	AAT CAT GTT GTG TGT CCA AGA CC TGC ATT AAG GGT GGT TAG ATA CC	56	500	SNP
	NL27[7]	f r	TAG AGA GCA TTA AGA AGC TGC TTT TGC CTA CTC CCG GCA TG	58	1200	SSCP-*Mse*I
	GP125[5]	f r	AGC AGC TCT GAT GGA AAT GC GAG CCT AGC TGC CCA GCT TC	57	1000	SSCP-*Rsa*I
	GP229[2]	f r	AGTCTTGGCATCAAATATTTG ATTATCTTCACAGCAGTAGAG	53	400	SSCP-*Rsa*I
	GP76[5]	f r	ATG AAG CAA CAC TGA TGC AA TTC TCC AAT GAA CGC AAA CT	53	1500	CAPS-*Rsa*I
XII	GP34[8]	1.1 1.2	CAA ACG TTG CTA GGT AAG CA TCG TTC CGT TGT TTT GTC AA	56	1250	CAPS-*Alu*I

[1] Rickert 2002; [2]group C.Gebhardt (MPIZ, Cologne); [3]Milbourne. 1999; [4]Ballvora et al. 2002; [4]Castillo-Ruiz; [5]Oberhagemann et al. 1999; [6] Ilarionova 2005 (in the Ph.D. thesis); [7]Marrczewski et al. 2001;[8]Bendahmane et al. 1997;[9]polymorphic marker band

Table B: List of primer pairs used for the expression study on chromosome V.

ORF №	primer	Primer sequence 5´→3`	Ta, °C	RT-PCR cycles	Genomic size,bp	cDNA size,bp
ORF2	F	GGT GGT GAT TTA CAA GGA ATG	55	45	700	-
	R	AGT GGA AAT ATG AAG ATG AAG C				
ORF3	F	TTG CTT GAA CAT GAT CCA CAC	59	43	807	649
	R	GCT TAG TTT CCT CTG GCA CC				
ORF4	F	TAG CTC ATA TGT TAC TTT CAA GTG	54	45	355	-
	R	CTT CTT CTT TGC TTC TAA ACA TG				
ORF8	F	AAG TAC TTG GCA GCA TTG TGT G	55	45	512	-
	R	TTT CAG ATG ATT GCT TTT ACA GAG				
ORF10	F	TTG GAG CTA AAT TGA GGA AC	55	45	653	-
	R	CAG CAG TAC ATT CCT CTA ATT G				
ORF13	F	CGA TGA ATA TGA AGA TGA AGC	57	36	426	426
	R	ACC TTT TTA GCT TTG ATA CGT TG				
ORF15	F	CTT GGA TTG TCG GTT CTG TC	57	45	600	-
	R	CTT TAG GAT GAT CGA CCG TG				
ORF17	F	ACA ACA CCC ATA TCT CAA TTT C	59	45	603	-
	R	CAG CAA CGT TAT CAT GAG GG				
ORF19	F	GAC TAA TAG AGA CAG CAT CAA TAC C	56	43	930	431
	R	TCA ATG GGA GTA ACC GAG C				
ORF21	F	TTC TAC ATA CCC GAG GAC ATA TG	58	36	949	465
	R	CAT ACG AGT TCA AAT GGT CAG				
ORF22	F	GCA AAA CAC CAT CAA GAC TAG	55	36	863	863
	R	GAC GTC CAG AAT ATG TAG TAG C				
ORF24	F	ACA AGT ACA GCT AAT AGA CCC	55	36	560	560
	R	GGC TTA TCG TCT TCA CTA CC				

Table B (continuation): **List of primer pairs used for the expression study on chromosome V.**

ORF №	primer	Primer sequence 5´→3`	Ta, °C	RT-PCR cycles	Genomic size,bp	cDNA size,bp
ORF36	F	TAC AAC AAT ATA TGC AGA ACC C	56	36	602	602
	R	TGT TGA TGT TAG ATA CTT GGC				
ORF37	F	ATC CTC GGA TGA ATA GTC ACC	56	43	1208	456
	R	AAG GCT TAT AAC AGA TCA TTC AC				
ORF38	F	AGA ACC CGT TAC TGA TCC TG	57	43	1065	464
	R	CAA TAA CCT CCG ATA CAT CAC C				
ORF40	F	GTG GAT TTG TTG GTT TGT GG	57	45	711	-
	R	AAT GGT TTG TCT GGA TTC ATT CTC				
ORF41	F	TGA AAC TAT TGA ATG GCT CTT AC	56	43	350	320
	R	CTT GAT CTT CTG ACC TCC GC				
ORF44	F	CAA AGG AGA TGT TGA CTG AG	55	45	531	-
	R	TCG TAT CCC TTT CAA TTT CAG				
ORF45	F	ATG TCG ACA GTT ACT ATA GAG CC	55	43	970	341
	R	CTA CAT CCC ACA GAG AGG TGC				
ORF46	F	CCC ACT GCT AAG GCT ATT TTG	58	43	1055	386
	R	CAT GTC AAA ATC CCG AAT GC				
ORF47	F	TAA ATC TAC GAA CAC AAG CAG AGA C	58	43	548	442
	R	GCT TCC TCG AGG CAT AAA TTC				
ORF48	F	GGTAATGATAAAGGAGGCCCAC	58	43	617	491
	R	ATCCACAAAGCCCATCGTATC				
P.infestans	08-3	GAA AGG CAT AGA AGG TAG A	53	30	258	-
P.infestans	08-4	TAA CCG ACC AAG TAG TAA A				

-not expressed ORFs

Table B (continuation): **List of primer pairs used for the expression study on chromosome V.**

ORF №	primer	Primer sequence 5´→3`	Ta, °C	RT-PCR cycles	Genomic size,bp	cDNA size,bp
*PR*1-b	F	TTA AAC CAA TCC AAA CTA TTC C	57	36	500	500
	R	CAA GTT ATA AAG TAC CAC CCG				
Tubulin	F	ATG GAT CTA GAG CCT GGT ACT ATG	58	28	1500	525
	R	CAA ACA GCA AGT AAC ACC ACT C				

Table C: List of primer pairs used for the QTL gradient experiment on chromosome V.

Marker[1]	primer	Primer sequence 5´→3`	Seq. primer	Ta,°C	Genomic size,bp
ORF36	F	CAA CAT ATC CTC AAT AGC TAA C	R	55	459
	R	ATG GTG GGT AGT GTG AAT ATT G			
ORF47	F	ACT TGA AAT GAT AAC AGG TAG GAG	F	58	589
	R	CCC TTT TGC CAG TTC ATC C			
GP21	F	CCC TGA ACC TCC TTA TCC CC	R2	55	754
	R	ACT ATT ATG TCT ATG AGG AAG TGG TC			
St*Pto*	F	TCA CAT TGG ATT GGG TGG C	F	55	670
	R	CGA GTC CAC TGC CCA TTC			

[1]Primer pairs for ORF 3 and 24 are the same as in Table B.

Table D: Digestion pattern of alleles descending from SR1, SR2, NK5 and NK6 for the SSCP markers

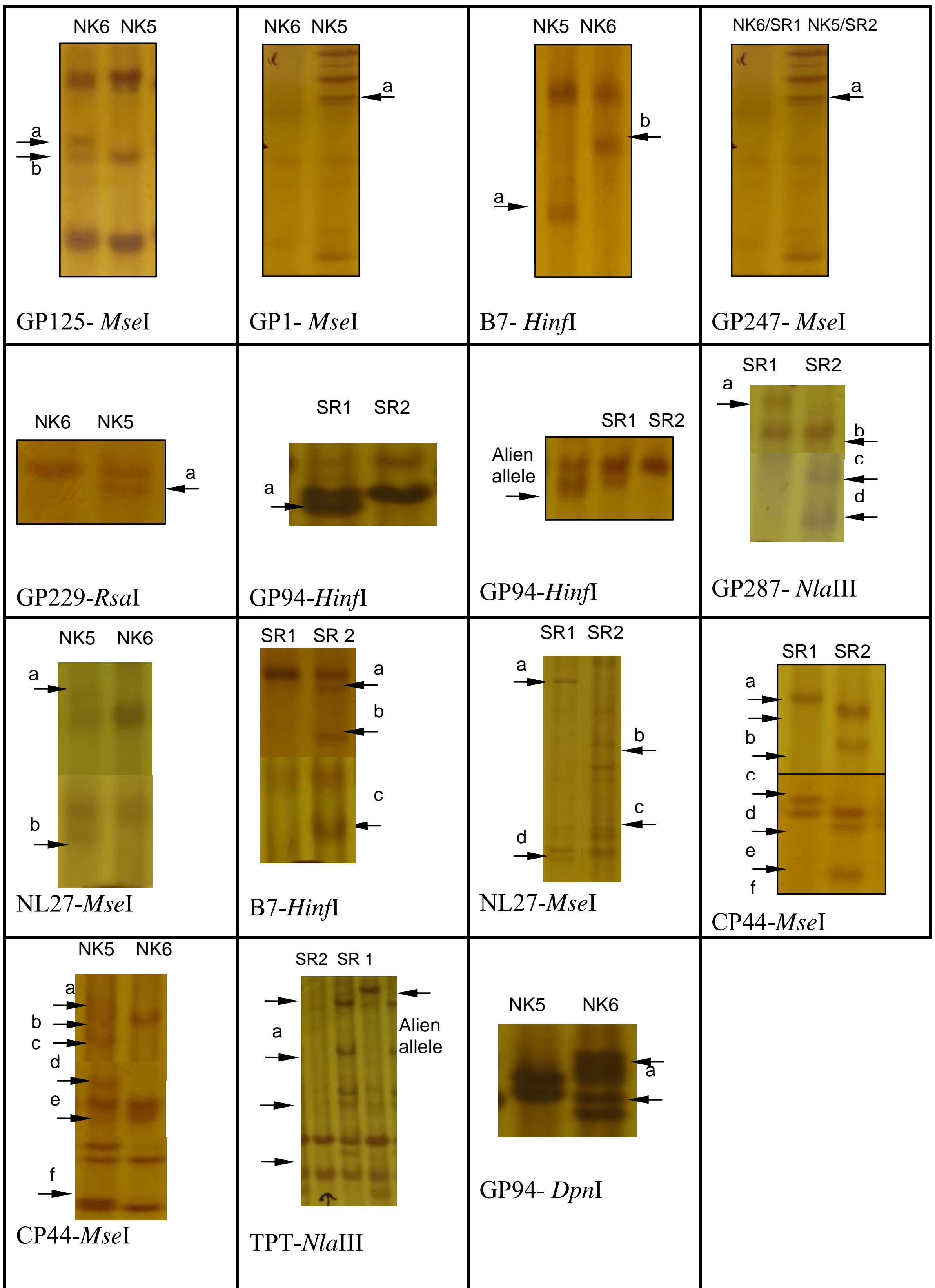


Table E: Varieties included in the QTL gradient experiment.

GL98[1]	GL00[2]	MPIZ[3]	CV [4]	Origin[5]	Year [6]	Maturity [7]	Foliage resistance [8]	Tuber resistance [9]	Group[10]
660			Deodara (1913)	deu	1913	1	7	-	0
829			Filli	brd	1974	1	7	7	0
812			Meta (1978)	sun	1978	1	7	-	0
813			Prikarpatskiy	sun	1978	1	7	-	0
816			Rosamunda	swe	1974	1	7	7	0
852a			Victor	esp	1954	1	7	7	0
820			Zvikov	csz	1984	1	7	7	0
874			Prosna	pol	1972	1	7	5	0
801			Elba	pol	1987	1	7	4	0
860			Bertita	mex	-	1	7	-	0
817			Belchip	usa	1979	1	7	-	0
861			Erendira	mex	1959	1	7	-	0
11			Delica	brd	1981	7	3	4	1
68			Dunluce	gbr	1976	7	2	2	1
76			Amazone	nld	1983	7	2	4	1
121			Bea	nld	1956	7	3	2	1
	99		Colmo	nld	1973	7	3	5	1
150a			Early Rose	usa	1867	7	3	-	1
66			Europa	fra	1989	7	3	-	1
64			Hankkijas Tanu	fin	1982	7	2	1	1
21			Iris	brd	1977	7	3	5	1
152a			Irish Cobbler	usa	1976	7	3	4	1
71			Maris Bard	gbr	1972	7	3	3	1
85			Minerva	nld	1988	7	3	4	1
138			Perkoz	pol	1984	7	2	3	1
141			Powirowez	sun	1974	7	3	-	1
80			Primura	nld	1963	7	3	4	1
142			Priobskiy	sun	1972	7	3	-	1
129a			Rode Eersteling	nld	1942	7	3	3	1
2			Romina	aut	1989	7	3	3	1
139			Ruta	pol	1985	7	2	2	1
74			Sharpes Express	gbr	1901	7	3	3	1
	866		Caribe	cdn	1983	7	3	3	1
901			Ulster Prince	gbr	1947	7	3	3	1
763			Aguti	brd	1977	1	7	7	0
778			Amyl	csk	1971	1	7	7	0
879			Ariadna	sun	1975	1	7	7	0

[1]GL98, [2]GL00, [3]MPIZ= years of DNA extraction; [4]CV= variety name; [5]Origin= country of origin; [6]Year= year of breeding; [7]Maturity = 1 (highly early mature varieties), 7 (highly late mature varieties); [8]Foliage resistance to *P. infestans*; [9]Tuber resistance to *P. infestans*; [10]Group= (Phenotype), 0 means (resistant to *P. infestans*/ late maturing),
1 means (susceptible to *P. infestans*/ early maturing varieties); - not available information

TableE (continuation): **Varieties included in the QTL gradient experiment.**

GL98[1]	GL00[2]	MPIZ[3]	CV4	Origin[4]	Year [5]	Maturity [6]	Foliage resistance [8]	Tuber resistance [9]	Group[10]
785			Artana	nld	1990	1	7	7	0
815			Elin	sun	1984	1	7	7	0
790			Karnico	nld	1987	1	7	5	0
804			Olza	pol	1988	1	7	4	0
775		12	Rebecca (1984)	brd	1984	1	7	7	0
760a			Bionta	aut	1993	1	8	-	0
796			CIP 38 31 17 06	per	-	1	8	4	0
145			Worotynskiy ranniy	sun	1981	7	1	-	1
87			Lotos	pol	1986	7	3	4	1
56		68	Toccata	brd	1983	7	3	4	1
84			Vindika	nld	1976	7	3	7	1
810			Berezka	sun	1968	1	9	9	0
798			Bzura	pol	1983	1	7	5	0
766a		41	Cosima	brd	1959	1	7	7	0
767			Donella	brd	1990	1	7	7	0
484		129 = XIII	Isola	brd	1958	3	7	5	0
			Berber	-	-	7	3	-	1
		20	Atica	brd	1971	7	3	-	1
		38	Christa	brd	1975	7	3	-	1
770		107	Franca ??	brd	1987	2	6		0
18		112	Gloria	brd		7	4	7	1
107		92	Hela	brd	1964	7	5	5	1
28		39	Leyla	brd	1988	7	4	6	1
825a		121 = V	Aula	brd	1974	1	5	7	0
476		47	Dinia	brd	1987	3	7	7	0
			SR1	-	-	-	-	-	0
-	-	-	SR2	-	-	-	-	-	1
787		124 = VIII	Darwina	nld	1981	1	4	6	0

[1]GL98, [2]GL00, [3]MPIZ= years of DNA extraction; [4]CV= variety name; [5]Origin= country of origin; [6]Year= year of breeding; [7]Maturity = 1 (highly early mature varieties), 7 (highly late mature varieties); [8]Foliage resistance to *P. infestans*; [9]Tuber resistance to *P. infestans*; [10]Group= (Phenotype), 0 means (resistant to P.infestans/ late maturing),
1 means (susceptible to *P. infestans*/ early maturing varieties); - not available information

SNP position for the markers included in the QTL gradient experiment on chromosome V.

Forward and reverse primers are shown in orange underlined sequence. Sequence in red shows the intron sequence. Numbers above each SNP are shown for every SNP position. SNP sequence is shown in parenthesis, bold and underlined. The description applies for all six analyzed marker. Primers sequence is shown in Tables B and C. The sequence of ORF36 and GP21 are reverse complement.

>ORF3 (sequencing primer F)

TTGCTTGAACATGATCCACACGAGTATCACACATATAACCAAGTTGCCGGAGAATATCAGCAATTTCAAG
1 2
TACTTTTACAAGATTTGTCGAGGATGAGCCATTTCAAGGACCACGAGTCCAAAA**(C/T)**T**(A/T)**TGTCAGGA
3 4 5 6 7
AGAG**(G/A)**TGCATTCTCCGTA**(G/A)**CTTTCTTCAAGTCCC**(G/A)**AGCAT**(A/G)**AGA**(G/A)**GTATGCATTTTTT
10 11 12 13 15 16
CATTGAGTTGTTATTAAAATACCT**(C/T)**GAC**(T/A)**CA**(C/T)(T/A)**GTAAC**G**AGGAAT**(A/T)**CTTTG ACT**(G/T)**
17 18 19 20 21
TCTAGCAGATGATCGTTT**(T/C)(A/C)**AGGGAAA**(A/G)**AAGGG**(C/T)**G**(A/G)**ACGAGAAGAGGAAAAAGATG
23 24
TCATTTCCATTGTGAAATTGGG**(C/G)**GAAGTTATTAAGGAGACA**(A/G)**TGCAAGTTTTCTGGGAGTTTCTT
25 26 27 28
(C/T)(G/A)TGCTGATAAACGTGAAGCTAATTTGGCCTTAAAGGG**(T/C)**GTTCAA**(A/G)**GAACTCAAATGGAC
29 30 31 32 33
AATGCAGAAATCGAGCTTTT**(T/C)**ATGAATGT**(C/T)**A**(A/G)**GTTAGAC**(C/T)**TTCAAAAGGTTAGTC**(T/C)**AT
34 35 36 37 38
CTTTATTCATAGATA**(C/T)**GCCA**(C/T)(A/G)**CCTTTCACAACAACAACAACA**(T/G)**GTTGATGAAGTT**(C/T)**
39 40
(A/T)TAGCTAATTCCAGTTTTTTT**(G/A)**TAGCGATTTTAGTTTCTAAACTTTGTGAATCACTTGATATTCTCT

ATGCAGAAGGAGAGGAAGTTGAAAGATGTACAAAGAAGTGGCAATTGCATAGTGAAGAAGTTCCAAAA

ACAACAAGAACGACGATTGAGTCATTCATTATTCGCGTCACTCGTGGAGCTAAAATTGGTATCAAGAGTG

CTAAGTTTACCAAGATTAAGGAGAGATCATTTGGTTTGGTGCCAGAGGAAACTAAGC

>ORF24 (sequencing primer 24F)

ACAAGTACAGCTAATAGACCCAATATTGTAGAAAACATAAAAGCTAAAACACCCCTAAGTACACTTTATAT
1 2 3
ATG(**T/C**)GT(**A/G**)TTCATCAATCTTTGCTA**(A/G)**CATCACCATTTTCCTTAACACCATTATCTTTTTCGTTTACA
4 5 8 10 11 12
(**C/T**)(**C/T**)GTCAG(**C/T**)ATTCTT(**G/A**)GCTTCTTCAGTA(**T/G**)GCTGTTACTTCTTTCTTTTCCTC(**G/A**)CGATCT
13 14 15 16 17 18
TTCTCTTCCT(**T/C**)CTCCTT(**C/T**)TCAGC(**C/T**)GCCTTTGCT(**T/C**)TCTCTTCTTCCTCAGCCTTCA(**G/A**)TTT(**C/T**)G
20 24 21
CTTCCTCTTTTGCAGTTTCAAGAGCTTTAATCAATCTCTC(**C/T**)AA(**A/G**)CAAGT(**G/T**)TCTGCATTTTCCTTT
22 23
GAAGACTT(**A/G**)GGCATCAAATTCTCAGCAATATCAGCAGG(**A/T**)GTCATATTAGTTTCCTCCAATAAACGAC

GAATCTCAGGAAAGTGAACATGAGATTCAACGATGTCAAGATAGTTATGTGCAAGAACTTTGAATGAC
26 25
TCAAAGCAACA(**G/A**)TA(**G/C**)GATAGGACAATATGTTTATCCATCCTCCCCCTCCTAATTAGAGCAGGATCAAG

CTTTTCCACGTAGTTGGTAGTGAAGACGATAAGCC

>ORF36 (sequencing primer 36R)

ATGGTGGGTAGTGTGAATATTGATCAACAGGGGAAGGGAGGATATGTGTCAGATGAAGTTTTTGGGACATT
1
TTTACCAATAGTTGTGTATTGGGTATATTCAGGATTGTATTTAATGCTTGGGA(**A/C**)TATGGATAATTATAGGTT
2
GCATTCTAAGAAAGA(**T/C**)AGGATGAGAAGAATTTGGTGTCAAAAAAAGAGGTTGTTAAAGGTGTTCTTCTCC
3 4 5 6 7
AACAGATTGTTCAGGCTGCTGTTGCTACCGT(**T/A**)CT(**T/C**)TT(**C/T**)G(**C/G**)GGTTAGTTACTT(**T/C**)GATCATCTC
8 9
TAGTTGCTGCAAAGTTTTG(**A/T**)TTTTTATTTGTTTTGGAGGTTATTATGCATCATGATGA(**A/G**)TTGGATTCATA
10 11 19 20 21 15 16
T(**G/A**)GTTTACTATT(**A/G**)AGG(**T/C**)(**T/G**)(**A/T**)AGATG(**T/G**)TATTGATGTATTATTATGCATATGCTTGTTT(**G/T**)
17 18
(**G/A**)TCTTCT(**C/T**)TTTATGCTCATAATGATATTTATATGGTTAGCTATTGAGGGATATGTTG

>ORF47 (sequencing primers 4F)

ACTTGAAATGATAACAGGTAGGAGATCGATGGACAAGAACCGACCAAATGGGGAGCACAACCTTGTTGA
2
TGGGCACGACCTCATCTTGGTGAAAGAACCATGTAAACAGATTGGTAGATCCTAGACTTGAAGG**(C/G)**CA
3 4
TTCTTCAATAAAAGGTGCTCAGAAAGCTGCACAGTTGGC**(C/T)**GCTCGTTGCCTTAG**(C/T)**GTGATCCCAA
5 6 7
AGCTAG**(G/A)**CC**(T/G)**ATGATGAGTGA**(C/T)**GTGGTTGAAGCCTTGAAGCCATTACCAAATCTTAAAGACA

TGGCCAGCTCATCCTACTATTTCCAGACAATGCAAGCAGACCGAGTTGGATCAAGTCCAAGTACCAAAAA
8
TGGCGTTAGAACACAGGGATCGTT**(C/T)**TCGAGGAATGGACAACAACATCCTAGAAGTCTTTCAATCCCA
9 10
AATGGTTCTCATGCTTCTCCATACCATCAGCA**(G/A)**TT**(C/T)**CCTCAGAACTCACCAAAACCAAACGGCAA

AACTTAGTATTATTGGATTGACAAGTAATCTGTTTCTACCATTCTTTTCGTTTTCTCTCCAGCTATGAATAT

ATTTTGTTGGCCACCTCCCGTTTTGTCGTTGGATGAACTGGCAAAAGGG

>GP21(sequencing primer R2)

ACTATTATGTCTATGAGGAAGTGGTCATAGTTCACCACATTAGTGAGCCAGCATAGCATTACTTGCTTG
1 2 3
CTCATTGACTTTCTAAATGGCCA**(C/T)**GTATTTGA**(T/C)**AGAAAGAACTG**(A/T)**TTTAAAATGCACTGCTCT
9 11 4
AATAGTATCAGAAATCAGCAAATTTGACACACTGAAGTT**(T/C)**GTTTTTGACGAG**(T/C)**ATC**(T/A)**CTGAA
5 12 13 6 7
GTCACTTA**(T/C)**ACTTAAATTAAGTATGAAAGTT**(T/C)**AC**(G/A)**TTT**(A/G)**CTAGTATAAA**(A/T)**CAAATGAG

AGAGACAAAAAATAGATTCAACCTGCAAAAGAAATATAGGGTATTAAACTACTGATGAATTTGTTACTCC
8
(A/G)TTGCTGATAATACATGTTACTTTCAGTCAGAGATTATGAATACAAAAGACTAATAAGAAATTTAAC

ACAGTAGGTGTCTTAGTATATGAAAGATTTAACAGATAGCATAGCTTATATCAGCTCAAGTCAGCCTAAT

TTACTTCATAACGTGGTGGTCGAAGCATCACCCGAGGAAGGCACCAGGATTAAACTTCCTCTATCGCTCA

CCCCACCCACCGGCGCTGGCGGTATAAATGAATTAGTCATTAACCAGGTTTAAGAATCAATGGACATTGA

ATTGTTTCCTTCAAAGCAAATCCGGCTAATTAATTCACAAAAGGAAACCCTGGTTGGTAAAAAAAAAAAA

TAAATTATTAAGGTGCATAAACAATGATTTTTTTTTATGGTGGCGCACAAAATTTTACCTCTCGGGGATA

AGGAGGTTCAGGG

>*St*Pto- sequencing primer F

TCACATTGGATTGGGTGGCTTTGGGAAAGTTTACAGGGGTGTTTTGCGTGATGGAACAAAGGTGGCCCTG

1
AAGAGGTGTAAGCGTGAGTCCTCACAAGGTATTGAAGAGTTCC**(G/A)**AACAGAAATTGAGATTCTCTCTT

2 3 4 5
T**(T/C)**TGCAGCCATCCGCAT**(T/C)**TGGTTTCATTGATAGGATACTGT**(G/C)**ATGAAA**(C/G)**AAATGAGATGA

6 7 8 9
TTCTAGTTTATGACTACAT**(T/G)**GAGAATGGGAACCTCAGGAGCCATTT**(G/A)**TATGG**(C/G)(C/T)**CAGATC

10 11 12
TACC**(C/A)**A**(C/G)**TATGAGCTGGGAGCAGA**(G/C)**GCTGGAGATATGCATCGGGGCAGCCAGAGGTCTGCA

13 14 15 16 17 18
(T/C)TA**(C/T)**CTTCATAC**(T/C)**AGCGCAGTTATACATCGTGA**(T/G)**GTCAAGT**(C/T)**TAT**(A/C)**AACATATT

19 20 21 22 23 24
(G/A)CTTGATGAGAATTTTGT**(G/A)**GCAAAAAT**(G/T)**ACTGA**(T/C)**TTTGGA**(A/C/G)**TA**(T/G)**CCAAGAAAG

25 26 27 28
GGACTGAGCTTGAT**(C/G)**AAACCCATCTTAGCACCCT**(T/A)**GTGCAAGGAA**(C/T)**TAT**(A/G)**GGCTACCT

29 30
(T/G)GACCCTGAATATTTTATACGGGGACAACTGACAGAAAAATCTGATGT**(T/C)**TATTCTTTCGGTGTTGT

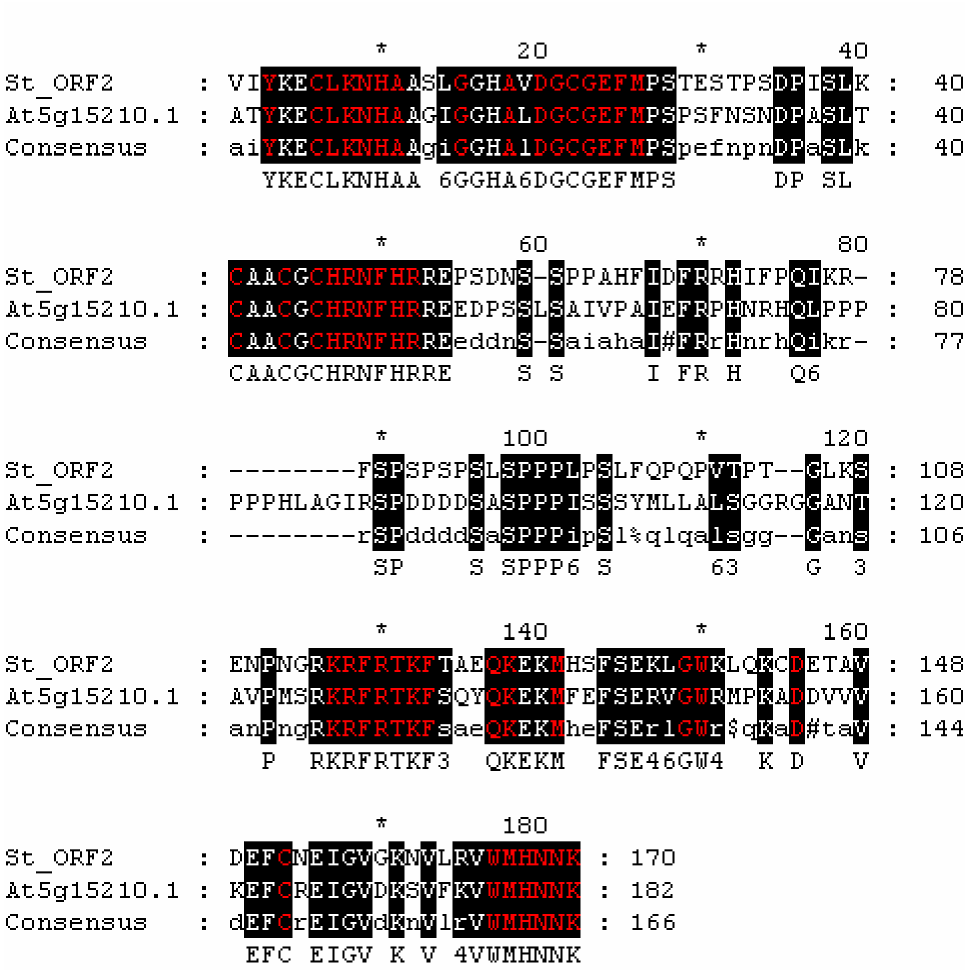
31 32
TTTATT**(C/T)**GAAGTTCTTTGTGCTAGGCCTGCCATAGTTCAATCTCTTCCAAGGGAGATGGTTA**(A/G)**TTT

AGCTGAATGGGCAGTGGACTCG

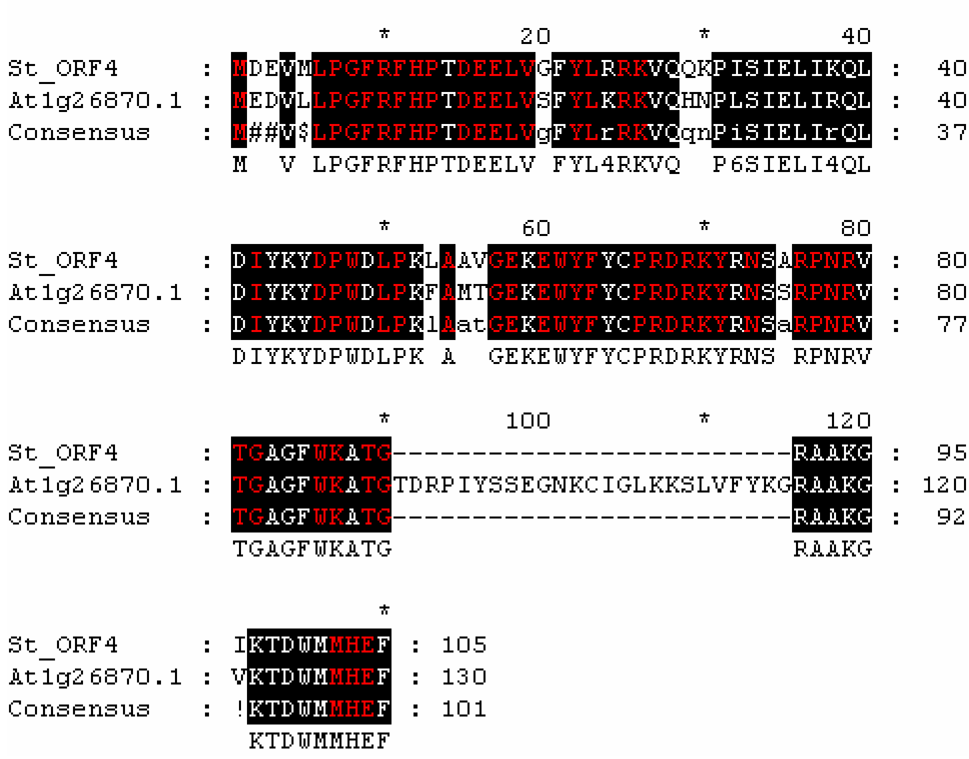
Domain structure of the predicted ORFs

Domain structure of all 14 putative candidate genes is highlighted in red amino acids. The domains were found in TAIR database; where for each gene alignment sequences with other eukaryotic organisms is shown. Only the highly conservative amino acids between all organisms are highlighted. Domain description is shown in Table 3.21, Results. The beginning of the alignment does not start with the start codon (Met) for all ORFs. Sequences for every putative candidate gene from *S.tuberosum* (St) and the corresponding *A.thaliana* (At) sequence are aligned.

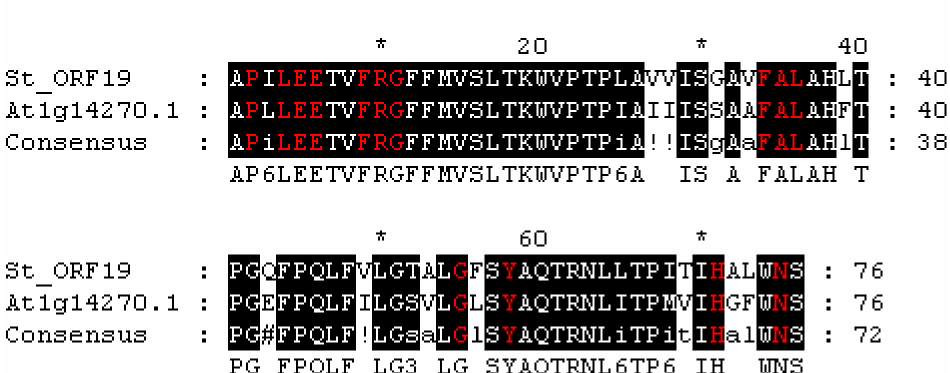
>**ORF2**



>ORF4



>ORF19



>**ORF21**

```
                                      *        20         *        40
St_ORF21     : YTDYTPCQDQKRAMTFPRENMNYRERHCPPQEEKLHCLIP :  40
At1g26850.1  : YTDYTPCQDQRRAMTFPRDSMIYRERHCAPENEKLHCLIP :  40
Consensus    : YTDYTPCQDQrRAMTFPR#nMnYRERHCaP##EKLHCLIP :  37
               YTDYTPCQDQ4RAMTFPR  M YRERHC P  EKLHCLIP

                                      *        60         *        80
St_ORF21     : APKGYVTPFPWPKSRDYVPYANAPYKSLTVEKAIQNWVQY :  80
At1g26850.1  : APKGYVTPFSWPKSRDYVPYANAPYKALTVEKAIQNWIQY :  80
Consensus    : APKGYVTPFpWPKSRDYVPYANAPYKaLTVEKAIQNW!QY :  76
               APKGYVTPF WPKSRDYVPYANAPYK LTVEKAIQNW QY

                                      *       100         *       120
St_ORF21     : EGNVFRFPGGGTQFPQGADKYIDQLASVVPIENGTVRTAL : 120
At1g26850.1  : EGDVFRFPGGGTQFPQGADKYIDQLASVIPMENGTVRTAL : 120
Consensus    : EG#VFRFPGGGTQFPQGADKYIDQLASV!PiENGTVRTAL : 114
               EG VFRFPGGGTQFPQGADKYIDQLASV P6ENGTVRTAL

                                      *       140         *       160
St_ORF21     : DTGCGVASWGAYLWKRNVIAMSFAPRDSHEAQVQFALERG : 160
At1g26850.1  : DTGCGVASWGAYLWSRNVRAMSFAPRDSHEAQVQFALERG : 160
Consensus    : DTGCGVASWGAYLWkRNVrAMSFAPRDSHEAQVQFALERG : 154
               DTGCGVASWGAYLW RNV AMSFAPRDSHEAQVQFALERG

                                      *       180         *       200
St_ORF21     : VPAVIGVLGTIKMPYPSKAFDMAHCSRCLIPWGAADGILM : 200
At1g26850.1  : VPAVIGVLGTIKLPYPTRAFDMAHCSRCLIPWGANDGMYL : 200
Consensus    : VPAVIGVLGTIK$PYPsrAFDMAHCSRCLIPWGAaDGil$ : 192
               VPAVIGVLGTIK PYP34AFDMAHCSRCLIPWGA DG6

                                      *       220         *       240
St_ORF21     : MEVDRVLRPGGYWVLSGPPINWKVNFKAWQRPKEDLEEEQ : 240
At1g26850.1  : MEVDRVLRPGGYWILSGPPINWKVNYKAWQRPKEDLQEEQ : 240
Consensus    : MEVDRVLRPGGYW!LSGPPINWKVN%KAWQRPKEDL#EEQ : 229
               MEVDRVLRPGGYW LSGPPINWKVN KAWQRPKEDL EEQ

                                      *       260         *       280
St_ORF21     : RKIEEAAKLLCWEKISEKGETAIWQKRKDSASCRSAQENS : 280
At1g26850.1  : RKIEEAAKLLCWEKKYEHGEIAIWQKRVNDEACRSRQDDP : 280
Consensus    : RKIEEAAKLLCWEKisEhGEiAIWQKRk#daaCRSaQ##p : 266
               RKIEEAAKLLCWEK  E GE AIWQKR     CRS Q
```

(continuation)

(continuation)

```
                     *       300         *       320
St_ORF21     : AARVCKPSDPDSVCVSGLGCVHIVSILKIKLMADPLFLPL : 320
At1g26850.1  : RANFCKTDDTDDV--------------------------- : 293
Consensus    : aArfCKpdDpDdV--------------------------- : 279
                A  CK  D D V

                     *       340         *       360
St_ORF21     : WFRYNKMEMCITPNNGNGGDES-----LKPFPERLYAVPP : 355
At1g26850.1  : W--YKKMEACITPYPETSSSDEVAGGELQAFPDRLNAVPP : 331
Consensus    : W--YnKMEaCITPnnenggd#e-----LqaFP#RLnAVPP : 310
               W   Y KME CITP              L   FP RL AVPP

                     *       380         *       400
St_ORF21     : RIANGLVSGVSVAKYQEDSKKWKKHVSAYKKINKLLDTGR : 395
At1g26850.1  : RISSGSISGVTVDAYEDDNRQWKKHVKAYKRINSLLDTGR : 371
Consensus    : RIanGl!SGVsVaaY##DnrqWKKHVkAYKrINkLLDTGR : 347
               RI  G  SGV3V  Y  D 4 WKKHV AYK4IN LLDTGR

                     *       420         *       440
St_ORF21     : YRNIMDMNAGLGGFAAALHNPKFWVMNVMPTIAEKNTLGV : 435
At1g26850.1  : YRNIMDMNAGFGGFAAALESQKLWVMNVVPTIAEKNRLGV : 411
Consensus    : YRNIMDMNAGlGGFAAALenqKlWVMNVmPTIAEKNrLGV : 387
               YRNIMDMNAG GGFAAAL   K WVMNV6PTIAEKN LGV

                     *       460         *       480
St_ORF21     : IFERGLIGIYHDWCEAFSTYPRTYDLIHASGLFSLYKDNN : 475
At1g26850.1  : VYERGLIGIYHDWCEAFSTYPRTYDLIHANHLFSLYKNK- : 450
Consensus    : !%ERGLIGIYHDWCEAFSTYPRTYDLIHAngLFSLYK#n- : 423
                 ERGLIGIYHDWCEAFSTYPRTYDLIHA  LFSLYK

                     *       500         *       520
St_ORF21     : LLKIKYGSLRVLWLSPPSFLLFAAVTSYAVIRNCTVLVEI : 515
At1g26850.1  : ---------------------------------CNA---- : 453
Consensus    : ---------------------------------Cna---- : 426
                                                 C

                     *       540         *       560
St_ORF21     : VMPAHILLEMDRILRPEGAVILRDNVDVLIKVKKIIGGMR : 555
At1g26850.1  : ---DDILLEMDRILRPEGAVIIRDDVDTLIKVKRIIAGMR : 490
Consensus    : ---adILLEMDRILRPEGAVIiRD#VDtLIKVKrIIaGMR : 462
                    ILLEMDRILRPEGAVI6RD VD LIKVK4II GMR
```

>ORF22

```
                         *         60          *
St_ORF22     : QPPRPTKTNNKSYGKK-FLARFGCNCYCKD : 69
At2g02950.1  : KKNSNGQIQKVTNNKKSFLANLGCKCACSD : 70
Consensus    : qknrngqi#nksnnKK-FLArlGCnCaCkD : 66
                          3   KK FLA  GC C C D
```

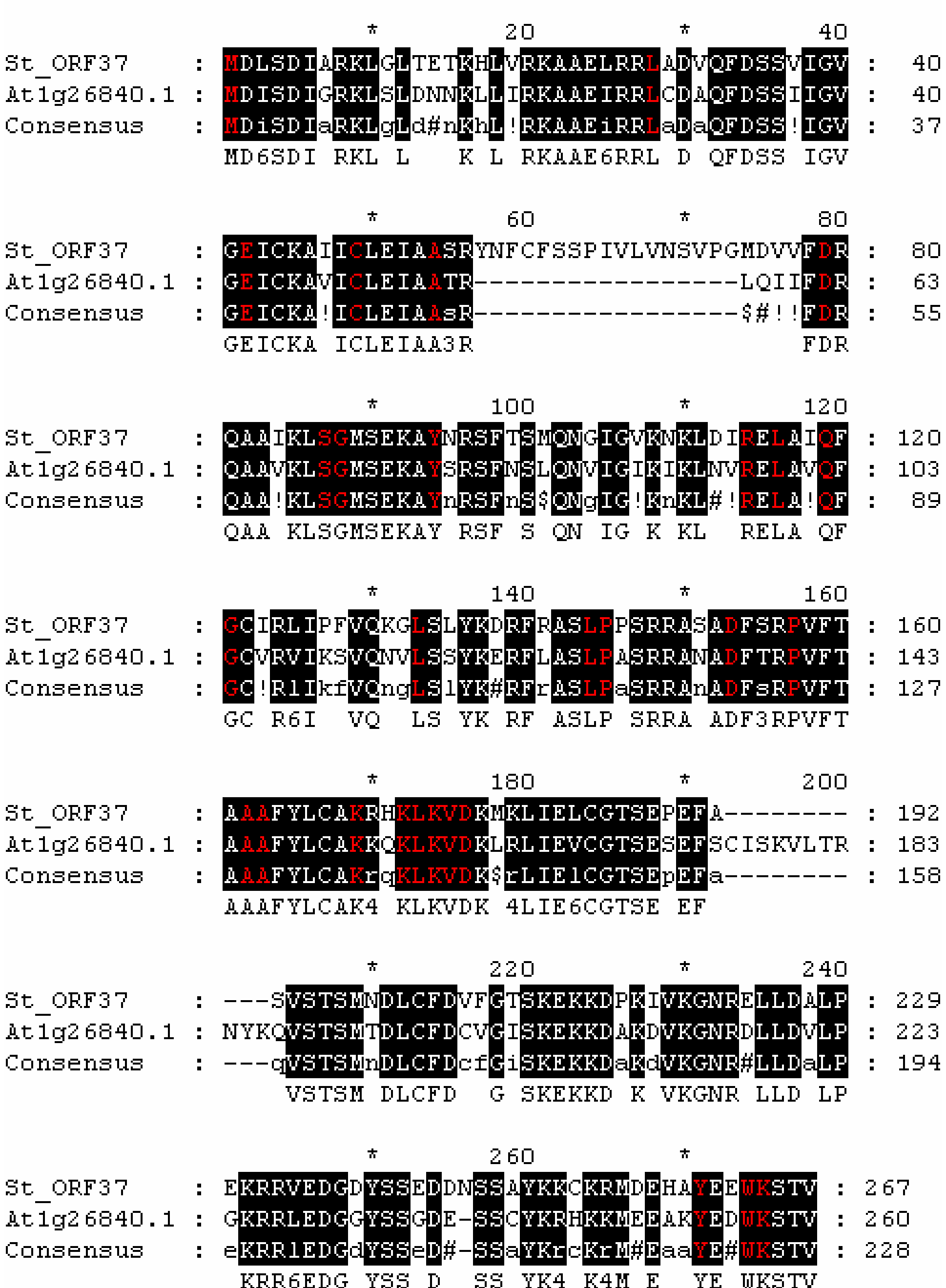
>ORF24

```
                        *        20        *        40
St_ORF24     : YAKIGKAWKRGYLLYGPPGTGKSSMIAAMANFLKYDVYDL : 40
At5g40010.1  : YKKIGKAWKRGYLLFGPPGTGKSTMIAAMANLLEYDVYDL : 40
Consensus    : YaKIGKAWKRGYLL%GPPGTGKSsMIAAMANlLeYDVYDL : 39
               Y KIGKAWKRGYLL GPPGTGKS3MIAAMAN L YDVYDL
```

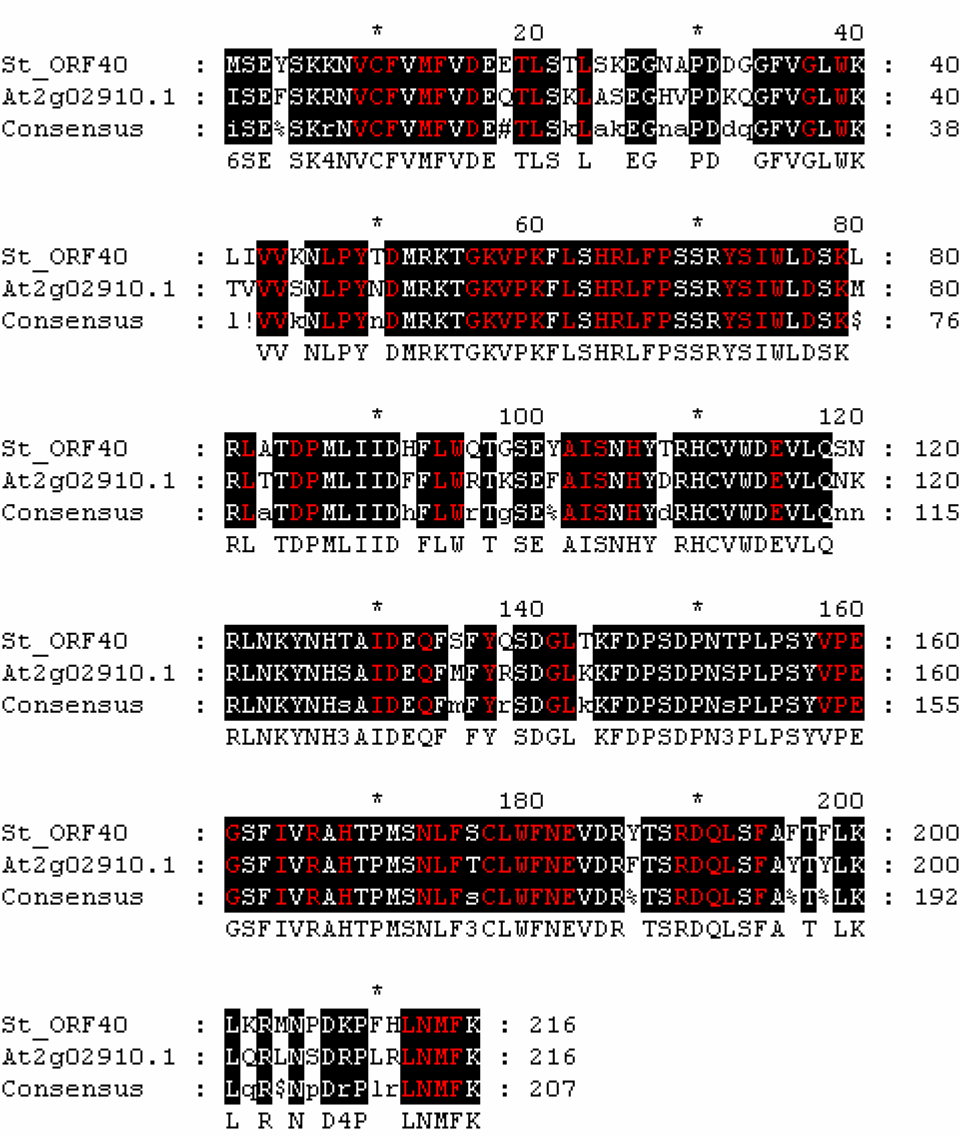
>ORF36

```
                        *        20        *        40
St_ORF36     : LDTWQYFMHRYMHQNKFLYKHIHAQHHRLIVPYAFGALYN :  40
At1g69640.1  : LDTWQYFMHRYMHQNKFLYKHIHSQHHRLIVPYAYGALYN :  40
Consensus    : LDTWQYFMHRYMHQNKFLYKHIHaQHHRLIVPYA%GALYN :  39
               LDTWQYFMHRYMHQNKFLYKHIH QHHRLIVPYA GALYN

                        *        60        *        80
St_ORF36     : HPLEGLILDTIGGALAFLVSGMSPRTSIFFFSFATIKTVD :  80
At1g69640.1  : HPVEGLLLDTIGGALSFLVSGMSPRTSIFFFSFATIKTVD :  80
Consensus    : HPlEGLiLDTIGGALaFLVSGMSPRTSIFFFSFATIKTVD :  79
               HP6EGL6LDTIGGAL FLVSGMSPRTSIFFFSFATIKTVD

                        *       100        *
St_ORF36     : DHCGLWLPGNLFHIFFKNNSAYHDIHHQLY : 110
At1g69640.1  : DHCGLWLPGNLFHMVFKNNSAYHDIHHQLY : 110
Consensus    : DHCGLWLPGNLFHifFKNNSAYHDIHHQLY : 109
               DHCGLWLPGNLFH6 FKNNSAYHDIHHQLY
```

>ORF37



>ORF40



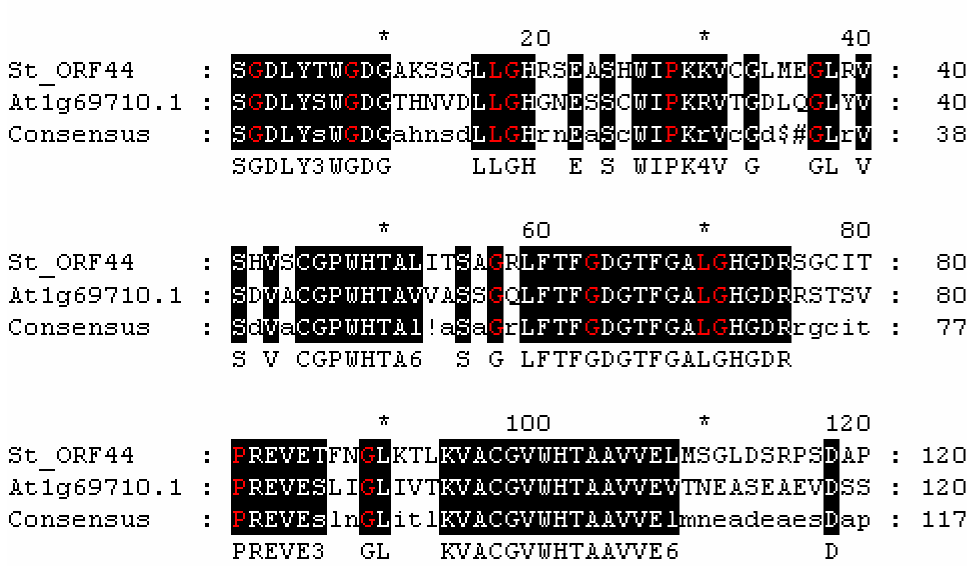
>ORF41

```
                         *        20          *        40
St_ORF41     : TKDRHTKVDGRGRRIRMPATCAARVFQLTRELGHKSDGET : 40
At1g58100.1  : TKDRHTKVDGRGRRIRMPALCAARVFQLTRELGHKSDGET : 40
Consensus    : TKDRHTKVDGRGRRIRMPAlCAARVFQLTRELGHKSDGET : 40
               TKDRHTKVDGRGRRIRMPA CAARVFQLTRELGHKSDGET

                         *
St_ORF41     : IEWLLQQAEPA : 51
At1g58100.1  : IEWLLQQAEPA : 51
Consensus    : IEWLLQQAEPA : 51
               IEWLLQQAEPA
```

>ORF43

```
                         *        20          *        40
St_ORF43     : VSLVYPLYASIKAIETKSRADDRQWLTYWVLYSLITLFEL : 40
At1g74520.1  : VSLVYPLYASVQAIETQSHADDKQWLTYWVLYSLLTLIEL : 40
Consensus    : VSLVYPLYAS!qAIETqSrADDrQWLTYWVLYSLiTLiEL : 39
               VSLVYPLYAS  AIET S ADD4QWLTYWVLYSL6TL EL

                         *        60          *
St_ORF43     : SFSKLIEWFPIWSYAKLGAICWLVLPYFNGACYVYE : 76
At1g74520.1  : TFAKLIEWLPIWSYMKLILTCWLVIPYFSGAAYVYE : 76
Consensus    : sFaKLIEWlPIWSYaKLgaiCWLViPYFnGAaYVYE : 75
               3F KLIEW PIWSY KL   CWLV6PYF GA YVYE
```

>ORF44



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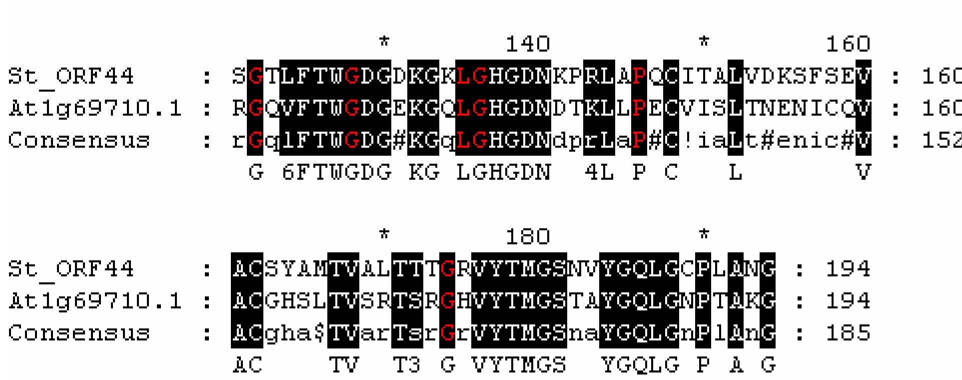
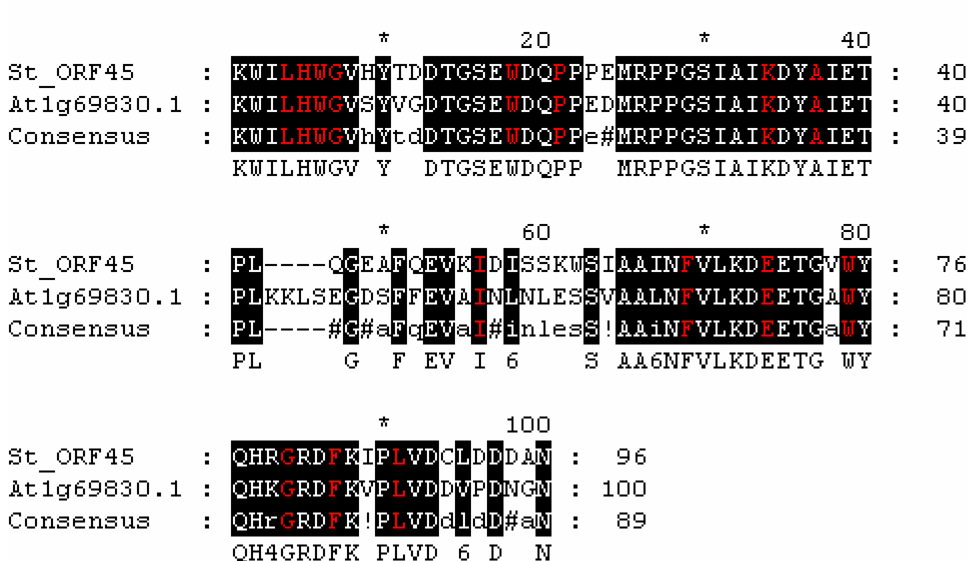
(continuation)



>ORF45



>ORF47

```
                          *        20         *        40
St_ORF47     : PESLLGEGGFGCVFKGWIEENGTAPVKPGTGLTVAVKTLN :  40
At5g15080.1  : PESLLGEGGFGCVFKGWIEENGTAPVKPGTGLTVAVKTLN :  40
Consensus    : PESLLGEGGFGCVFKGWIEENGTAPVKPGTGLTVAVKTLN :  40
               PESLLGEGGFGCVFKGWIEENGTAPVKPGTGLTVAVKTLN

                          *        60         *        80
St_ORF47     : HDGLQVLSIWQAEVNFLGDLVHPNLVKLIGYCIEDDQRLL :  80
At5g15080.1  : PDGLQGHKEWLAEINFLGNLLHPNLVKLVGYCIEDDQRLL :  80
Consensus    : hDGLQghkeWqAE!NFLG#LlHPNLVKL!GYCIEDDQRLL :  77
                DGLQ     W AE NFLG L6HPNLVKL GYCIEDDQRLL

                          *       100         *       120
St_ORF47     : VYEFMPRGSLENHLFRRSMPLPWSIRMKIALGAAKGLAFL : 120
At5g15080.1  : VYEFMPRGSLENHLFRRSLPLPWSIRMKIALGAAKGLSFL : 120
Consensus    : VYEFMPRGSLENHLFRRS$PLPWSIRMKIALGAAKGLaFL : 116
               VYEFMPRGSLENHLFRRS PLPWSIRMKIALGAAKGL FL

                          *       140         *       160
St_ORF47     : HEEAERPVIYRDFKTSNILLDADYNAKLSDFGLAKDGPEG : 160
At5g15080.1  : HEEALKPVIYRDFKTSNILLDADYNAKLSDFGLAKDAPDE : 160
Consensus    : HEEAerPVIYRDFKTSNILLDADYNAKLSDFGLAKDaP#e : 155
               HEEA 4PVIYRDFKTSNILLDADYNAKLSDFGLAKD P

                          *       180         *       200
St_ORF47     : DKTHVSTRVMGTYGYAAPEYVMTGHLTSKSDVYSFGVVLL : 200
At5g15080.1  : GKTHVSTRVMGTYGYAAPEYVMTGHLTSKSDVYSFGVVLL : 200
Consensus    : dKTHVSTRVMGTYGYAAPEYVMTGHLTSKSDVYSFGVVLL : 195
                KTHVSTRVMGTYGYAAPEYVMTGHLTSKSDVYSFGVVLL
```

ERKLÄRUNG

“Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von PD Dr. Christiane Gebhardt betreut worden.”

Köln, den 11.11.2005 Evgeniya Valentinova Ilarionova

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