Isolation and characterization of potato homologues of *Arabidopsis thaliana* genes operating in defense signal transduction

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Dedicated to my Parents

Małgorzata and Zbigniew Pajerowscy

Abbreviations and Acronyms

Amp	ampicillin
ATP	adenosine 5-triphosphate
At	*Arabidopsis thaliana*
Avr	avirulence
BAC	Bacterial Artificial Chromosome
bp	base pair
c	centi
Carb	carbenicillin
cDNA	complementary DNA
CHORD	cysteine- and histidine- rich domain
Col-0	Columbia 0
CS	CHORD and SGT1 motif
cv.	cultivar
^{o}C	degree Celsius
ddH_2O	deionised and distilled water
db	database
DEPC	diethylpolycarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
dsRNAi	double-stranded RNA interference
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
EtBr	ethidium bromide
EtOH	ethanol
g	gram
g	gravity constant
Gent	gentamycin
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour(s)
hpi	hours post inoculation
hpRNA	hairpin RNA
HR	hypersensitive response

HSP90	heat shock protein 90
ihpRNA	intron-containing hairpin RNA
InDel	insertion/deletion polymorphism
ISR	induced systemic resistance
JA	jasmonic acid
Kan	kanamycin
kb	kilobase(s)
kDa	kilodalton (s)
L	litre
LB	Luria-Bertani medium
LRR	leucine-rich repeat
LZ	leucine-zipper
m	mili
M	molar
MAPK	Mitogen-Activated Protein Kinase
min	minute(s)
mmol	milimolar
MeJA	methyl jasmonate
mRNA	messenger ribonucleic acid
nahG	salicylcate hydroxylase
NaOH	sodium hydroxide
NBS	nucleotide binding site
ng	nanogram
Nb	*Nicotiana benthamiana*
Nt	*Nicotiana tabacum*
OD	optical density
O/N	over night
OPDA	12-oxo-phytodienoic acid
ORF	open reading frame
Os	*Oryza sativa*
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDF1.2	Plant Defensin 1.2
pH	negative decimal log. of the H^+ concentration
PR	pathogenesis related
PTGS	post-transcriptional gene silencing
pv.	pathovor

QTL	Quantitative Trait Locus
QRL	Quantitative Resistance Locus
R	Resistance
RGA	Resistance Gene Analog
Rif	rifampicin
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SCF	SKP1/CULLIN/F-box protein
SDS	sodium dodecyl sulphate
s	second(s)
SKP1	suppressor of kinetochore protein
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SNP	Single Nucleotide Polymorphism
smGFP	soluble modified GFP
SSC	sodium chloride-sodium citrate buffer
St	*Solanum tuberosum*
ssp.	subspecies
T-DNA	transfer DNA
TIR	*Drosophila* Toll and human interleukin-1 receptor
TRIS	Tris-(hydroxymethyl)-aminomethane
U	unit
UTR	untranslated region
UV	ultraviolet
V	Volt
VIGS	virus induced gene silencing
%(v/v)	volume-percent
%(w/v)	weight-percent
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abbreviations for nucleic acids

Abbreviation	Base
A	Adenine
C	Cytosine
G	Guanine
T	Tymidine
U	Uracyl

Abbreviations for amino acids

One-letter-code	Three-letter-code	Full Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophane
Y	Tyr	Tyrosine

1. INTRODUCTION

1.1. The potato

Potato (*Solanum tuberosum* L.) originated in the highlands of South America, where it has been consumed for more than 8000 years. The first potato used by humans was the wild coastal potato, *Solanum maglia*, which is the ancestor of those still found in the moister river valleys along the north-central Peruvian coast. Spanish explorers brought the plant to Europe in the late 16th century as a botanical curiosity. By the 19th century it had spread throughout the continent, providing cheap and abundant food.

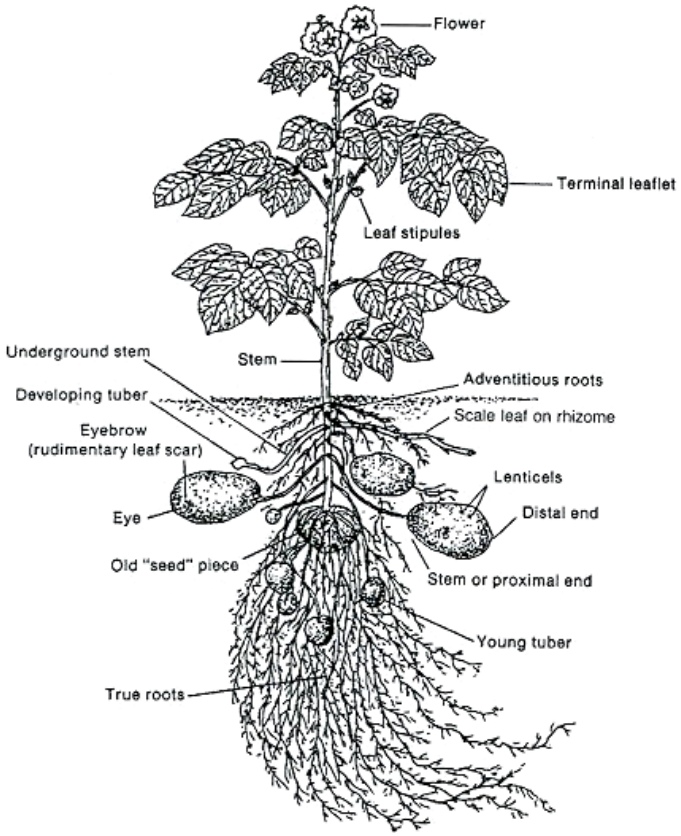


Fig. 1. Diagram of a potato plant. For simplicity, one main stem is shown. Productive plants may have two or more main stems. The stolons and tubers arise from the stem tissue, and the true roots arise at the base of the stem (Courtesy of Alberta, Canada, Food and Rural Development, http://www.gov.mb.ca/agriculture/crops)

Potato is propagated vegetatively. The shoots develop from the "eyes" of a "seed" tuber. The new tuber generation is formed at the tips of underground stolons which are adventitious shoots formed at the base of the stem (Fig. 1).

The potato is, in terms of quantity, after rice, wheat and corn fourth on the list of the crop species that are most important for the human nutrition worldwide. More than one-third of the global potato output now comes from developing countries, comparing to just 11 percent in the early 1960s. According to the latest FAO data (FAOSTAT, September 2005), potato production worldwide stands at 327 million tons and covers more than 18 million hectares. China is now the world's largest potato producer (yearly 70 million tons). Although potato production in Europe has fallen since the early 1960s (now 140 million tons), this decline has been more than offset by the growth in Asia, Africa and Latin America, thereby explaining the rise in global potato tonnage.

1.2. The potato genome and transcriptome

One complement of the potato genome consists of twelve chromosomes with estimated DNA content in the order of 1 x 10^9 base pairs. Cultivated potato is a tetraploid plant having, therefore, four genome complements and 48 chromosomes. The four homologous chromosomes pair and segregate independently (tetrasomic inheritance). An allele at any given genetic locus can occur in four allelic states: simplex (present one time at the locus), duplex (present two times), triplex (present three times) and quadruplex (present four times, homozygous state). Therefore, at any given genetic locus, up to four different alleles are possible. However, the majority of wild potato species are diploid. At the diploid level, potato genetics directly corresponds to human genetics: both are self-incompatible, outcrossing species.

Recently, significant progress has been made in the potato genome sequencing initiative. The **P**otato **G**enome **S**equencing **C**onsortium (PGSC, http://www.potatogenome.net/) aims to elucidate the complete DNA sequence of the potato genome by the end of year 2008. This sequencing initiative will be carried out by a number of international partners, currently including the Netherlands, China,

Canada, India, New Zealand, Poland, United Kingdom, USA and Brazil. The Dutch potato genotype RH, a parent of the potato Ultra-High Density mapping population, was chosen for the genome sequencing (Van Eck et al. 2005). Physical maps of all the twelve chromosomes are available for this genotype and two BAC (Bacterial Artificial Chromosome) genomic libraries were generated, with an average insert size of ~130 kb. Currently, the minimal tiling paths for all the chromosomes are being constructed (Van Eck et al. 2005). The availability of the complete sequence of potato DNA, currently estimated to be of 840 MB in size, will enable potato breeders and related industries to fully exploit the genetic potential of this important field crop.

In parallel, a high-throughput generation of potato Expressed Sequence Tags (ESTs), coordinated by the two major consortia: the Institute of Genomic Research (http://www.tigr.org/tdb/potato/) and the Solanaceae Genomics Network (http://www.sgn.cornell.edu/), has been initiated. Currently, vast collections of potato ESTs are available (approximately 190,000 and 135,000 sequences in TIGR and SGN database, respectively) (September 2005), generated from various potato tissues, both healthy and *P. infestans* challenged (compatible and incompatible interactions).

In addition, the Solanaceae Gene Expression (SGE) (http://www.tigr.org/tdb/potato/SGED_index2.shtml) offers a ~12,000 clones potato cDNA microarray for the transcriptional profiling. Experiments already performed are made publicly available through the Solanaceae Gene Expression database.

1.3. The major potato diseases

Numerous diseases of potato have been reported up to date. The 2nd edition of *Compendium of Potato Diseases* (German 2001) lists approximately 60 diseases caused by bacteria, fungi, nematodes, viruses, viroids, and phytoplasmas. Late blight disease, caused by an oomycete *Phytophthora infestans* (Mont.) De Bary is the most important disease of potatoes on a worldwide basis. Another serious threat to the potato yields is *Erwinia carotovora* subspecies *atroseptica* (Eca) (van Hall) Dye, also

called *Pectobacterium atrosepticum*, a bacterium that infects potato in temperate regions. Eca is the most damaging bacterial pathogen of potatoes worldwide.
Control of the potato diseases is in the focus of modern farming technologies; for some pathogens (e.g. Eca) control depends largely on sanitation. However, for the majority of diseases chemical sprayings remain the most efficient means of protection.
At the same time, the toxicity of chemical fungicides, the persistence of their breakdown products and the frequency with which they are sprayed poses health and environmental risks that are of concern. Particularly worrisome are the potential effects on reproduction and increased cancer risk triggered by some of the synthetics. Moreover, the prevention efficiency continuously decreases as the fungicide resistant *P. infestans* populations are building up with the use of some chemicals (Rommens and Kishore 2000).

Importantly, the vegetative propagation makes potato cultivation particularly vulnerable to diseases, because, in contrast to propagation by true seeds, infected plants will transmit the disease to the next tuber generation.

1.3.1. *Phytophthora infestans* and the late blight disease

Phytophthora infestans is an oomycete (Phylum *Oomycota*, Order *Peronosporales*, Family *Pythiaceae*), the causal agent of the late blight disease of potato and tomato plants. *Phytophtora infestans* is distributed worldwide, with the A2 mating type being the most popular and having significant impacts on disease severity and incidence (Grünwald and Flier 2005).

The late blight disease is famous for the destruction of the potato crop in Ireland in the 1840s and the resulting famine and death of over a million people. Today, epidemics still recur in disease favorable environments (Fig. 3).

The development of leaf rots or lesions varies with environmental conditions. The relative humidity, temperature, light intensity, and the kind of potato cultivar (resistant, susceptible) all affect symptom development. Leaf lesions begin as small, irregularly

shaped, light green to gray spots. In cool (20ºC or less), moist (95-100% relative humidity) environments, lesions expand rapidly to form large black rots (blights), spreading throughout the leaf, into petioles and finally the stem of the plant. Apical tips, flowers, and young stems are very also susceptible (Fig. 2A). Large sections of the plant are rotted and plants become eventually killed.

Fig. 2. The late blight symptoms development on aerial parts (A) and tubers (B) of the potato plant.

Rots of the tuber also develop (Fig. 2B) and are characterized by slightly sunken areas having a brown to purplish skin. The infected tuber areas are dark, reddish brown and 5 to 15 mm deep. Secondary fungal and bacterial invasion frequently occurs and the entire tuber decays.

In temperate climate areas, soil or plant debris are believed to harbor the pathogen between seasons. The oomycete will also survive in infected tubers that remain in soil from the previous season. In addition, the “seed potatoes” pieces can be infected and harbor the pathogen. When new shoots are produced from infected “seed” pieces or older tubers, the oomycete infects, and then sporulates on this new growth. The sporangia are then spread through the air or by water. When the environment is highly conducive for disease, sporangia are airborne and spread for large distances. Sporangia land on healthy foliage, zoospores are formed, released and start to germinate in the presence of moisture. The germ tube formed by zoospores penetrates the leaf epidermis at multiple sites causing small spots. These small rots expand rapidly and

leaf spots and blights (large rots) develop. At close to 100% relative humidity, *Phytophthora infestans* produces sporangia in abundance on the surface of the leaves. Sporangial masses are white on dark colored leaf lesions. These are blown to healthy plants and continue the disease cycle. Still, the most predominant way of disease prevention are the chemical spray programs, implemented before disease is observed, especially during cool and wet periods. Once foliar infection develops, epidemics can become uncontrollable (Shattock 2002).

Two major types of late blight resistance are recognized in potato: 1) specific resistance (also called vertical, race specific, or monogenic resistance), and 2) general resistance (also called horizontal, race nonspecific, or polygenic resistance). Specific resistance was discovered in the *R*-genes from *Solanum demissum* and potato breeders incorporated those into new cultivars. Unfortunately, *Phytophthora infestans* is highly variable and all new cultivars with specific resistance eventually succumb to this pathogen. However, some cultivars with moderate levels of general resistance are available.

Although potato yields are more affected by absence of rain than by the presence of the oomycete, nevertheless, if blight prevention and control are neglected, the potential for blight epidemics increases, threatening the crop's yield. Early defoliation resulting from a neglected blight attack translates into a high percentage of small, undeveloped potato tubers.

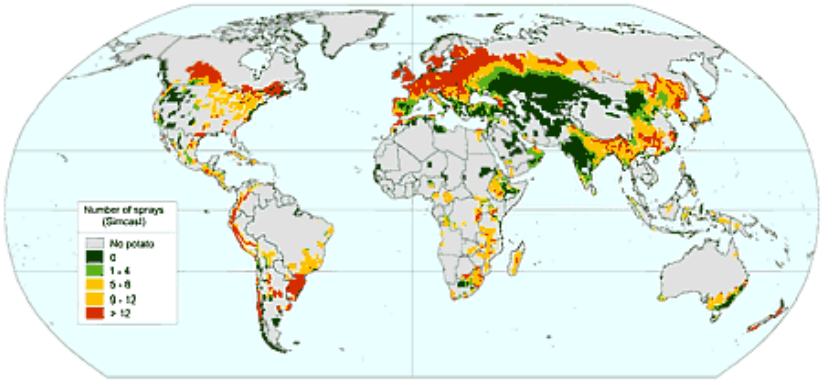


Fig. 3. Distribution map of *Phytophthora infestans* and intensities of fungicide sprayings. Source: The CIP webpage (http://www.cipotato.org)

1.3.2. *Erwinia carotovora* ssp. *atroseptica.* The blackleg/soft rot disease

Erwinia carotovora subsp. *atroseptica* is an important bacterial plant pathogen, causing soft rot and blackleg in potato. As a member of the *Enterobacteriaceae*, it is related to *Escherichia*, *Shigella*, *Salmonella* and *Yersinia*. *E. c. atroseptica* is associated mostly with potatoes and currently, together with *Ralstonia solanacearum*, is the most devastating bacterial pathogen on cultivated *S. tubesosum*. The bacteria do not survive well in soil for more than one year, unless they are contained on or within diseased tubers or other potato plant debris. The blackleg disease is usually caused by *E. c. atroseptica* carried on contaminated tubers. Most lots of "seed" potato tubers are contaminated to some degree, but the bacteria are usually dormant and do not cause disease unless environmental conditions are favorable. The aerial stem rot is usually caused by *E. c. atroseptica* persistent in infested soil or introduced to the crop by irrigation water, wind-blown rain, and insects. Development of the disease is largely controlled by favorable moisture and temperature conditions (Toth et al. 2003a, 2003b).

The soft rot Erwinia appears to be an opportunistic pathogen, surviving and growing both (epiphytically) on and (endophytically) within the plant. Blackleg symptoms can occur at several stages of plant development. The disease often develops after plants are well grown up or even in flower. The stem bases of infected plants typically show

a black to light-brown decay that can extend up the stem. Leaves of infected plants tend to roll upward at the margins, become yellow, wilt, and consequently die. Stem infection (aerial blackleg) can occur through wounds or through natural openings of leaves. Lesions on diseased stems first appear as irregular brownish to inky-black areas. These enlarge into a soft, mushy rot that causes entire stems to wilt and die. Potato tubers with soft rot have tissues that are very soft and watery, having a slightly granular consistency. The diseased tissue is cream- to tan-colored, and often has a black border separating diseased from healthy areas. In the early stages, soft-rot decay is generally odorless, but later a strong odor usually develops as secondary decay bacteria invade infected tissues. Most internal tuber tissues may be consumed by soft rot organisms, sometimes leaving only a shell of skin remaining in the soil.

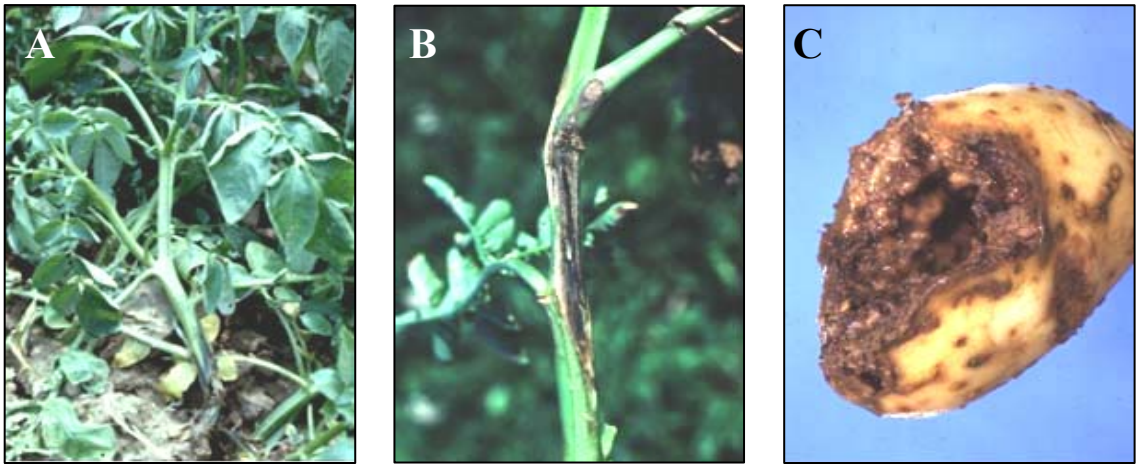


Fig. 4. A. Inky black to light-brown stem decay of blackleg originating from the potato tuber piece and extending above ground through up the stem, accompanied by a general wilting of the entire plant. B. Irregular, brownish or black, soft stem lesions characteristic of blackleg on potato stems. C. Potato tuber mostly consumed by soft rot bacteria, resulting in a soft cream- to tan-colored decay (http://ohioline.osu.edu).

Recently, the genome sequencing project of *Erwinia carotovora* subsp. *atroseptica* strain SCRI1043 (ATCC BAA-672) has been completed (Bell et al. 2004). The genome is 5.064 Mb in length with a G+C content of 50.97%. This opened a new

chapter, facilitating a better insight into the bacterial virulence activities, by application of numerous genetic and biochemical approaches.

1.4. The genetic map of potato

Currently, the potato map is one of the most highly marker-saturated maps among the crop plant species. Initially, two potato maps were constructed concurrently using RFLP markers on different genetic backgrounds (Bonierbale et al. 1988, Gebhardt et al. 1989). These maps were then compared with each other and also aligned with the tomato RFLP map (Gebhardt et al. 1991, Tanksley et al. 1992). With the development of new molecular markers, the potato map was enriched, and at the moment it is based on more than 350 markers, which cover approximately 90% of the potato genome (Gebhardt and Valkonen 2001), making it a valuable tool for localizing genes that control the expression of useful traits.

The existence of highly saturated potato maps allowed to localize many genes on the 12 potato chromosomes, and markers linked to these genes can be used to perform positive marker-assisted selection. The first gene to be localized on the map was *Gro1* (Barone et al. 1990, Paal et al. 2004), a gene that confers resistance to pathotype *Ro1* of the nematode *Globodera rostochiensis*. The mapping work was performed on a diploid F_1 progeny (100 genotypes) using 58 RFLP markers (Barone et al. 1990).

Many other genes have since been mapped, including the self-incompatibility gene (Gebhardt et al. 1991), three genes for flower color determination (Van Eck et al. 1993), and several single loci controlling tuber traits such as skin color (Gebhardt et al. 2001), flesh color (Bonierbale et al. 1988), or tuber shape (Van Eck et al. 1994).

However, the majority of mapping work was dedicated to various resistance genes. Currently, there are 22 single dominant resistance genes (*R* genes) localized on 10 chromosomes. The position of 20 *R* genes on the potato map was reviewed in 2001 by Gebhardt and Valkonen. Two more resistance genes have since been mapped: the gene *Rpi1* from *S. pinnatisectum* conferring resistance to *Phytophthora infestans* and

mapped on chromosome VII (Kuhl et al. 2001) and the gene *Ns* for resistance to PVS, that is localized on chromosome VIII (Marczewski et al. 2002).

1.5. Quantitative Trait Loci (QTL)

The majority of physiological characters, like disease resistance, show continuous or quantitative phenotypic variation because phenotypic expression of the character is controlled by more than one gene, and in addition can be largely regulated by environmental influences. The precise number of the genes involved is usually not known. The loci where such genes are located in the genome are called "quantitative trait loci" (QTL, Gelderman 1975). DNA markers with Mendelian segregation patterns and linkage maps based on them have made possible for the first time the dissection and mapping of QTL for quantitative characters.

Structure and function of the proteins encoded at QTL are unknown. It is assumed, however, that DNA polymorphisms must exist in the gene(s) which are responsible for the observed QTL effect. These DNA polymorphisms are the molecular basis for phenotypic selection of superior genotypes through breeding. In contrast to many mutations with drastic effects on the phenotype, the molecular and functional variability present in genes controlling QTL operates under field conditions and should not have, therefore, severe effects on fitness.

Some genes that control quantitative resistance traits or yield and tuber quality were mapped as QTLs. Among QTLs involved in resistance to biotic stresses, the first mapped were those conferring resistance to insects (Leonards-Schippers et al. 1994, Yencho et al. 1996). Afterwards, other QTLs for resistance to various pathogens were mapped, involved in resistance to *Phytophthora infestans*, *Erwinia carotovora*, and *Globodera* spp. as reviewed by Gebhardt and Valkonen (2001), and to Potato Leafroll Virus (Marczewski et al. 2001). As for tuber characteristics (size, shape, starch content, etc.), these are mostly polygenic traits and a lot of mapping work has been carried out by various researchers to localize the related QTLs on the potato map, using different segregating progenies and marker systems (Chen et al. 2001).

More recent technical progress in the area of molecular biology and genomics have made possible the cloning of QTLs, i.e. the identification of the DNA sequences (coding or non-coding) responsible for QTL effect. Although the identity of the gene(s) present at a QTL is not known, they may correspond to genes that have already been identified and characterized when studying the phenotype (for example, resistance to pathogens) at the physiological, biochemical and/or molecular level. The candidate genes approach plays an increasingly important role in the search for loci underlying the QTL effect. Genes are candidates for participating in the control of a quantitative character when they fulfill the following criteria (Pflieger et al. 1997a):

A) The gene operates in a metabolic network related to the quantitative character, based on knowledge in biochemistry, physiology and/or molecular biology.

B) The gene is genetically linked to a QTL having an effect on the quantitative trait studied.

C) Specific molecular variants (alleles) of the gene are present in different frequencies in samples of plants which have significantly different mean values for the quantitative trait.

So far, twelve plant-derived Quantitative Trait Loci have been cloned and described in the literature (listed in Table 1).

Species	Trait	QTL	Gene	Function	Molecular identification	Candidate gene[a]	R^2 (%)[b]	Plants (no.)[c]	Resolution (kb)[d]	ORF (no.)[e]	Identification of QTN	Functional proof
Arabidopsis	Flowering time	*ED1*	*CRY2*	Crypt.	Pos. cloning	Yes (L)	28–56	1822	45	15	Amino acid substitution	Transformation
		FLW	*FLM*	TF	Pos. cloning	Yes (E)	27	NA	138	38	Deletion of whole gene	Transformation
	Gluc. structure	*GS-elong*	*MAM*	MAM synthase	Pos. cloning	Yes (E)	NA	4600	NA	NA	Nucleotide and gene indels	No
	Root morphology	*BPX*	*BRX*	TF	Pos. cloning	No	80	860	45	10	Premature stop codon	Transformation
Maize	Plant architecture	*Tb1*	*Tb1*	TF	Transp. tagging	Yes (E)	17–31	NA	NA	NA	No, possibly regulatory	Complementation
Rice	Heading time	*Hd1*	*Se1*	TF	Pos. cloning	Yes (L)	67	1505	12	2	No	Transformation
	Heading time	*Hd3a*	*Hd3a*	Unknown	Pos. cloning	Yes (L)	NA	2207	20	4	No	Transformation
	Heading time	*Hd6*	*αCK2*	Protein kinase	Pos. cloning	No	NA	2807	26	1	Premature stop codon	Transformation
	Heading time	*Ehd1*	*Ehd1*	B-type response regulator	Pos. cloning	No	NA	>2500	16	3	Amino acid substitution	Transformation
Tomato	Fruit sugar content	*Brix9-2-5*	*Lin5*	Invertase	Pos. cloning	Yes (L)	NA	7000	0.5	1	Amino acid substitution	Complementation
	Fruit shape	*Ovate*	*Ovate*	Unknown	Pos. cloning	No	48–67	3000	55	8	Premature stop codon	Transformation
	Fruit weight	*fw2.2*	*ORFX*	Unknown	Pos. cloning	No	30	3472	92	4	Unknown regulatory variant	Transformation

Table 1. Summary of the main characteristics of the QTLs cloned in plants (cited after Salvi and Tuberosa 2005), (Cong et al. 2002, Doebley et al. 1995, 1997, Doi et al. 2004, El-Din El-Assal et al. 2001, Frary et al. 2000, Fridman et al. 2000, 2004, Kojima et al. 2002, Kroymann et al. 2003, Mouchel et al. 2004, Takahashi et al. 2001, Werner et al. 2005, Yano et al. 2000).
Abbreviations: crypt., cryptochrome; gluc. structure, glucosinolate structure; MAM synthase, methylthioalkylmalate synthase; NA, not applicable or not available; ORF, open reading frame; pos. cloning, positional cloning; QTL, quantitative trait locus; QTN, quantitative trait nucleotide; TF, transcription factor; transp. tagging; transposon tagging.

[a]Evidence for candidate gene: (E) indicates early evidence, after primary QTL analysis; (L) indicates late evidence, after physical mapping and/or sequencing.
[b]Proportion of phenotypic variance explained by the QTL in the primary cross.
[c]Dimension of the population used for fine mapping.
[d]DNA physical interval completely linked with the QTL.
[e]Number of ORFs completely linked with the QTL.

Majority, except just one of these QTL, were isolated using the positional cloning method. However, for seven out of twelve, the candidate gene approach was applied in the later stages of cloning, after sufficient narrowing down of the chromosomal region harboring putative QTL.

1.6. The application of *A. thaliana* model system for crops improvement

Over the last years, plant functional genome research has been driven mainly through the model organism *Arabidopsis thaliana*. Nowadays, the trends in plant genomics are mostly oriented towards development of enhanced productivity, quality, improved pests resistance and sustainability of the food production systems.

Development, reproduction, stress responses and other fundamental physiological processes are expected to be controlled by similar molecular mechanisms in all flowering plants. However, most of them are not well accessible to direct functional analysis, due to large and complex genomes, long living cycles, limited collections of sequenced genes, etc. Even though Arabidopsis is a wild, non-commercial member of mustard family, yet it has several properties which promote it as an ideal system for plant research. The small size (approximately 15-20 cm tall) and short life cycle (about 6-8 weeks) makes research possible in limited space and time. It naturally self-pollinates, but cross-pollination can be performed, as well (Redei 1975). Uncomplicated *A. tumefaciens* – mediated transformation by the inflorescence-dipping method is well established and efficient. Each plant readily produces up to ten thousands of seeds and the large progeny size allows reliable statistical analyses of segregation ratios for extensive genetic experiments. It has a small, almost completely sequenced genome, consisting of five chromosome pairs (about 125Mb of DNA excluding telomeric, nucleolar and centromeric tandem repeats) (Bevan et al. 2001). A relatively small amount of repeated sequences (approximately 10% of the total genome) has been reported by Dean and Schmidt (1995).

The *Arabidopsis* Genome Initiative (2000), by completing the sequencing of 115.4 megabases of the *A. thaliana* genome, and annotating the genes functions, contributed a valuable tool allowing a deeper insight and a better understanding of complex plant physiological processes. The powerful model system *Arabidopsis thaliana* can be, therefore, used for the identification of structural and functional gene orthologues and unravelling gene function in other plant species (Glazebrook et al. 1997).

1.7. Defense responses in *Arabidopsis thaliana*

Many genes functional in pathogenesis and defense have been already identified in *Arabidopsis*. A set of major resistance genes, conferring resistance to model pathogens of *Brassicaceae*, such as *Pseudomonas syringae* and *Peronospora parasitica*, and their downstream effectors involved in signal transduction and expression of defense reactions have been discovered and complex cross-talk mechanisms between different components of resistance have been elucidated (Glazebrook 1999, Glazebrook 2001, Parker 2000) (Fig. 5). In addition, the results of research reported over the past few years offer clear evidence that *Arabidopsis thaliana* can serve as a rich source of candidate resistance genes, which are supposed to have direct structural, and possibly also functional equivalents in other flowering plants (Martin 1999).

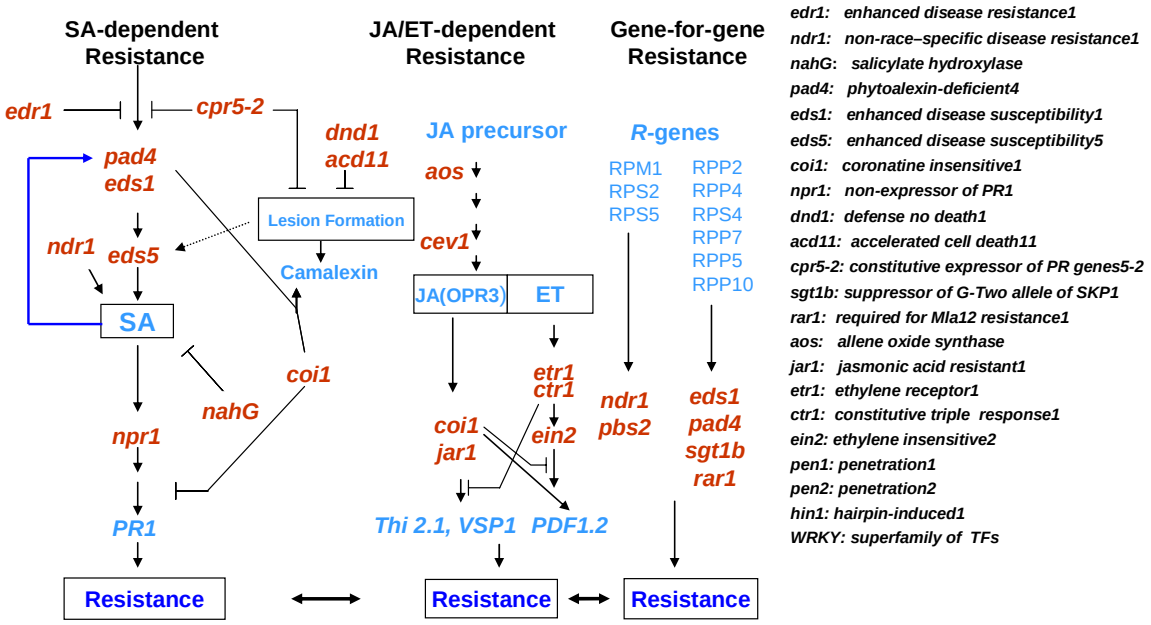


Fig. 5. Model for the Arabidopsis signal transduction network in activation and fine-tuning of defense responses (after Glazebrook 2001, modified).

Knowledge gained from *Arabidopsis* model system can be then extrapolated to search for potato orthologs (i.e. homologous genes with a common ancestor that have been separated during speciation) involved in certain pathways of defense responses. This

could promote the genetic engineering of resistance-controlling genes or provide valuable markers for Marker-Assisted Selection (MAS), therefore facilitating the breeding of cultivars with improved resistance, and consequently, significantly contributing to the development of superior varieties of potato.

In potato, positional and/or sequence information is available for a number of major resistance genes for *P. infestans*, viral pathogens (*Potato virus X*, *Potato virus Y*, *Potato Leaf Roll Virus*) and root cyst nematodes of the genus *Globodera*, as well as genes involved in expression of defense reactions (Pathogenesis-Related (PR) proteins, chitinases, osmotins, proteinase inhibitors, etc.) (Gebhardt and Valkonen 2001). However, as mentioned above, plant pathogen resistance at the phenotypic level often does not behave as resulting from a single *R* gene, but rather as a quantitative trait, controlled concurrently by more than one gene and by the environment. The molecular basis of quantitative resistance is not known so far. Search for its putative sources draws attention to genes active in signal processing and transduction from the receptor to nucleus; however, molecular identity of these genes in potato still remains to be elucidated.

The aim of this study was to make use of the Arabidopsis model system as a tool to identify and initially characterize some of potato homologous genes putatively involved in signal transduction during host/pathogen interactions, as a pre-requisite for further characterization of selected candidates in the context of qualitative effects on disease resistance.

A candidate gene (CG) approach combined with genetic mapping was chosen to search for novel genes that can possibly harbor quantitative resistance in potato. CG analysis in QTL studies is based on the hypothesis that known-function genes (the candidate genes) could correspond to loci controlling traits of interest in other species. In plant genetics, one way to identify a CG is to look for map co-segregation between CGs and loci affecting the trait. As a next step, statistical association analyses between molecular polymorphisms of the CG (SNPs, InDels) and variation in the trait of interest need to be performed. The final validation of a candidate gene is provided through physiological analyses, genetic transformation (silencing of CG) and/or

complementation studies (Byrne and McMullen 1996, Fridman et al. 2004, Pfieger et al. 2001a).

So far, this field is still in its infancy, although the candidate gene approach has been already applied successfully in several species as a tool for finding resistance-linked target genes, and turned out to be a very promising strategy leading to the development of molecular markers facilitating the selection of superior genotypes (Gedil et al. 2001, Liu et al. 2004, Ramalingam et al. 2003).

In this study, the selection of candidates was based on their functions in signal transduction during pathogen invasion responses in *Arabidopsis*. The candidate genes were acquired from the current model for the signal transduction network in activation of defense responses (Fig. 5, reviewed by Glazebrook 2001). A number of Arabidopsis genes acting downstream of *R* genes, as well as the effector molecules of salicylic acid (SA)-dependent, and jasmonic acid (JA)/ethylene (ET)-mediated signaling pathways, derived from this network, were selected for further analyses. The experimental approaches encompassed the functional characterization using the RNAi-based post-transcriptional gene silencing technology in the potato and complementation studies in corresponding *Arabidopsis thaliana* knock-out mutants, along with the investigation of natural variation and statistical analyses of linkage to pathogen resistance in *S. tuberosum* populations.

1.8. The goals of the study

The goals in the scope of this work were:

1. To identify a number of potato homologues of *Arabidopsis thaliana* genes operating in defense signaling and determine their chromosomal positions in the potato genome

2. To characterize them at the molecular level as candidate genes for quantitative resistance characters. The resistance characters targeted are (i) field resistance to *Phytophthora infestans* causing the late blight disease, (ii) resistance to the soft rot bacterium *Erwinia carotovora* ssp. *atrosepica*, (iii) resistance to the Potato Leaf Roll

Virus (PLRV), and (iv) resistance to the potato root cyst nematodes *Globodera pallida* and *Globodera rostochiensis*.

3. To clone and functionally characterize at the molecular level natural candidate gene alleles affecting the target characters in extreme manner, i.e. in a positive and negative direction (superior and inferior alleles).

Knowledge on number, genomic position, coding function, naturally existing molecular and functional variants of genes affecting quantitative resistance will make possible the precise genotypic selection of plants with superior resistance alleles. Moreover, when those variants of genes controlling quantitative resistance characters can be identified which are responsible for increased or decreased resistance, this will open up a new route for the design of crop plants with improved field performance.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plants

Arabidopsis thaliana L. WT cultivar Col-*6, gl-1*, N8155 (provided by the NASC)
Arabidopsis thaliana L., *aos* knock-out line #1180 from the T. Jack T-DNA lines collection, Col-*6* background (Park et al. 2002)
Solanum tuberosum L. diploid cultivars H79.691/37, H80.601/4, and H83.385/14
Solanum tuberosum L. diploid cultivars DG 83-2025 and DG 81-68 (Zimnoch-Guzowska et al. 2000)
Solanum tuberosum L. diploid cultivars P18, P40, P3 and P38 (Gebhardt et al. 1989)
Solanum tuberosum L. diploid cultivars G87D2.4.1 ([(DH Flora x PI 458.388) x (DH Dani x PI 230.468)] and I88 (Oberhagemann et al. 1999)
Solanum tuberosum L. tetraploid cultivars Desirée, Escort, Nikita, Leyla, NK5, NK6
Solanum tuberosum L. mapping populations: F1840, K31 and Erwinia
Collection of *Solanaceae* species (listed in Table 2)

2.1.2. Plant propagation

Seeds of *Arabidopsis thaliana* lines were sown in plastic pots (diameters 8 x 8 x 8.5 cm) with *Minitray* soil (Gebr. Patzer GmbH & co. KG, Sinntal-Jossa, Germany) and incubated for 3d at 2-5°C in the dark to break dormancy. Plants were grown in a greenhouse under the following conditions: day period of 16h with 21°C and PFD of 80 mmol photons m^{-2} s^{-1}; night period of 8h with 18°C.

2.1.3. Bacteria

E. coli DH5α:
F⁻ supE44, Δ(lacZYA-argF)U169, (Θ80dlacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1, deoR (Hanahan et al. 1983)

E. coli DB3.1:
*F⁻ gyrA462 end*A1 Δ(sr1-*rec*A) *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *sup*E44 *ara*⁻14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^R) *xyl*-5 λ-l*eu mtl*1 (Invitrogen)

A. tumefaciens PGV3101:
C58C1, Rif^R, $Gent^R$ carrying a helper plasmid *pMP90RK* (Kan^R)

Abbreviation	Species	Accession	Alternate accession
acl a	*S. acaule*	BGRC 018627	EBS 2664
acl b	*S. acaule*	BGRC 016835	EBS 3052
acl c	*S. acaule*	BGRC 024555	
aln A1	***S. alandiae***	**BGRC 018521**	**EBS 3090**
aln E2	***S. alandiae***	**BGRC 031187**	
aln 028489	***S. alandiae***	**BGRC 028489**	
adg A3	*S. andigena*	BGRC 007464	
adg B3	*S. andigena*	BGRC 007506	
adg 007768	*S. andigena*	BGRC 007768	
ber a	***S. berthaultii***	**BGRC 010063**	**EBS 1846**
ber b	***S. berthaultii***	**BGRC 018548**	**EBS 1271x1288**
ber D2	***S. berthaultii***	**BGRC 028033**	
brc A1	*S. brevicaule*	BGRC 008207	
brc B3	*S. brevicaule*	BGRC 024571	
brc D3	*S. brevicaule*	BGRC 028023	
buk A1	***S. bukasovii***	**BGRC 007993**	
buk C1	***S. bukasovii***	**BGRC 015424**	**EBS 2152**
can 007165	*S. canasense*	BGRC 007165	EBS 1896
can 024572	*S. canasense*	BGRC 024572	
can 7166	*S. canasense*	BGRC 007166	EBS 1921
chc A3	***S. chacoense***	**BGRC 008025**	
chc b	***S. chacoense***	**BGRC 016979**	
chc C3	***S. chacoense***	**BGRC 027357**	
dms a	***S. demissum***	**BGRC 010022**	
dems 256	*S. demissum*		GLKS 256
dems 325	*S. demissum*		GLKS 325
dul 2	***S. dulcamara***		
tomato	*L. esculentum*		
tomato	*L. esculentum (Heir fein)*		prov. by Dr. D. Bellin
etb a	***S. etuberosum***	**BGRC 28476**	
etb b	***S. etuberosum***	**BGRC 53007**	
grl 007185	*S. gourlayi*	BGRC 007185	
grl 024600	*S. gourlayi*	BGRC 024600	
grl a	*S. gourlayi*	BGRC 007180	EBS 3048
hdm B3	***S. hondelmannii***	**BGRC 024710**	
hdm D1	***S. hondelmannii***	**BGRC 027317**	
kur b	*S. kurtzianum*	BGRC 017585	
ktz B3	*S. kurtzianum*	BGRC 017580	
ktz 017620	*S. kurtzianum*	BGRC 017620	
lph D1	***S. leptophyes***	**BGRC 027269**	
lph E3	***S. leptophyes***	**BGRC 008211**	
lph 018582	***S. leptophyes***	**BGRC 018582**	**EBS 3096**

Abbreviation	Species	Accession	Alternate accession
lignica A2	***S. lignicaule***	**BGRC 008106**	
maglia A3	***S. maglia***	**BGRC 023571**	**EBS 1059**
meg a	*S. megistacrolobum*	BGRC 008113	
meg b	*S. megistacrolobum*	BGRC 027262	
eggplant	***S. melongena***		**prov. by Dr. D. Bellin**
mcd 027354	*S. microdontum var. gigantophyllum*	BGRC 027354	
mcd A3	*S. microdontum*	BGRC 024644	
mcd C3	*S. microdontum*	BGRC 007197	EBS 3202
morelli A1	***S. morelliforme***	**BGRC 007200**	**EBS 3026**
ngr	*S. nigrum*		prov. by Dr. D. Bellin
nrs B2	***S. neorossii***	**BGRC 050197**	
opl A2	*S. oplocense*	BGRC 016868	
opl C1	*S. oplocense*	BGRC 024650	
opl 027345	*S. oplocense*	BGRC 027345	
phu 7907	***S. phureja***	**BGRC 007907**	
phu 7915	***S. phureja***	**BGRC 007915**	
phu 51240	***S. phureja***	**BGRC 051240**	
pnt a	*S. pinnatisectum*	BGRC 008168	
sis	***S. sisymbriifolium***		**prov. by Dr. D. Bellin**
spl B1	*S. sparsipilum*	BGRC 024678	
spl 018595	*S. sparsipilum*	BGRC 018595	EBS 3089
spl 027229	*S. sparsipilum*	BGRC 027229	
spg b	***S. spegazzinii***	**BGRC 016929**	
spg D3	***S. spegazzinii***	**BGRC 024694**	
spg 8220	***S. spegazzinii***	**BGRC 008220**	
stn A3	*S. stenotomum*	BGRC 051242	
stn C2	*S. stenotomum*	BGRC 053633	
stn 027167	*S. stenotomum*	BGRC 027167	
sto a/2	***S. stoloniferum***	**BGRC 007229**	**EBS 2942**
sto b	***S. stoloniferum***	**BGRC 007230**	**EBS 2626**
sto 55189	***S. stoloniferum***	**BGRC 055189**	
vrn A1	*S. vernei*	BGRC 008241	
vrn C1	*S. vernei*	BGRC 024729	
vrn D3	*S. vernei*	BGRC 017536	
verru B2	***S. verrucosum***	**BGRC 008250**	
verru C3	***S. verrucosum***	**BGRC 008255**	**EBS 2664?**
verru 008245	***S. verrucosum***	**BGRC 008245**	
tobacco	*N. tabaccum*		prov. by Dr. D. Bellin
pepper	***C. annuum***		**prov. by Dr. D. Bellin**
petunia	*P. hybrida*		prov. by Dr. D. Bellin

Table 2. Species of *Solanaceae* used for the study on *AOS2* gene sequence polymorphism.

2.1.4. Oligonucleotides sequences

Synthetic oligonucleotides were purchased from Qiagen Operon and Invitrogen.

Gene sequence	Forward primer 5' → 3' sequence	Reverse primer 5' → 3'
StAOS1	*aattcatcgtctcatcgtgttag*	*gcttcatcaagaacggaagttg*
StAOS2	*tctcttcctcttccttctc*	*gaccgagagtgagtacagg*
StAOS3	*accaaagactcataccacat*	*atatccttcatggagttatag*
StDND1	*tccctcacatgcattattatgttgcc*	*agcgatctctcgacacgtaagc*
StEDS1	*actcatttcctctacatttcatcc*	*ttcagattacatgcagcatagc*
StEDS5	*ggacctttgatgagtcttattg*	*catgccaagcctcgaatctg*
StHIN1	*cggagcctattatggtccatcc*	*gatctgccactggactccaaag*
StNDR1	*tctcaggcttaacagctctc*	*tttataatctcgtcgtaacg*
StNPR1	*gagcgagcttctcactcattgcgttg*	*agggaccaataatcgtgcaaatgcc*
StPAD4-1	*gaattttatgcaatttgaattttc*	*cggcatggaccattgccggatc*
StPAD4-2	*tgttgaaaaaatatgttatactag*	*taaactggaaagaacatgatgggg*
StPEN1	*atgggagataccggtggtgtc*	*ttgtaattgttgagcacctcctc*
StRAR1	*caatggagagacttcgatgtcag*	*acaaaagaatccctggtggcatc*
StSGT1-1	*gccgttgacctctacactc*	*ccacctcctctggtttctg*
StSGT1-2	*ttctatatcatgtgcatgaatctcg*	*acattagattagcccatgttctcc*
StWRKY1	*gccgggttcttgggactaatgg*	*tcaatgggatgtgaatgcatgccttc*
StPAD4-5´RACE		*gcagacggcagagaagccagagag*
StPAD4-3´RACE	*cgccgttgctggaggagtcatggaag*	
StEDS1-5´RACE		*gtgctcaggccaaggtcattcagtgctg*
StEDS1-3´RACE	*ggcagctctctggtgtctggaatggtgc*	
StSGT1-5´RACE		*gctcttctggtgtcctcgtcagcatc*
StSGT1-3´RACE	*cctcagctagtgtcgttgcacctcctgc*	
StSGT1 GW-F	*(GWF)taatggcgtccgatctggagactag*	
StSGT1 GW-R (Δstop)		*(GWR)cgatctcccatttcttcagctccatg*
StEDS1 GW-F	*(GWF)taatggtgaaaattggagaaggaattg*	
StEDS1GW-R(Δstop)		*(GWR)caggagttattttccttgatacccaag*
StPAD4 GW-F	*(GWF)taatggaatcggaagcttcatcgttc*	
StPAD4 GW-R (Δstop)		*(GWR)caggaaactgaggttggagcagctg*
StAOS2-snp	*ttgatgttcttccttctttcttc*	*gccagcggattttacttccgatc*
StAOS2-snp-GW	*(GWF)ttgatgttcttccttctttcttc*	*(GWR)gccagcggattttacttccgatc*
StAOS2 GW	*(GWF)taatggcattaacttcatcttttttc*	*(GWR)cagctttttttcaaagaagttatag*
StAOS2 GW-1	*(GWF)taatggctttaacttcatttttttc*	*(GWR)cagctttttttcaaagaagttatag*
StAOS2 5´UTR GW	*(GWF)tacatcaaacaccttttgtatcac*	
StAOS2 3´UTR GW		*(GWR)acccctttttgtgaaacaattactg*
AtTubulin-F	*acgtatcgatgtctatttcaacga*	*atatcgtagagagcctcattgtcc*
StTubulin-F	*acgtatcaatgtttatttcaatga*	*atatcatatagagcttcgttgtca*
StAOS2-1 RNAi Gw	*(GWF)tagggaagatcacgatgtcggcgatg*	*(GWR)ccttctccaacgaaccgatcggc*
StAOS2-2 RNAi Gw	*(GWF)tagatgttcttccttctttcttctcg*	*(GWR)ccacctcgaaaacggcgtcgtgtg*
StAOS2-3 RNAi Gw	*(GWF)tagatgttcttccttctttcttctcg*	*(GWR)ctgccggagtttaaaccagctg*
synth. gene RNAi GW	*(GWF)tagtctctgaatcagaaatccttctatc*	*(GWR)ccatgtcaaatttcactgcttcatcc*

Table 3. Oligonucleotides used for PCR, RACE-PCR and RT-PCR reactions described below.

The universal Gateway – compatible extensions for the BP recombination reactions (between an *att*B-flanked PCR product and a donor vector containing *att*P sites to create an entry clone) were:

GWF (*att*B1) 5´ *ggggacaagtttgtacaaaaaagcaggctta*3´,
GWR (*att*B2) 5´*ggggaccactttgtacaagaaagctgggtc*3´.

2.1.5. Plasmids

Plasmids used for the generation of constructs described in this thesis are listed below:

pCR TOPO2.1 (Invitrogen, Heidelberg) amp^R kan^R
pDONR™ 201 (Invitrogen, Heidelberg) kan^R
pDONR™P4-P1R (Invitrogen, Heidelberg) kan^R
pENTR™P2-RP3 terminator (kindly provided by L Deslandes and IE Somssich) kan^R
pENTR™201 smGFP (kindly provided by MS Mukhtar and IE Somssich) kan^R
pAM-PAT 35S GW terminator (kindly provided by IE Somssich) amp^R
pAM-Kan 35S GW myc (kindly provided by IE Somssich) amp^R
pAM-Kan 35S GW GFP (kindly provided by IE Somssich) amp^R
pAM-PAT Multi based on pDEST™R4-R3 (kindly provided by IE Somssich) amp^R
pJawohl17 RNAi (kindly provided by IE Somssich) amp^R

2.1.6. Buffers and solutions

Buffer/Solution	**Composition**
DNA loading buffer	67% (w/v) Sucrose, 50 mM EDTA, pH 8.0 0.42% (w/v) Bromphenolblue, 0.42% (w/v) Xylenecyanol, 0.42% (w/v) Orange G
DNA extraction buffer	0.1 M NaCl, 0.01 M Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS
3x RNA loading buffer	50% Formamid, 10% 10x MOPS, 0.45% Formaldehyd, 7% Glycerin, 0.5% Bromophenolblue
Spermidine solution (0.1 M)	Spermidine 73.0 mg, ddH_2O ad 50.0 ml

20x SSC	2 M NaCl, 0.3 M Sodium citrate, pH to 7.0 with HCl
20x TAE	800 mM Tris, 20 mM EDTA, 2.3% (v/v) glacial acetic acid
1x TBE	0.9 M Tris-HCl, 0.9 M boric acid, 25 mM EDTA

5 x OLB	100 µl solution A 250 µl solution B 150 µl solution C
Solution A	1 ml solution O 18 µl 2-mercaptoethanol 5 µl dATP, 5 µl dTTP, 5 µl dGTP (each 100 mM, pH 7.0)
Solution B	2 M Hepes, pH 6.6
Solution C	2.5 mg hexadeoxyribonucleotides pdN6 250 µl TE
Solution O	1.25 M Tris-HCl pH 8.0 1.27 g $MgCl_2$ x $6H_20$ sterile H_2O up to 50 ml

2.1.7. Enzymes

If not indicated otherwise, enzymes used for experiments described in this thesis were obtained from Roche and New England Biolabs. 10 x concentrated buffers for restriction enzymes were accompanied with the enzymes and supplied by the manufacturers.

Nucleic acid modifying enzymes

Taq DNA Polymerase	Invitrogen
TAKARA LA *Taq* polymerase	Takara, Otsu, Japan

Klenow fragment exo-	MBI Fermentas
RNase A (DNase-free)	MBI Fermentas
Ribonuclease Inhibitor	MBI Fermentas

2.1.8. Chemicals and radiochemicals

If not indicated otherwise, chemicals and radiochemicals used for experiments described in this thesis were obtained from Amersham Buchler GmbH & Co KG, J.T. Baker Chemicals, BioRad, Difco Laboratories, Fluka, Merck AG, Serva Feinbiochemica GmbH & Co, Sigma Aldrich GmbH.

2.1.9. Other materials

Parafilm M - American National Can.
Hybond N - Amersham Pharmacia Biotech
Reaction tubes - Eppendorf
Petri dishes - Greiner GmbH
Pipette tips - Greiner GmbH
Autoradiofilm XOMAT AR - Kodak
Sterile filtration units - Millipore
Qiagen Gel Extraction kit - Qiagen
Plasmid Isolation Midi kit - Qiagen
Whatman 3MM paper - Whatman
ExoSAP-IT - USB
BP-Clonase - Invitrogen, Heidelberg
LR-Clonase - Invitrogen, Heidelberg
LR-Clonase plus - Invitrogen, Heidelberg
RNAwiz extraction reagent - Ambion
Miniprep® Kit - QIAGEN
Gel Extraction Kit - QIAGEN
First Strand cDNA Synthesis Kit - MBI Fermentas
RNeasy Plant Mini® Kit - QIAGEN

2.1.10. Media for plants

Media were diluted in 1L deionized H_2O.

MS FL - liquid MS (for potato transformation): 4.7g MS salt supplemented with vitamins, 30g glucose, pH 5.7-5.8

MSp (for calli induction): 4.7g MS salt containing vitamins, 16g glucose, 1g MES, 8g agar agar, supplemented with hormones and antibiotics:
NAA (1mg/ml) – 5ml
BAP (1mg/ml) – 100 µl
Claforan (100mg/ml) – 2.5ml
Kanamycin (100mg/ml) – 500 µl
pH 5.7-5.8

MSII (for calli cultivation and root/shoot induction):
4.7g MS salt containing vitamins, 16g glucose, 8g agar agar, supplemented with hormones and antibiotics:
NAA (1mg/ml) – 20 µl
GA3 (1mg/ml) – 20 µl
Zeatin (1mg/ml) – 2ml
Claforan (100mg/ml) – 2.5ml
Kanamycin (100mg/ml) – 500 µl
pH 5.7-5.8

MSIII (for plants cultivation):
4.7g MS salt containing vitamins, 20g sucrose, 8g agar agar, supplemented with antibiotics:
Claforan (100mg/ml) – 2.5ml
Kanamycin (100mg/ml) – 500 µl
pH 5.7-5.8

Induction medium (1 L)

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH4)_2\ SO_4$	1 g
NaCitrate, $2H_20$	0.5 g
$MgSO_4$ (1M stock solution)	1 ml
Glucose	1 g
Fructose	1 g
Glycerol	4 ml
MES	10 mM

pH=5.6 and mild autoclavation (to avoid sugars caramelization)
Supplemented with appropriate antibiotics and 50 µg/ml Acetosyringone.

Infiltration medium (1 L)

10 mM MES, pH 5.3-5.5
10 mM $MgCl_2$
150 μg/ml Acetosyringone added prior to use.

2.1.11. Media for bacteria

Media were diluted in deionized 1l H_2O. For solid media 15 g of agar agar was added.

LB medium (Sambrook et al. 1989) 5g yeast extract, 10g trypton, 10g NaCl, pH=7.5

SOC medium (Sambrook et al. 1989) 5g yeast extract, 20g trypton, 20 mM glucose, 0.5g NaCl, 2.5 mM $CaCl_2$, pH=7.5

YEB medium 10g yeast extract, 10g peptone, 5g NaCl, 10g sucrose, pH=7.2

When required, antibiotics were supplemented to the following final concentration:
Ampicillin 100 mg/l
Carbenicillin 100 mg/l
Gentamycin 10 mg/l
Rifampicin 100 mg/l
Kanamycin 50 mg/l

Transformation buffer I (for preparation of the chemo-competent *E. coli*):

For 300 ml: 0.883 g KaCl, 2.969 g $MnCl_2x4H_2O$, 3.627 g $RbCl_2$, 0.441 g $CaCl_2x2H_2O$, 45 ml 15 % Glycerin.
Adjust pH to 5.8 with HCl

Transformation buffer II (for preparation of the chemo-competent *E. coli*):

For 150 ml: 0.45 g MOPS, 1.65 g $CaCl_2x2H_20$, 0.18 g $RbCl_2$, 22.5 ml 15 % Glycerin.
Adjust pH to 7.0 with NaOH

2.1.12. Jasmonic acid solution

The stock solution was prepared using 100mg of (±)-jasmonic acid (Sigma) dissolved in 5ml of acetone, and stored at 4°C. The working solution for sprayings of *A. thaliana*

aos plants was prepared freshly each time using 1ml of stock solution in 50ml of water, with addition of 5 µl of Silwet L-77.

2.1.13. Microscopes

Fluorescence microscope: Leica MZ12 with Mercury HBO 50 W/Ac lamp and FITC filter.

Confocal laser scanning microscope: Leica DMIRBE, TCS4D, with digital imaging processing, a 530+/-15nm band pass filter for FITC specific detection and a 580 nm band pass filter for autofluorescence detection.

2.1.14. Online softwares and databases

MultAlin software (Corpet, INRA Toulouse, France)
(http://prodes.toulouse.inra.fr/multalin/multalin.html)
GeneDoc (Nicholas, Pittsburgh Supercomputing Center, PA, U.S.A)
(http://www.psc.edu/biomed/genedoc/)
ExPASy Translate and ProtParam Tools http://www.expasy.org/tools
InterProScan Sequence Search tool (http://www.ebi.ac.uk/InterProScan/)
iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/)
TargetP (http://www.cbs.dtu.dk/services/TargetP/)
ChloroP (http://www.cbs.dtu.dk/services/ChloroP/)
Predotar (http://genoplante-info.infobiogen.fr/predotar/predotar.html)
NCBI (http://www.ncbi.nlm.nih.gov/)
TIGR (http://tigrblast.tigr.org/tgi)
SGN (http://www.sgn.cornell.edu/cgi-bin/tools/blast/simple.pl)
GABI PoMaMo Database (https://gabi.rzpd.de/PoMaMo.html)

2.1.15. Photographical data processing

Pictures were taken with assistance of Mrs. Maret-Linda Kalda, MPI-Photo Laboratory. Photos were processed using Adobe Photoshop 6.0 (Adobe Systems Inc.).

2.2. Methods

2.2.1. Potato genotypes used for identification of defense signaling homologs, genetic mapping and assessing the linkage to the quantitative resistance

Genomic DNA (purified according to Gebhardt et al. 1989) of three diploid potato genotypes (H79.691/37, H80.601/4, H83.385/14) was used for initial PCR amplification of potato genomic fragments with high sequence similarity to *A. thaliana* genes. These genotypes were not parents of mapping populations. For mapping of the candidate genes, genomic DNA of parents and progeny of three diploid potato mapping populations were used. Populations F1840 and K31 consisted of 100 and 113 individuals, respectively. Molecular maps based on RFLP (restriction fragment length polymorphism) and CAPS (cleaved amplified polymorphic sequence) markers have been constructed for the twelve chromosomes of both parents in these populations (Chen et al. 2001, Gebhardt et al. 2003, Leister et al. 1996, Schäfer-Pregl et al. 1998). In the K31 population, QTL for resistance to late blight caused by *Phytophthora infestans* have been mapped (Oberhagemann et al. 1999). The 'Erwinia' population, consisting of 158 diploid genotypes, has been developed at the Plant Breeding and Acclimatization Institute in Młochów, Poland. The individuals segregate for resistance against the most dangerous bacterial pathogen of potato, *Erwinia carotovora* ssp. *atroseptica*, causing both, the soft rot of tubers and the blackleg disease of potato stems. Currently, no monogenic resistance against *Erwinia* species is available. The seed parent DG 83-2025 (further referred as DG83), a hybrid of wild species *S. chacoense*, known as an excellent source of quantitative resistance against *Erwinia* spp., with *S. tuberosum* and *S. yungasense*, was highly resistant to tuber soft rot and

blackleg. The pollen parent DG 81-68 (further referred as DG81) was fully susceptible. The pedigree of this clone for the seed parent was *tbr* x [(*chc* x *chc*) x *tbr*] and for the pollen parent *tbr* x [(*chc* x *yun*) x *tbr*]. Molecular maps of maternal and paternal chromosomes have been constructed based on AFLP (amplified fragment length polymorphism) and RFLP markers (Zimnoch-Guzowska et al. 2000).

The population tested for linkage of *StAOS2* to quantitative late blight resistance, named 'GC', is derived from a cross of clone G87D2.4.1 [(DH Flora x PI 458.388) x (DH Dani x PI 230.468)], a hybrid of *S. tuberosum*, and wild species *S. kurtzianum*, *S. vernei* and *S. tarijense* (further referred as G87), as a female parent, with a diploid clone of unknown origin and pedigree, referred to as PX, as a male parent. The population consisted of 80 diploid individuals. The clone G87 is moderately resistant to late blight (average score 6.0 for foliage and 7.0 for tuber resistance), while foliage and tubers of PX were not evaluated for resistance to late blight (Oberhagemann et al. 1999). No known major *R* genes were present in this material; however, progeny of the cross segregates for quantitative resistance to *P. infestans*.

2.2.2. PCR

Routine PCRs were carried out according to a standard protocol. Fifty ng genomic DNA were amplified in 30 µl of 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP and dTTP (Carl Roth & Co. KG, Karlsruhe, Germany), 0.25 µM of each primer (Qiagen Operon Biotechnologies, Cologne, Germany), and 0.03 U/µl of *Taq* DNA polymerase (Invitrogen, Life Technologies, Karlsruhe, Germany). Reaction conditions were as follows: initial sample denaturation (3 min, 95°), 35 cycles of denaturation (20 s, 94°), primer annealing (40 s, annealing temperatures listed in Table 3) and elongation (1 min per 1 kb, 72°C), terminated by final elongation (72°C, 10 min).

2.2.3. Purification of gel-extracted DNA fragments

PCR products were purified using Qiagen PCR Fragments Purification Kit or Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

2.2.4. Purification of PCR products for sequencing

ExoSap (USB) reagent was used in order to remove primers and other compounds, potentially interfering with the sequencing reactions. 2 µl of ExoSap were added to 5 µl of PCR product and the mixture was incubated for 15 min at 37°C. Subsequently, alkaline phosphatase was inactivated by incubation at 80°C for another 15 min.

2.2.5. DNA sequencing

DNA sequences were determined by the DNA core facility (ADIS) of Max-Planck Institute for Plant Breeding Research on Abi Prism 377, 3100 or 3730 sequencers (Applied Biosystems, Weiterstadt, Germany) using BigDye-terminator v3.1 chemistry (Sanger et al. 1977). Premixed reagents were from Applied Biosystems.

2.2.6. Gene sequence analyses

BLAST searches with specific gene sequences of *Arabidopsis thaliana* (Table) were performed on the websites of the Institute of Genomic Research (TIGR) and of the Solanaceae Genomic Network (SGN) containing sequences of approximately 125.000 and 80.000 potato expressed sequence tags (ESTs), respectively. ESTs originated from various potato tissues including healthy leaf tissue and leaves after challenge with *P. infestans* (compatible and incompatible interactions) (Ronning et al. 2003). Exon-intron boundaries of potato genomic fragments were predicted using the DNASTAR software package (DNASTAR, Madison, WI, USA). Sequence multialignments were computed on-line with the MultAlin software and shaded in GeneDoc. Amino acid sequences of cloned genes, molecular masses and isoelectric points of deduced polypeptides were obtained using the ExPASy Translate and ProtParam Tools. Profile searches were made using the InterProScan Sequence Search tool on the website of EMBL-EBI. The subcellular localization was predicted using PSORT and TargetP.

2.2.7. Genetic mapping of candidate genes

PCRs were performed using gene specific primers (Table 3) and genomic DNA of parents and progeny as template. PCR products from parents of the mapping population that were polymorphic for a given gene marker were re-sequenced in order to confirm their identity and specificity. CAPS markers were developed for 14 candidate genes that segregated in at least one mapping population. Seven µl of PCR product (sizes varying from 624 bp to 2864 bp) were digested with 2 units of the appropriate restriction enzyme (Table 4) according to the description below. Restriction enzyme-digested PCR amplicons were separated agarose gels as described below. Two genes (*StNDR1* and *StPAD4-1*) were mapped as SSCP (Single Strand Conformation Polymorphism) markers. SSCP analysis was performed as described (Bormann et al. 2004). Segregating CAPS and SSCP fragments were scored as present (1) or absent (0). Fragments that could not be scored reliably were declared as missing values (2). The fragments were mapped in collaboration with Dr. C. Gebhardt relative to the existing marker database using the MAPRF software package (Ritter et al. 1990).

Candidate gene	Product size (bp)	Mapping population	Marker
StAOS1	860	F1840	CAPS/*Rsa*I
StAOS2	741	F1840	CAPS/*Taq*I
StAOS3	803	F1840	CAPS/*Taq*I
StDND1	874	Erwinia	CAPS/*Dde*I
StEDS1	1288	F1840	CAPS/*Alu*I
StEDS5	1787	Erwinia	CAPS/*Taq*I
StHIN1	624	K31	CAPS/*Hpa*II
StNDR1	198	F1840	SSCP direct
StNPR1	1452	F1840	CAPS/*Msp*I
StPAD4-1	323	F1840	SSCP direct
StPAD4-2	1676	F1840	CAPS/*Rsa*I
StPEN1	1239	F1840	CAPS/*Msp*I
StRAR1	1415	K31	CAPS/*Alu*I
StSGT1-1	2864	K31	CAPS/*Taq*I
StSGT1-2	1887	F1840	CAPS/*Dde*I
StWRKY1	2010	K31	CAPS/*Msp*I

Table 4. Markers developed for mapping of candidate genes.

2.2.8. Southern analysis

Southern analyses were performed according to Sambrook et al. (1998). 5.5 µg purified genomic DNA were digested to completion with *Eco*RI, *Eco*RV or *Hind*III for 6 h at 37°C, and a probe complementary to *StAOS2* gene was used for hybridization. Primers for probe amplification were same as those used for genetic mapping (*StAOS2*F+R) (Table 3).

2.2.9. DNA separation and blotting onto membranes

Digested genomic DNA was subsequently mixed with loading buffer, loaded and separated overnight via electrophoresis in 0.7% agarose gels with 0.5 x TBE buffer. The DNA was depurinated with 0.125 M HCl for 10 minutes. The gel was soaked for 45 minutes in denaturing solution (1.5 M NaCl + 0.5 M NaOH) and then for 30 minutes in neutralizing solution (1.5 M NaCl + 1 M Tris-Cl, pH 7.5). After washing in 20 x SSC, the DNA was transferred to a Nylon N membrane (Amersham) according to the standard capillary transfer procedure (Sambrook et al. 1989). The DNA was crosslinked to the filter with a UV crosslinker (Stratagene) by applying 120,000 J x cm^{-2} of energy. The filter was baked in an oven at 80°C for 1 h.

2.2.10. DNA hybridization

Pre-hybridization and hybridization were carried out in hybridization solution in glass tubes (30 cm x 4 cm) at 65 °C under continuous rotation in a hybridization oven (Bachofer, Reutlingen, Germany). The pre-hybridization was performed overnight. Upon adding the denatured radioactive probe, the hybridization was performed for at least 16 hrs.

After hybridization the filter was washed accordingly:

1. twice 50 ml 2 x SSC + 0.1 % SDS at RT for 10 minutes
2. twice 50 ml 1 x SSC + 0.1 % SDS at 65 °C for 10 minutes
3. once 50 ml 0.1 x SSC + 0.1 % SDS at 65 °C for 15 minutes

The filter was wrapped in thin plastic foil (Saran wrap) and exposed overnight to a phosphoimager screen (Molecular Dynamics) in a cassette at room temperature.

2.2.11. Preparation of radioactively labeled probe

50-100 ng of gel-purified PCR product were diluted up to final volume of 12 µl and denatured at 95°C for 5 min. Then, insert was immediately cooled down on ice and mixed with 4 µl 5 x OLB buffer, 3 µl of P^{32} α-dCTP (=30 µCi) and 1 µl (=2U) Klenow enzyme. The random primer - labeling reaction was running over night at room temperature. Probe was subsequently purified on a Sephadex G25 column.

2.2.12. Evaluation of *Arabidopsis thaliana* pollen viability by FDA staining

The fluorochromatic reaction (FCR) procedure (Heslop-Harrison et al. 1984, Shivanna and Rangaswamy 1992) was used for determining the viability of pollen produced by the *2xCaMV35S::StAOS2* complementation lines. 0.02 g of fluorescein diacetate (FDA) (Sigma-Aldrich GmbH, Munich, Germany), was mixed with 10 ml of acetone. A 20% sucrose solution was made up and 5 ml removed into a separate container. The FDA solution was added drop by drop to these 5 ml of sucrose until persistent turbidity. This solution was used within 30 min of mixing. One 10 µl drop of the mixture was applied on a clean microscope slide and subsequently pollen was added. Each slide was incubated at room temperature for 15 min. Then a cover slip was placed over the sample. Viability of pollen grains was examined under a fluorescence microscope (Leica MZ12, excitation filter 450–490nm). Using the fluorochromatic reaction test for the complementation lines' pollen, the viable pollen grains fluoresced in a bright green color, whilst the non-viable grains were of a dull yellow color. A hundred grains visible on the slide were scored and the percentage viability calculated.

2.2.13. Cloning strategies

Cloning strategies performed in the course of this thesis are described below. Plasmids and primers used for cloning procedures are listed in 2.1.4 and 2.1.5.

Basic TOPO reaction approach

The key to TOPO® Cloning is the enzyme, DNA topoisomerase I, which functions both as a restriction enzyme and as a ligase. Its biological role is to cleave and rejoin DNA during replication. *Vaccinia* virus topoisomerase I specifically recognizes the pentameric sequence 5´-(C/T)CCTT-3´ and forms a covalent bond with the phosphate group of the 3´ thymidine. It cleaves one DNA strand, enabling the DNA to unwind. The enzyme then religates the ends of the cleaved strand and releases itself from the DNA. To harness the religating activity of topoisomerase, TOPO® vector is linearized with topoisomerase I covalently bound to each 3´ phosphate. This enables the vector to readily ligate DNA sequences with compatible ends (www.invitrogen.com).

Composition of the ligation reaction

PCR product, gel-purified (20 ng in 4µl)	4 µl
pCR TOPO2.1® salt solution	1 µl
pCR TOPO2.1® topoisomerase/vector	1 µl

Gateway Technology-based cloning

Gateway® is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda. The Gateway® Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression and can be schematically represented as follows:

*att*B1-gene-*att*B2 × *att*P1-*ccd*B-*att*P2 ⇔ *att*L1-gene-*att*L2 × *att*R1-*ccd*B-*att*R2
(expression clone) (*pDONR*) (entry clone) (destination vector)

The *att*B × *att*P reaction is mediated by Gateway® BP Clonase™ enzyme mix; the *att*L × *att*R reaction is mediated by Gateway® LR Clonase™ enzyme mix. *ccd*B is the F plasmid-encoded gene that inhibits growth of *E. coli* and "gene" represents any DNA segment of interest (e.g. PCR product, cDNA, genomic DNA) (www.invitrogen.com).

Basic BP reaction approach

Gateway® BP Clonase® enzyme mix is a proprietary enzyme formulation containing the bacteriophage lambda recombination protein Integrase (Int) and the *E. coli*-encoded protein Integration Host Factor (IHF). Gateway® BP Clonase™ enzyme mix promotes *in vitro* recombination between an *att*B-PCR product (or *att*B containing expression clone) and an *att*P-containing donor (i.e., *pDONR*™) vector to generate *att*L-containing entry clones. "Genes" in entry clones can then be transferred into any number of *att*R-containing destination vectors using Gateway® LR Clonase™ enzyme mix (www.invitrogen.com).

Composition of the recombination reaction

*att*B-PCR product (50 ng/µl)	1 µl
GATEWAY® BP clonase	1 µl
BP reaction buffer (5x)	1 µl
pDONR™201 vector (50 ng/µl)	1 µl
ddH_2O	1 µl

Basic LR reaction approach

Gateway® LR Clonase™ enzyme mix is a proprietary enzyme formulation containing the bacteriophage lambda recombination proteins Integrase (Int) and Excisionase (Xis), and the *E. coli*-encoded protein Integration Host Factor (IHF). Gateway® LR Clonase™ enzyme mix promotes *in vitro* recombination between an entry clone (*att*L-flanked "gene") and any number of *att*R-containing destination vectors to generate *att*B-containing expression clones (www.invitrogen.com).

Composition of the recombination reaction

Entry clone (50 ng/µl)	1 µl
GATEWAY® LR clonase	1 µl
LR reaction buffer (5x)	1 µl
Destination vector (50 ng/µl)	1 µl
ddH_2O	1 µl

Basic LR Plus (MultiSite) reaction approach

MultiSite Gateway™ is an extension of the Gateway® site-specific recombinational cloning technology, which is based on the recombination properties of bacteriophage lambda. MultiSite Gateway allows simultaneous cloning of multiple DNA fragments in a defined order and orientation, providing a rapid and highly efficient way to recombine specific DNA elements into vector systems for functional analysis and protein expression. The three-fragment reaction using MultiSite Gateway® Technology can be represented as:

5' element × gene × 3' element × *ccd*B ⇒ 5' element-gene-3' element
(entry clone 1) (entry clone 2) (entry clone 3) (pDEST® R4-R3) (expression clone)

The recombination reaction occurs between specific attachment sites (*att*L and *att*R) on the entry clones and the pDEST·R4-R3 destination vector and is mediated by Gateway™ LR Clonase™ Plus enzyme mix. *ccd*B is the F plasmid-encoded gene that inhibits growth of *E. coli* (2,3), and the "gene" and 5' and 3' elements represent the DNA elements that are of interest, usually a promoter and a C-terminal epitope tag (www.invitrogen.com).

Composition of the recombination reaction

5' element *p4-P1R* Entry clone (20 ng/µl)	1.2 µl
Entry clone *pENTR201* (20 ng/µl)	1.2 µl
3' element *p2-RP3* Entry clone (20 ng/µl)	1.2 µl
GATEWAY® LR Plus clonase	2.0 µl
LR Plus reaction buffer (5x)	2.0 µl
Destination vector (20 ng/µl)	1.4 µl
ddH_2O	1.0 µl

Typically, reactions were carried out in 1.5 ml Eppendorf tubes. Reactions were incubated at 25ºC for at least 1 h (for TOPO TA cloning) or 12 h (for Gateway cloning), before the entire reaction volume was transformed into *E. coli* strain DH5α.

Small scale plasmid isolation from *E. coli*

Small scale plasmid isolation from *E.coli* was performed by alkaline lysis according to Sambrook et al. 1989, using Plasmid Isolation Mini kit (Qiagen).

Plasmid DNA cleavage by digestion with restriction endonucleases

Isolated plasmids were cleaved at diagnostic sites using restriction enzymes in order to confirm the accuracy of the construct. For the digestion of plasmid DNA with

restriction endonucleases, buffers supplied by manufacturers were used. Cleavage of DNA was performed at recommended optimal temperatures, usually at 37°C. 5-10 U of enzyme were used. Digestion of plasmid DNA was performed for 1-3 h. Enzyme reactions were stopped by heat inactivation of restriction enzymes upon transfer of the restriction mix to 65° for 20 min.

Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were mixed with DNA loading buffer and analyzed by agarose gel electrophoresis. The agarose concentration depended on the size of fragments to be resolved (Sambrook and Fritsch, 1998). Electrophoresis was performed at 5 V/cm using TBE buffer. 1kb ladder DNA size marker (Invitrogen) was used to estimate the size of DNA fragments. After electrophoresis, DNA was visualized on a transilluminator under UV light (254 nm).

2.2.14. RACE-PCR-based cloning of cDNA and genomic DNA of candidate genes

To obtain full-length cDNA and genomic sequences of *StSGT1, StPAD4* and *StEDS1*, RACE-PCR (Rapid Amplification of cDNA Ends) was employed.

RNA isolation and mRNA purification

Total RNA was isolated from 100 mg fresh, healthy leaf tissue of potato plants (cv. Desirée) grown in the greenhouse. The tissue was flash-frozen and ground in liquid nitrogen. Total RNA was extracted with 1 ml RNAwiz extraction reagent (Ambion, Huntingdon, Cambridgeshire, UK) following the supplier's protocol. DNaseII treatment was performed using the DNA-*free* reagent (Ambion) for 20 min at 37ºC, and reaction composition was as suggested by the producer. Poly(A)$^+$ RNA was purified using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech GmbH, Hamburg, Germany)

according to the supplier's instructions. Poly(A)$^+$ RNA was eluted in 20 µl of DEPC-treated water.

RACE-PCR

The BD SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech, East Meadow Circle, CA, USA) was used for the synthesis of RACE-ready cDNA and for the subsequent RACE experiments. Two independent populations of 5′-RACE-Ready and 3′-RACE-Ready cDNAs were synthesized, each using 250 ng of poly(A)$^+$ RNA, according to the supplier's protocol.

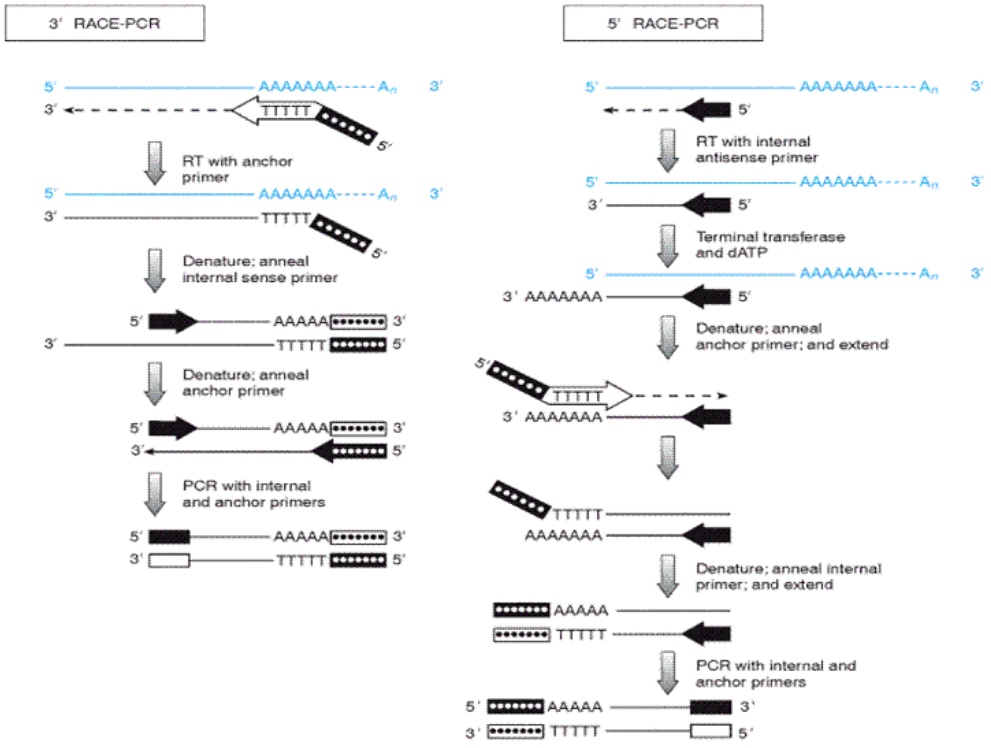


Fig. 6. The principle of the RACE-PCR reactions on 5′-RACE-Ready and 3′-RACE-Ready cDNAs using Universal Primer Mix and Gene Specific Primers (http://www.zoo.utoronto.ca)

RACE-PCR reactions were performed on the RACE-ready cDNA templates (Fig. 6), using the Universal Primer Mix provided in the kit, and gene-specific primers listed above (2.1.4). The RACE-PCR Touch Down program was as follows: 5 cycles 94°C 30 sec, 72°C 3 min, 5 cycles 94°C 30 sec, 70°C 30 sec, 72°C 3 min, 23 cycles 94°C 30 sec, 68°C 30 sec, 72°C 3 min. Six µl of each PCR reaction were loaded on 1.5% agarose gel and in each case a clear, single product was observed, except for *StSGT1* 3´RACE, where two fragments were amplified. RACE products were cloned into the *pCR2.1*-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced.

Cloning of full-length cDNA and genomic fragments of candidate genes

5' RACE and 3' RACE sequences were assembled *in silico* to obtain the full-length cDNA sequence for *StPAD4*, *StEDS1* and *StSGT1*. Only the larger 3´RACE product of *StSGT1* (ca. 950 bp) was found to be specific. Gateway® Technology (Invitrogen) was used to generate full-length cDNA clones that could be used as universal Entry clones in subsequent experiments, e.g. helpful for quick generation of constructs for complementation or over-expression analyses. Gateway® Technology – compatible primers flanking the deduced full-length cDNA sequences were designed as described above (2.1.4). Full-length cDNA sequences were amplified on 50 ng of 5´-RACE-ready cDNA template using high fidelity proof reading TAKARA LA *Taq* polymerase (Takara, Seta 3-4-1, Otsu, Japan), with the following PCR protocol: initial denaturation for 2 min at 93°, 29 cycles of denaturation (15 sec, 93°), primer annealing (30 sec, 64°C) and elongation (1 min per 1 kb of expected product size, 68°C), terminated by final elongation (68°C, 5 min). PCR Master Mix was prepared according to the supplier's protocol. PCR products were cloned into the *pDONR201* Gateway vector (Invitrogen) and three positive entry clones of each gene were re-sequenced to confirm product specificity and obtain a consensus sequence. The same conditions were used for PCR reactions with potato genomic DNA (cv. Desirée) to amplify full-length genomic fragments. Similarly, PCR products were cloned into the *pDONR201* vector (Invitrogen) and sequenced on both strands as described above.

2.2.15. RT-PCR

Reverse transcription – polymerase chain reactions (RT-PCR) were carried out with mRNA templates, isolated and purified as described above (2.2.11.). 250 ng template mRNA were combined with 1µl 10µM oligo dT_{18}, samples were incubated at 65°C for 10 minutes and then immediately cooled on ice. Subsequently the reaction was set up to 20µl by adding the following components: 4µl 5× first strand buffer (250mM Tris pH 8.3; 375mM KCl; 15mM $MgCl_2$), 2µl 0.1M DTT, 1µl 10mM dNTPs mix and proper amount of DEPC treated water. The mix was incubated at 42°C for 2 min before adding into 1µl (200U) Reverse Transcriptase Superscript II. Subsequently, the reaction proceeded at 42°C (50 min), followed by inactivation the enzyme at 70°C for 15 min.

For subsequent RT-PCR analyses, 2 µl of the above mixture was used as template and reactions were standardized using tubulin primers, specific to tubulin β-subunit of Arabidopsis and potato (according to the material analyzed).

2.2.16. Methods for the cultivation and transformation of bacteria

Preparation of chemo-competent *E. coli* cells

5 ml of an over night culture of *E. coli* strains DH5α or DB3.1 were added to 100 ml of LB broth and shaken at 37°C until the bacterial growth reached an OD_{600} 0.5-0.6. The bacteria were pelleted at 5000 x g for 10 minutes at 4°C. The pellet was gently re-suspended in 30 ml ice-cold TfBI (Transformation Buffer I) solution and subsequently incubated for 10 minutes on ice. The cells were pelleted as before and re-suspended in ice-cold TfBII (Transformation Buffer II) solution. 1.5 ml Eppendorf reaction tubes containing 50 µl aliquots of cells were frozen in liquid nitrogen and stored at -80°C until use.

Transformation of chemo-competent *E. coli* cells

50 µl aliquot of chemo-competent cells was thawed on ice. For Gateway vector transformation, 20 to 50 ng of plasmid DNA were mixed with the aliquot of *E. coli* DB3.1 cells. In case of TOPO TA cloning and Gateway recombination reactions, entire reaction mixture (6 µl or 5 µl, respectively) were added to an aliquot of *E. coli* DH5α competent cells. The cells were incubated on ice for 30 minutes. The mixture was heat-shocked for 30 seconds at 42°C and again incubated on ice for 2 minutes. 900 µl of SOC medium was immediately added to the Eppendorf tube and incubated at 37°C for 1 hour with continuous shaking at 950 rpm. A fraction (~ 150-200 µl) of the transformation mixture was plated out onto selection media plates. Transformed colonies were isolated.

Preparation of electrocompetent *A. tumefaciens*

A single colony of *A. tumefaciens* was inoculated into 5 ml of YEB medium and grown over night at 28°C. The over night culture was used to inoculate 400 ml of YEB medium and grown to A_{600nm}=0.5. Cells were harvested by centrifugation at 4.9 krpm and successively resuspended in 200 ml, 100 ml and 10 ml of ice-cold 1 mM Hepes (pH=7.5). Finally cells were resuspended in 800 µl of 1 mM Hepes (pH=7.5), 10% glycerol v/v, aliquoted into 0.5 ml Eppendorf tubes and frozen at –70°C.

Electroporation of *A. tumefaciens* cells

An 50 µl aliquot of frozen electrocompetent *A. tumefaciens* was thawed on ice and mixed with 450 µl of 10% glycerol. The electroporator was set to 25 µF, 2.5 kV and 200 Ω. A single electroporation pulse was given and 1 ml of YEB medium was immediately added. After incubation at 28°C for 2 hrs, cells are plated on selective YEB medium and incubated for 2 d at 28°C. Transformed colonies were isolated.

2.2.17. Complementation of the Arabidopsis *aos* mutant

Cloning of *StAOS2* alleles

Selected alleles of *StAOS2* were cloned by a PCR-based method. As the *StAOS2* gene is composed of a single exon, a genomic DNA template could be used as template for the amplification and cDNA cloning. The following potato genotypes were selected for the purpose of allele cloning:

- G87 (alleles 6 and 7)
- DG83 (allele 1)
- Erwinia 14 – an F_1 progeny from the cross DG83 x DG81 (alleles 8 and 12).

Full-length alleles sequences were amplified on 50 ng of genomic DNA template using high fidelity proof reading TAKARA LA *Taq* polymerase (Takara, Japan), with the following PCR protocol: initial denaturation for 2 min at 93°, 27 cycles of denaturation (15 s, 93°), primer annealing (30 sec, 64°C) and elongation (1 min per 1 kb of expected product size, 68°C), terminated by final elongation (68°C, 5 min). PCR Master Mix was prepared according to the supplier's protocol. PCR products were cloned into the *pDONR201* Gateway vector (Invitrogen) and ten positive entry clones of each gene were re-sequenced to confirm product specificity and to obtain a consensus sequence.

2.2.18. Silencing of *StAOS2* in potato genotype G87

Silencing of *StAOS2* gene in potato G87 background was performed using a set of Gateway-based vectors. Initially, three regions of *StAOS2* gene were selected, that could be suitable targets for silencing, being of sizes 253, 150 and 128 bp, respectively. These fragments were amplified on G87 cDNA template using Gateway *att*B1- and *att*B2-extended primers. Subsequently, fragments were cloned to

pJawohl17 vector, supplied with two Gateway cassettes of opposite orientation, with a 287bp intron of an *AtWRKY* gene cloned in between them. The entire construct is driven by *CaMV 35S* promoter and carries the *NptII* gene for plant kanamycin selection. As a result, three different RNAi panhandle constructs were obtained. For a negative control, a 308 bp artificial sequence (Synthetic GeneAmplimer pAW109 RNA purchased from PerkinElmer, Wellesley, MA, USA) displaying no homology to any plant gene identified so far, was amplified with Gateway-compatible extensions and cloned first into *pDONR201*, then into *pJawohl 17*. Obtained destination constructs were transformed into *Agrobacterium tumefaciens* strain PGV3101 carrying the *MP90RK* plasmid.

2.2.19 Quantification of the *StAOS2* transcript levels in the potato dsRNAi lines

Leaf material was taken from plants grown on MS medium under tissue culture conditions. Extracted RNA was further purified by DNase II treatment and subsequent poly(A^+) RNA isolation. Complementary DNA was synthesized on mRNA template and used for subsequent RT-PCR experiments. The potato *AOS2* transcript was monitored in RNAi transgenic lines using specific primers. *Solanum tuberosum* tubulin β-subunit specific primers were used as an internal control for the experiment, in order to adjust the loading volumes and exclude the possibility of genomic DNA contamination in the template.

Agarose gels, Ethidium Bromide-stained, were scanned on a PhosphoImager Typhoon 8600, followed by analysis of obtained images in ImageQuant 5.2 software (Molecular Dynamics). The intensities of tubulin loading controls were measured and compared. Subsequently, intensities of *StAOS2* transcript were adjusted according to the tubulin transcript levels. This experiment was repeated twice, using independent RNA preps as starting material; moreover, for each cDNA sample three rounds of RT-PCRs were performed to obtain the standard error values.

2.2.20. Methods for the cultivation and transformation of plants

The *Agrobacterium* strain PGV3101 was used for all transformations. The strain has a C58C1 chromosomal background marked by a rifampicin resistance mutation, and carries *pMP90RK*, a helper Ti plasmid encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells (Koncz et al. 1990).

Transformation of Arabidopsis *aos* plants

Agrobacterium clones carrying the respective plasmid were grown in 5 ml of YEB medium with gentamycin (25 mg/l), kanamycin (25 mg/l), carbenicillin (50 mg/l) and rifampicin (50 mg/l) over night at 28°C. The o/n culture was used to inoculate 400 ml of YEB medium and grown for another 16-20h. Cells were harvested by centrifugation at 4.9 krpm and resuspended in 50 ml of 5% sucrose solution. The resuspended culture was brought to A_{600nm}=0.8 by dilution with 5% sucrose solution. Before transformation, benzylaminopyrine (10 µl/l) and Silwet L-77 (500 µl/l) were added to the *A. tumefaciens* culture. Arabidopsis *aos* plants were grown under greenhouse conditions at a density of 5 plants/pot (9 cm diameter). The first emerging floral bolts were monitored for male sterility phenotype and cut off to remove the apical dominance, and therefore to encourage growth of multiple secondary bolts. Transformation was performed 5-10 days after clipping. Starting from the 3rd day before transformation, plants were daily sprayed with 1.5 mM solution of jasmonic acid (Sigma) in order to restore male fertility and allow seed set. The plants were dipped for 30 s into *A. tumefaciens* culture and covered with a plastic dome for 24 hrs to maintain high humidity. After removal of the plastic domes, plants were transferred to a growth-chamber with high humidity conditions for two days and then to the greenhouse until seeds were harvested. Starting at the 2nd day after transformation, another series of daily JA sprayings was applied to support seed production.

Transformation of potato G87 plants

Strong big leaves of potato G87 plants, grown on MS medium under sterile culture conditions, in a cycle of 16 h light/8 h dark at 22°C, were used for *A. tumefaciens* – mediated transformation. *A. tumefaciens* cultures were grown in 10 ml YEB medium, supplemented with antibiotics over night at 28°C. Potato leaves were harvested immediately before transformation and wounded with a scalpel at the leaf tip and base and scratched all over the surface. 10-15 wounded leaves were placed in a Petri dish containing 12 ml of liquid MS medium (MS-FL). Subsequently, 50 µl of Agrobacterium culture were added to the medium. Petri dish was closed with Parafilm and left for 2 days at 22°C in darkness. Transformed leaves were carefully dried on sterile filter paper and embedded in MSp plates to trigger callus induction. After one week, leaves were shifted onto MSII plates (shoot/root induction) and grown in a phytochamber with 16h/8h light/dark cycle at 22°C. Medium was exchanged every 10-14 days. When calli appeared, the transformants were moved from Petri dishes to glass jars. The best developed single calli with shoots were transferred on MSIII medium, numbered and cultivated under constant antibiotic selection.

Transient transformation of *N. benthamiana* leaves

2 ml overnight culture of Agrobacterium carrying the binary construct *2x35S::StAOS2-GFP* were grown at 28°C in liquid YEB medium containing antibiotics. Bacteria were pelleted by centrifgation and resuspended in 1 ml induction medium. After transferring to new test tubes, 3-5 ml induction medium (with antibiotics) were added. Culture was grown for another 4-6 hrs and bacteria were harvested by centrifugation. Subsequently, bacteria were resuspended in infiltration medium to OD_{600nm}=0.4-0.6. Healthy, fresh-looking leaves of young *N. benthamiana* plants were infiltrated with a needleless syringe on the underside. Plants were examined under confocal laser scanning microscope 72 hrs after infiltration.

2.2.21. OPDA and jasmonic acid extraction

Plant material was fine-ground in a mortar under liquid nitrogen and 0.5 g was taken for the extraction procedure. 100 ng of deuterated (D6) jasmonic acid and 100 ng of deuterated (D5) OPDA were added to each sample as internal controls. The material was further homogenized for 60 s in 10 ml methanol using the Ultra Turrax homogenizer.

The homogenate was filtered and the eluate was applied on a Sephadex column. Under these conditions, both JA and OPDA are bound to the column. The flow-through was discarded and the column was washed with 3 ml methanol and 3 ml 0.1 M acetic acid in methanol. The final elution was done using 3 ml 1 M acetic acid in methanol and 1.5 M acetic acid in methanol. Subsequently, the eluate was dried under nitrogen flow and the pellet was dissolved in 100 µl methanol. Samples were re-filtered and applied on High Performance Liquid Chromatography (HPLC) in order to purify the jasmonic acid and OPDA fractions. Each sample was run at the flow rate of 0.8 ml/min for 35 min, using methanol as mobile phase. Jasmonic acid and OPDA-containing fractions were collected at 13-14 min and 26-27 min of each run cycle, respectively. The HPLC-enriched fractions were merged and dried under nitrogen flow. Subsequently, the fraction was derivatized in chloroform, N-ethyldiisopropylamine, with addition of highly reactive 2,3,4,5,6-pentafluorobenzylbromide, for 1 hour at 60º C. Derivatized fractions were again dried under nitrogen flow and dissolved in 5 ml of n-hexane. The fractions were then applied to pre-washed (2 x methanol, 2 x diethyleter, 2 x n-hexane) silica columns. At this stage, both JA and OPDA are bound to the column. The flow-through was discarded and the JA – and OPDA – enriched fractions were eluted with 7 ml of a solution of n-hexane and diethylether (2:1). Fractions were again dried under nitrogen flow and dissolved in 70 µl acetonitrile. The samples were then subjected to Gas Chromatography – Mass Spectroscopy (GC-MS) analyses in order to determine the content of jasmonic acid and OPDA.

3. RESULTS

3.1. Database sequence similarity searches

Twenty *A. thaliana* genes were selected on the basis of current knowledge of their roles in signal transduction pathways linking pathogen recognition with defense responses (Glazebrook 2001, Hammond-Kosack and Parker 2003, Turner et al. 2002, Wang et al. 2002) (Table 6). Complementary DNA (cDNA) sequences corresponding to these genes were subjected to nucleotide BLAST analyses against EST databases of potato, tomato and/or tobacco. Twenty five ESTs with the highest similarity to the Arabidopsis genes were retrieved independently from different databases. Twenty three ESTs were from potato and corresponded to 20 Arabidopsis genes included in the search.

For *AtNDR1*, only a tomato-derived EST sequence was present in the databases. In four cases, multiple hits with highly significant similarities to the target Arabidopsis gene were found: for *StHIN1,* a potato and a tobacco EST were retrieved, and identification of *StRAR1* and *StNPR1* was equally supported by potato and tomato ESTs. For *AtAOS*, three different potato ESTs were identified, annotated as putative members of a gene family. Primer pairs were designed for these ESTs and PCR reactions performed using genomic DNA of three diploid *S. tuberosum* genotypes as templates (results not shown).

For eight genes (*AtACD11*, *AtCEV1*, *AtCOI1*, *AtCPR5*, *AtEDR1*, *AtEIN1*, *AtETR1* and *AtJAR1*), no homologous gene fragments were obtained from potato DNA for various reasons. In these cases either no PCR product was amplified, the primers generated multiple products, or sequencing the amplicons resulted in overlapping sequences, most likely due to the presence of gene families in the potato genome. For the 12 remaining Arabidopsis genes, existing partial potato genomic fragments were amplified, sequenced and compared to the sequence databases. All deduced amino acid sequences of potato exhibited a high level of sequence conservation with the

dicotyledonous species Arabidopsis, tomato and soybean, and the monocotyledonous species barley and rice (Table 7). EST accession numbers (TIGR), e-values for amino acid similarities and the GenBank accession numbers of the potato genomic fragments are also listed in Table 7.

At gene	Full name	At locus	Annotation
ACD11	**A**ccelerated **C**ell **D**eath 11	At2g34690.1	Transporter of the glycolipid precursor sphingosine between membranes (Brodersen et al. 2002).
AOS	**A**llene **O**xide **S**ynthase	At5g42650.1	Allene oxide synthase, catalyses dehydration of the hydroperoxide to an unstable allene oxide in the jasmonic acid biosynthetic pathway (Laudert et al. 1996).
CEV1	**C**onstitutive **E**xpression Of ***V****sp1* 1	At5g05170.1	Cellulose synthase CeSA3 (Ellis et al. 2002).
COI1	***Co***ronatine **I**nsensitive 1	At2g39940.1	An F-box protein required for response to jasmonates, which regulate defense against insects and pathogens, wound healing, and pollen fertility (Xie et al. 1998).
CPR5	**C**onstitutive expressor of **P**athogensis **R**elated genes 5	At5g64930.1	A transmembrane protein regulating expression of pathogenesis-related (PR) genes. Participates in signal transduction pathways involved in plant defense (systemic acquired resistance - SAR) (Clarke et al. 2001).
DND1	**D**efense, **N**o **D**eath 1	At5g15410.1	Cyclic nucleotide-gated ion channel, also known as *CNGC2* (Clough et al. 2000).
EDR1	**E**nhanced **D**isease **R**esistance 1	At1g08720.1	A mitogen-activated protein kinase kinase kinase (MAPKKK) that confers resistance to powdery mildew disease caused by fungus *Erysiphe cichoracearum* (Frye et al. 2001).
EDS1	**E**nhanced **D**isease **S**usceptibility 1	At3g48090.1	Component of *R* gene-mediated disease resistance in *Arabidopsis thaliana* with homology to eukaryotic lipases (Falk et al. 1999; Parker et al. 1996).
EDS5	**E**nhanced **D**isease **S**usceptibility 5	At4g39030.1	Member of the MATE-transporter family, essential for salicylic acid-dependent signaling during defense responses, also known as *SID1* (Nawrath et al. 2002).
ETR1	***Et***hylene **R**eceptor 1	At1g66340.1	A putative ethylene receptor containing a histidine kinase and a response regulator domain, membrane component capable of ethylene binding, also known as *EIN1* (Chang et al. 1993).
JAR1	***Ja***smonate **R**esponse 1	At2g46370.1	An auxin-induced gene encoding a cytoplasmic localized phytochrome A signaling component protein similar to the GH3 family of proteins (Staswick et al. 2002).

HIN1	***H***arpin-***In***duced 1	At5g06320.1	An *NDR1*-like gene, potentially functional in plant response to pathogens downstream of signal recognition (Gopalan et al. 1996; Varet et al. 2002).
NDR1	***N***on-Race-Specific ***D***isease ***R***esistance 1	At3g20600.1	Required for non-race specific resistance to bacterial and fungal pathogens. Mediates systemic acquired resistance (SAR) response (Century et al. 1995).
NPR1	***N***on-Expressor of ***PR*** genes 1	At5g45110.1	Adaptor molecule containing ankyrin repeats, controls systemic acquired resistance (SAR), also known as *NIM1* and *SAI1* (Cao et al. 1997).
PAD4	***P***hyto***a***lexin-***D***eficient 4	At3g52430.1	A lipase-like gene important for salicylic acid signaling (Glazebrook et al. 1997b; Jirage et al. 1999).
PEN1	***Pen***etration 1	At3g11820.1	Plant syntaxin *AtSYP121* (Collins et al. 2003; Sanderfoot et al. 2000, 2001).
RAR1	***R***equired for Ml***a***12 ***R***esistance 1	At5g51700.1	Resistance signaling gene, encodes a protein with two zinc binding (CHORD) domains that are highly conserved across eukaryotic phyla, also known as *PBS2*, *RPR1* (Azevedo et al. 2002; Shirasu et al. 1999).
SGT1	***S***uppressor of ***G***-***T***wo allele of SKP1	At4g23570.1	Component of the ubiquitin ligase complex, phosphatase-like protein, required for *Peronospora parasitica* resistance in Arabidopsis (Austin et al. 2002).
WRKY75	**WRKY**-domain (Trp-Arg-Lys-Tyr)	At5g13080.1	Transcription factor from the WRKY superfamily (group IIc), carrying a zinc-finger-like motif and binding specifically to the W-box (Eulgem et al. 2000).

Table 6. *Arabidopsis thaliana* defense-related genes that were selected for search of potato homologues (Pajerowska et al. 2005).

3.2. Development of PCR-based markers and genetic mapping

CAPS or SSCP markers which segregated in at least one of the three mapping populations considered were developed for 16 potato genes (Figure 7). All genes were mapped relative to RFLP loci of known map position (Gebhardt et al. 1989, 1991, 1993). One locus was identified for each gene, except *StSGT1* for which two unlinked loci were identified, and *StPAD4*, where two co-segregating, highly sequence-related copies were found (see below). The sixteen candidate loci were distributed on ten of the twelve potato chromosomes (Figure 8). Based on linkage to RFLP anchor loci,

which mark the same positions in different potato mapping populations, five candidate loci mapped to segments of the potato genetic map harboring previously mapped QRL (Figure 8). The locus *StSGT1-1* on chromosome III co-localized with QTL Pin3B for resistance to late blight (Bormann et al. 2004, Leonards-Schippers et al. 1994, Oberhagemann et al. 1999). The loci *StPAD4-1*, *StPAD4-2*, *StSGT1-2* and *StAOS2* on chromosomes II, VI and XI, respectively, co-localized with QTL for resistance to late blight and *Erwinia carotovora* spp. *atroseptica* (Oberhagemann et al. 1999, Zimnoch-Guzowska et al. 2000).

***A. thaliana* gene**	**EST accession number (TIGR)**	**E-value for amino acid similarity**	***S. tuberosum* gene**	**GenBank Accession No. of potato homologue**	**Amino acid similarities [%]**				
					A. thaliana	**Tomato**	**Soybean**	**Rice**	**Barley**
AOS	NP451990	1.5e-126	*StAOS1*	AJ457080[3]	78	83	83	77	71
AOS	TC128063	5.9e-128	*StAOS2*	AJ457081[3]	69	91	77	67	69
AOS	TC127849	1.2e-22	*StAOS3*	AY615276	65	97	73	64	65
DND1	TC129362	4.0e-89	*StDND1*	AY615277	54	80	- [4]	59	59
EDS1	TC111810	1.6e-10	*StEDS1*	AY679160[3]	57	86	67	61	58
EDS5	TC132023	8.0e-40	*StEDS5*	AY615278	78	-	-	76	69
HIN1	TC116297 TC1573 [2]	5.8e-15 8.1e-18	*StHIN1*	AY615279	60	91	67	55	51
NDR1	BF114006 [1]	3e-008	*StNDR1*	AY615280	62	95	66	-	-
NPR1	TC116672 TC164925[1]	4.4e-30 1.5e-29	*StNPR1*	AY615281	73	94	84	80	78
PAD4	TC118477	7.5e-98	*StPAD4-1*	AY753546[3]	61	87	77	56	68
PAD4	TC118477	3.2e-99	*StPAD4-2*	AY753547[3]	62	86	77	52	67
PEN1	TC122378	1.4e-76	*StPEN1*	AY616763	79	91	83	65	62
RAR1	TC121848 TC159170 [1]	1.2e-41 2.7e-41	*StRAR1*	AY615275	79	95	81	73	74
SGT1b	TC115479	7.3e-108	*StSGT1-1*	AY615272[3]	66	92	76	70	70
SGT1b	TC115479	3.8e-79	*StSGT1-2*	AY615274	67	96	69	67	69
WRKY75	TC121153	9.5e-41	*StWRKY1*	AY615273	97	98	98	90	91
					Mean = 69	**Mean = 91**	**Mean = 77**	**Mean = 67**	**Mean = 68**

[1] Tomato EST.
[2] Tobacco EST.
[3] Complete deduced amino acid sequence was used for the comparison.
[4] No corresponding EST was found.

Table 7. GenBank accession numbers of *Solanaceae* ESTs with the highest similarity to Arabidopsis defense response genes, and percent amino acid sequence similarities of the potato homologues to the most closely related genes of Arabidopsis, tomato, soybean, rice and barley.

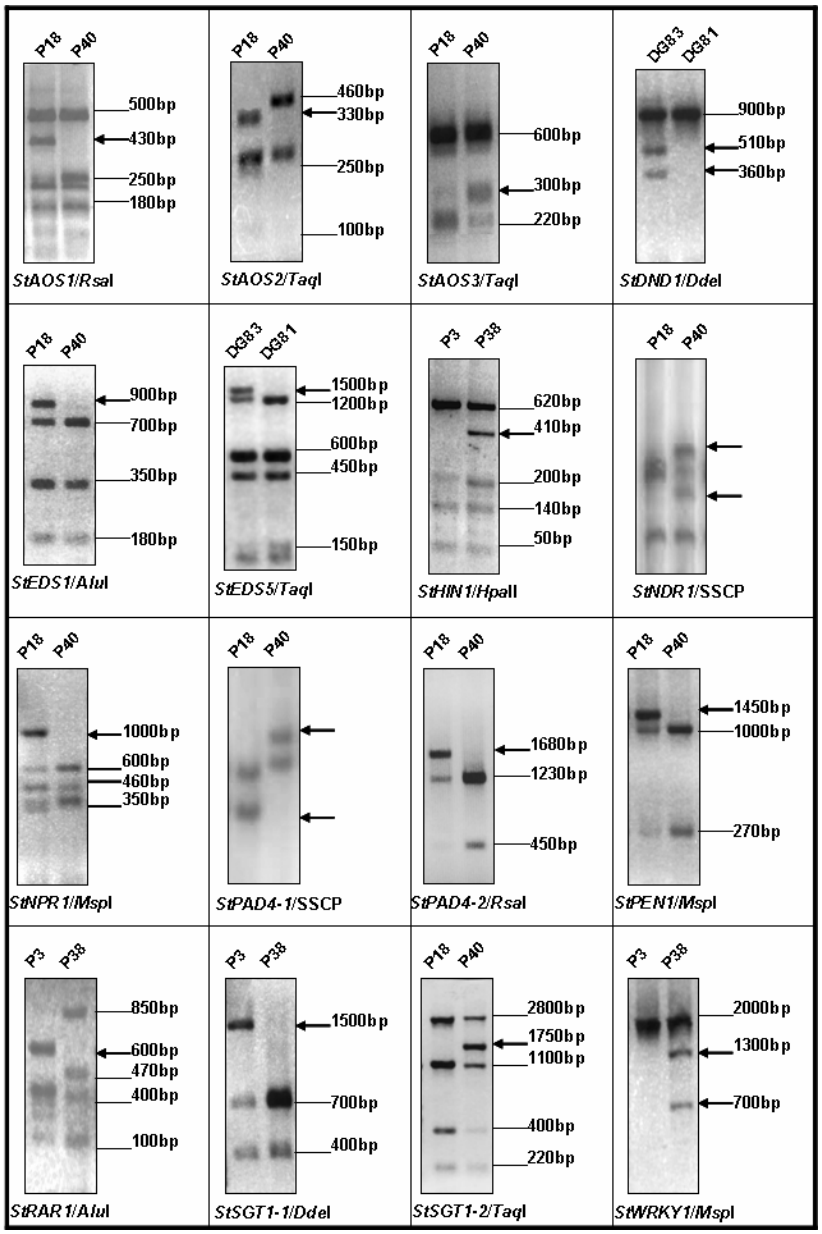


Fig. 7. CAPS or SSCP marker phenotypes. Sixteen markers polymorphic between the parental genotypes of three mapping populations were developed. Parents of the F1840 population: P18 (line H82.337/49, seed parent) and P40 (line H80.696/4, pollen parent) (Gebhardt et al. 1989, 2003). Parents of the K31 population: P3 (line H80.577/1, seed parent) and P38 (line H80.576/16, pollen parent) (Gebhardt et al. 1989; Oberhagemann et al. 1999). Parents of the "Erwinia" population: DG83 (line DG 83-2025, seed parent), and DG81 (line DG 81-68, pollen parent) (Zimnoch-Guzowska et al. 2000). Estimated sizes of restricted CAPS fragments are shown to the right. For loci *StNDR1* and *StPAD4-1* mapped as SSCP markers, no information regarding the size of segregating fragments is included, since the polymorphism is based on nucleotide composition and, consequently, conformation of undigested PCR products. Arrows indicate the polymorphic fragment(s) used for scoring the marker in the entire population.

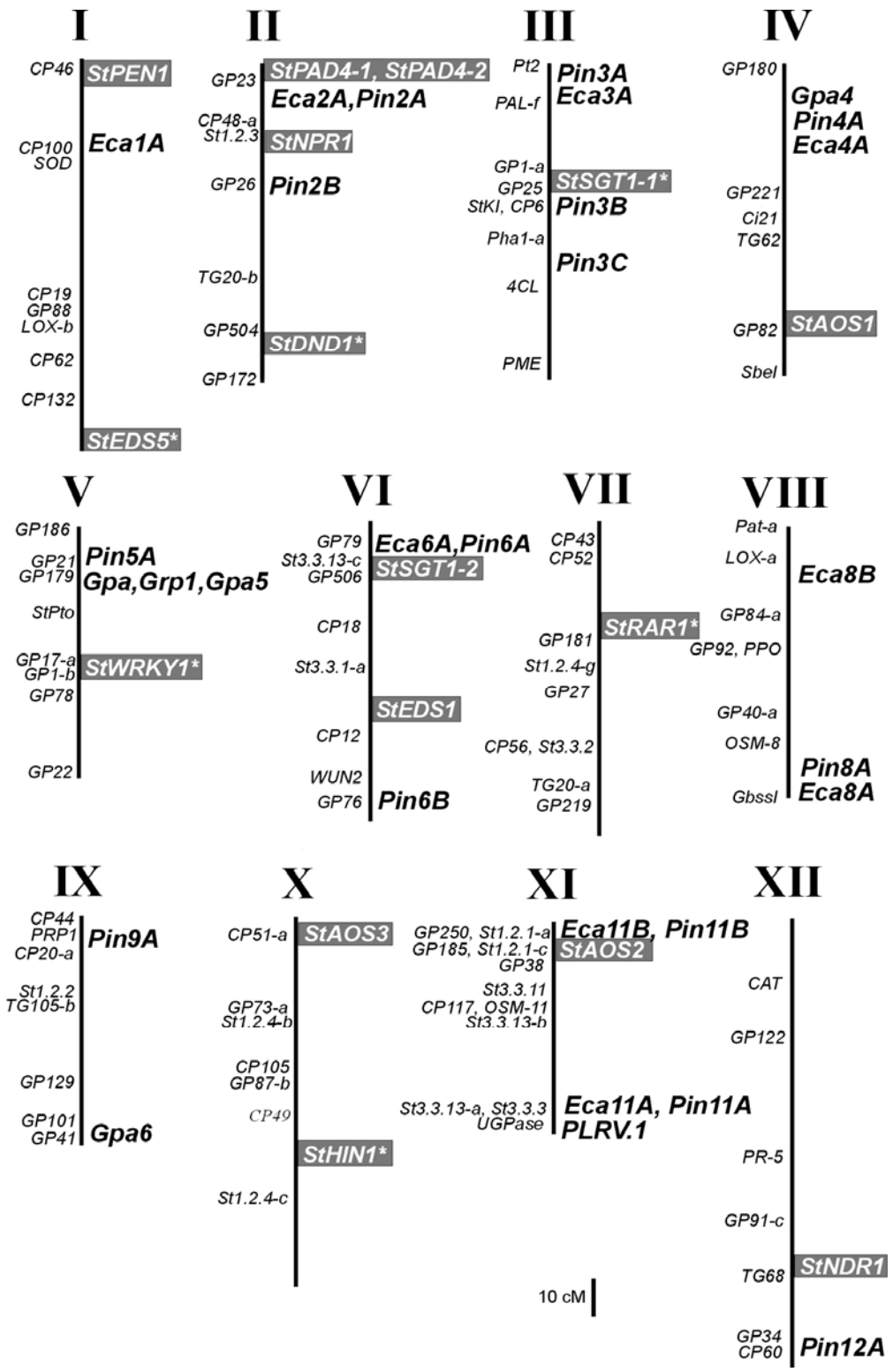


Fig. 8. Map positions of defense signaling (DS) loci in potato. The twelve linkage groups of the F1840 mapping population are shown, displaying to the left of each linkage group a subset of all RFLP loci

(GABI PoMaMo Database). RFLP loci *St1.2.1, St1.2.2, St1.2.3, St1.2.4, St3.3.1, St3.3.2, St3.3.11, St3.3.13* and *StPto* are sequence related to known *R* genes (Leister et al. 1996). The RFLP loci detected by known defense related genes in population F1840 are: *SOD* = superoxide dismutase, *LOX* = lipoxygenase, *PAL* = phenylalanine ammonia-lyase, *StKI* = Kunitz-type proteinase inhibitor, *4CL* = 4-coumaryl CoA ligase, *WUN2* = wound induced, *PPO* = polyphenol oxidase, *OSM-8* and *OSM-11* = basic osmotin-like, *PRP1* = pathogenesis related glutathione S-transferase 1, *CAT* = catalase, *PR-5* = acidic osmotin-like (Castillo Ruiz et al. 2005). Lower case extensions of RFLP loci indicate that the same probe detected more than one RFLP locus. The newly mapped DS loci are shown to the right of the linkage groups. DS loci that were mapped in populations other than F1840 (Table 4) are labeled with *. They were positioned relative to the closest anchor RFLP markers shared between the maps. Map segments having QRL (quantitative resistance loci) are indicated to the right of the linkage groups: *Pin*** = QRL to *P. infestans* (Bormann et al. 2004; Leonards-Schippers et al. 1992, 1994; Oberhagemann et al. 1999), *Eca*** = QRL to *E. carotovora* ssp. *atroseptica* (Zimnoch-Guzowska et al. 2000), *Gpa** and *Grp1* = QRL to root cyst nematodes *Globodera pallida* and/or *Globodera rostochiensis* (Kreike et al. 1994; Rouppe van der Voort et al. 1998, 2000), *PLRV.1* = QRL to *Potato Leafroll Virus* (Marczewski et al. 2001).

3.3. RACE-PCR-based isolation of the potato candidate genes

Potato genes homologous to *AtPAD4*, *AtEDS1* and *AtSGT1b* were characterized further. In order to obtain full length cDNA sequences of the candidates, RACE-PCR approach was employed.

3.3.1. *StSGT1*

RACE amplification and cloning of *StSGT1* resulted in a 1113 bp full length cDNA. Sequencing revealed a high level of conservation with *SGT1* genes from other plants. The isolated potato genomic fragment was 4482 base pairs long (accession AY615272). Ten exons encoded a putative protein of 370 amino acids (Figure 9) with a predicted molecular mass of 41.2 kDa and an isoelectric point (pI) of 5.11. Alignment of *StSGT1* with other *SGT1* genes supported the predicted open reading frame (Figure 9). No signal peptide or transmembrane domains were identified when

using the prediction programmes for sub-cellular localization, while typical domains present in previously described SGT1 proteins could be found: an N-terminal tetratricopeptide repeat (TPR) protein-protein interaction domain, a central CHORD specific (CS) domain involved in interactions with CHORD domain-bearing proteins and Hsp90, and a so-called SGT1-specific domain (SGS) that in yeast mediates interaction with the LRR domain of adenylyl cyclase and is also found in calcyclin binding proteins (Breen and Tang 2003, Muskett and Parker 2003, Schulze-Lefert 2004, Shirasu and Schulze-Lefert 2003) (Fig. 9). The locus *StSGT1-1* on potato chromosome III was identified with primers designed from exons 2 and 5 of the full length gene, whereas locus *StSGT1-2* on chromosome VI was identified with different primers corresponding to the original EST sequence (Table 3). This indicates that there are at least two *SGT1* genes in the potato genome and that the fully sequenced gene *StSGT1-1* is likely encoded at the locus on potato chromosome III. For gene *StSGT1-2,* the complete genomic sequence was not determined.

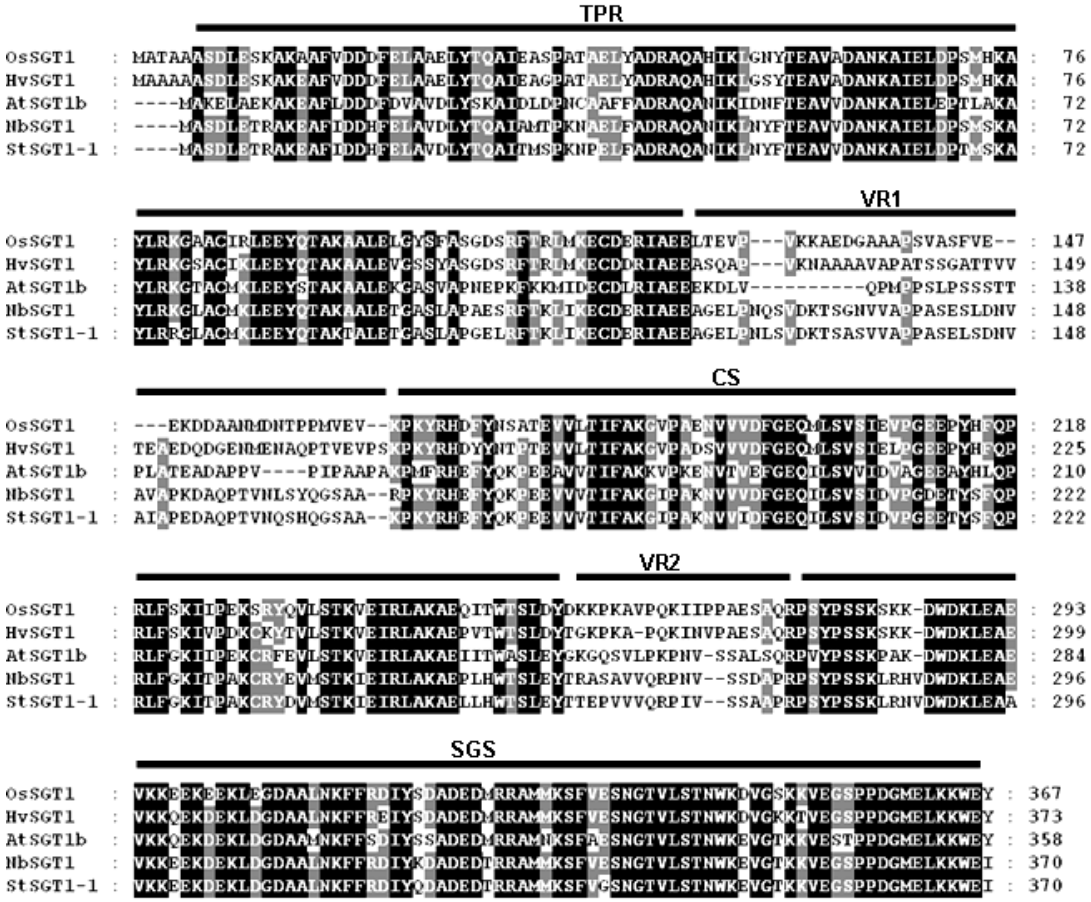


Fig. 9. Alignment of deduced amino acid sequence of *St*SGT1-1 with rice (*Os*), barley (*Hv*), Arabidopsis (*At*) and *Nicotiana benthamiana* (*Nb*) SGT1 proteins. GenBank accession numbers: *Os*SGT1, AAF18438; *Hv*SGT1, AF439974; *At*SGT1b, AAL33612; *Nb*SGT1, AAO85509; *St*SGT1-1, AY615272. The black and grey boxes represent 100% and 70% amino acid sequence conservation, respectively, between the deduced proteins compared. TPR: tetratricopeptide repeat domain, VR1 and VR2: Variable region 1 and 2, CS: CHORD protein and SGT1-specific motif, SGS: SGT1 specific domain.

3.3.2. *StPAD4*

A full-length cDNA of *StPAD4* was 1737 base pairs long. Sequencing of three cDNA clones revealed two highly similar, but distinct *StPAD4* transcripts. PCR using the same set of primers, performed on genomic DNA template, resulted in two amplicons

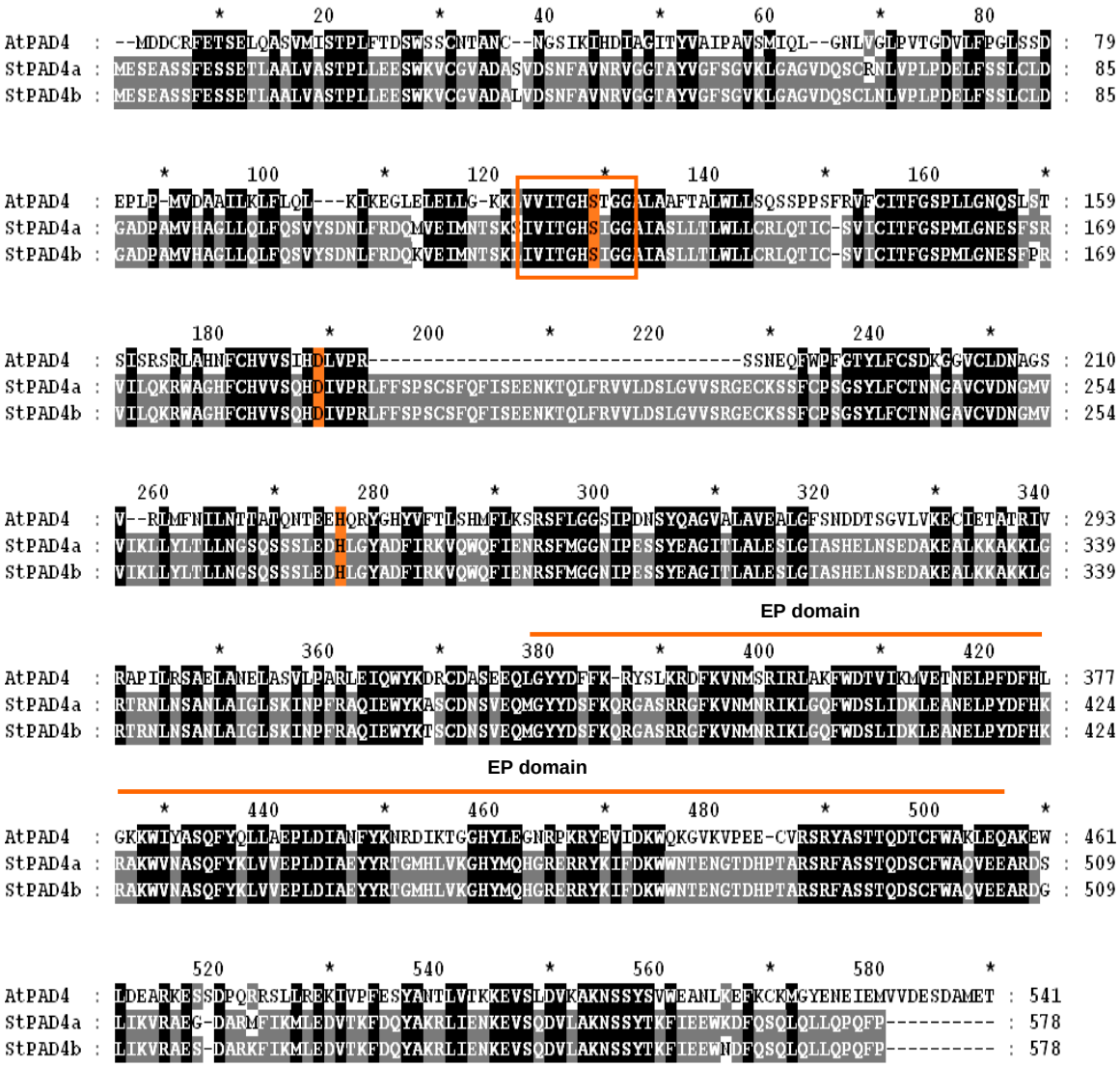


Fig. 10. Alignment of deduced amino acid sequences of *St*PAD4-1 (*St*PAD4a) and *St*PAD4-2 (*St*PAD4b) with Arabidopsis (*At*) PAD4 protein. The black and grey boxes represent 100% and 70% amino acid sequence conservation, respectively, between the deduced proteins compared. GenBank accession numbers: *At*PAD4, AAF09479; *St*PAD4-1, AY753546; *St*PAD4-2, AY753547. The class 3 lipase consensus sequence around the predicted catalytic serine (S) is boxed. The three predicted lipase catalytic residues, a serine (S), an aspartate (D), and a histidine (H) are highlighted in orange. The EP domain lies between amino acids 332 and 457 in Arabidopsis and between amino acids 378 and 505 in potato.

of 4637 (accession AY753546) and 5300 (accession AY753547) base pairs. Both products contained a gene composed of four exons and three introns, the first exon being small (25 bp) and the second intron large (2309 bp in *StPAD4-1* and 2985 bp in *StPAD4-2*). Differences between both genes were mostly confined to the introns with numerous insertions/deletions up to 700 base pairs and few single base pair exchanges present in the coding sequence. Based on sequence analysis, both genes are functional *StPAD4* copies, having defined start and stop codons and uninterrupted reading frames. The cDNA sequences deduced from the genomic clones perfectly matched the cDNAs obtained from RACE experiments, confirming the presence of two *PAD4* genes in the potato genome. The copies were named *StPAD4-1* (shorter) and *StPAD4-2* (longer). Genetic mapping, performed with help of copy-specific primers revealed genetically closely linked loci located on chromosome II (Fig. 8). This could classify them as paralogues that arose from gene duplication. The deduced polypeptides of both *St*PAD4 sequences comprise 578 amino acids and share 98% identity with a predicted molecular mass of 65 kDa and a pI of 6.49. Domain searches identified a class 3 lipase motif in the N-terminal part, a catalytically active serine (S^{129}) residue surrounded by the L3 consensus sequence (amino acids 123-132), and the two other residues of the catalytic triad of serine hydrolases: an aspartate (D^{188}) and a histidine (H^{275}) (Falk et al. 1999, Jirage 1999). In the C-terminal region (amino acids 378-505) high similarity was detected with the EP (EDS1 and PAD4 specific) domain of Arabidopsis PAD4 that is shared with *At*EDS1 and *At*SAG101 and may direct interactions between these proteins (Feys et al. 2001) (Fig. 10).

3.3.3. *StEDS1*

Molecular cloning of *StEDS1* resulted in isolation of a single 1824 base pair cDNA clone with high similarity to Arabidopsis and tobacco *EDS1* genes. The genomic sequence of *StEDS1* is composed of 3 exons and 2 introns with a total size of 2597 bp. The gene encodes a putative 607 amino acid protein of 69 kDa and pI = 6.72. Like PAD4 proteins, *St*EDS1 possesses characteristic features of a class 3 lipase with a defined catalytic triad: S^{125} surrounded by the L3 consensus between amino acids 119 and 128, D^{190} and H^{325} (Falk et al. 1999). The EP interaction domain was detected in the region of residues 426-544 and contains a "KNEDT" motif specific to EDS1 sequences identified so far (amino acids 499-503), with a conserved substitution of threonine to serine (Peart et al. 2002) (Fig. 11).

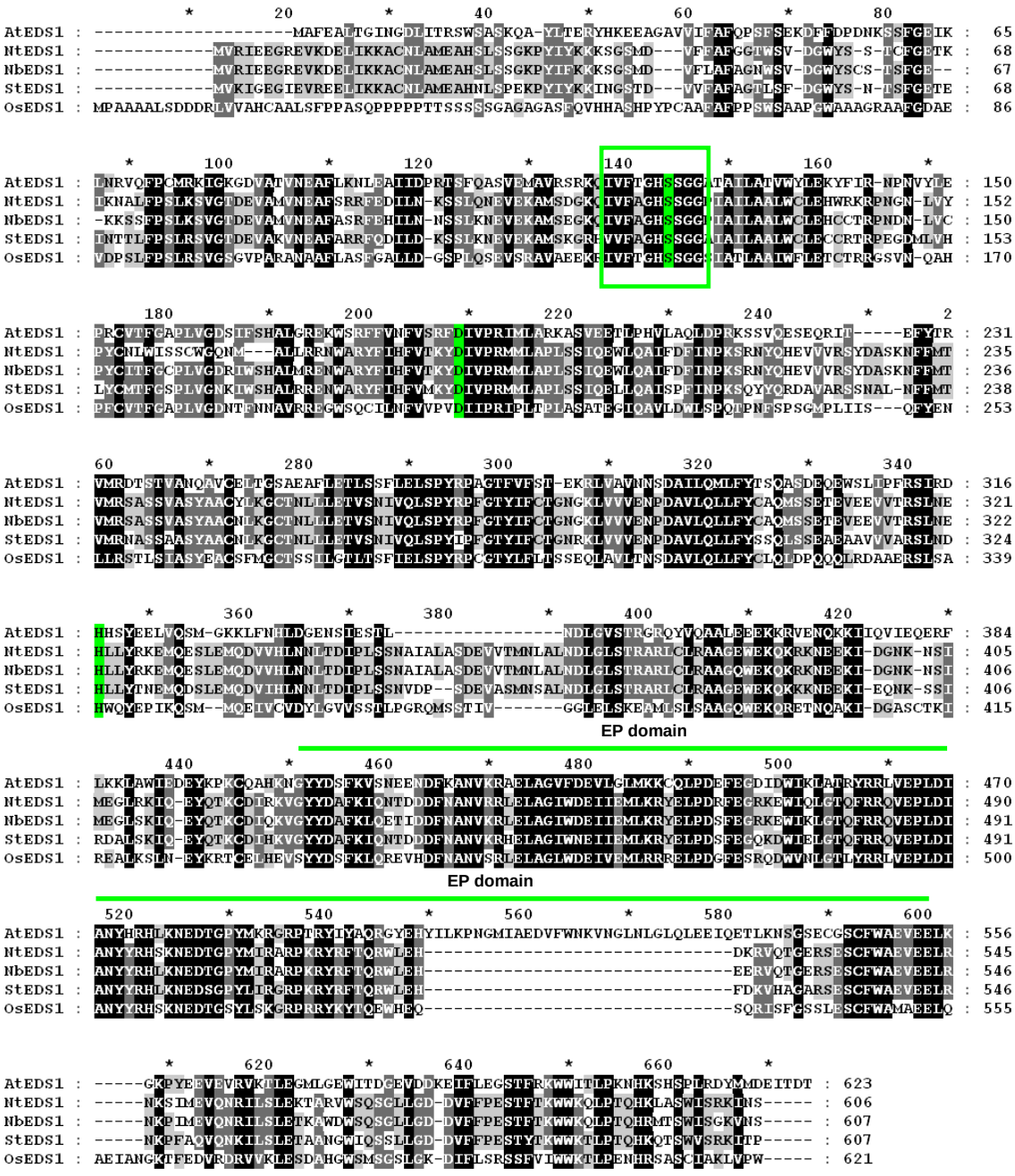


Fig. 11. Alignment of deduced amino acid sequence of *St*EDS1 with Arabidopsis (*At*), tobacco (*Nt*), *Nicotiana benthamiana* (*Nb*) and rice (*Os*) EDS1 proteins. The black and grey boxes represent 100% and 70% amino acid sequence conservation, respectively, between the deduced proteins compared. GenBank accession numbers: *At*EDS1, NP_190392; *Nt*EDS1, AAM62411; *Nb*EDS1, AAL85347; *St*EDS1, AY679160; *Os*EDS1, XP_450883. The class 3 lipase consensus sequence around the predicted catalytic serine (S) is boxed. The three predicted lipase catalytic residues, a serine (S), an aspartate (D), and a histidine (H) are highlighted in green. The EP domain lies between amino acids 405 and 554 in Arabidopsis and between amino acids 426 and 544 in potato.

3.4. Functional characterization of *StAOS2*

Basing on the positional selection criterion, the *StAOS2* was the most interesting candidate for a Quantitative Resistance Locus in potato. Therefore, a more detailed functional characterization of this gene has been attempted.

3.4.1. The family of Allene Oxide Synthases in potato

*St*AOS2 (EC 4.2.1.92) is not the only allene oxide synthase present in the potato genome. The family of *AOS* genes, basing on the EST data available, includes at least 3 members. *AOS1* and *AOS2* share a high overall homology and are believed to have overlapping substrate specificities (13-hydroperoxy-derivatives of linolenic acid). For *St*AOS3, the homology with other members is confined only to limited regions of cytochrome P450 domain. This gene is expressed exclusively in roots and the AOS3 enzyme preferentially processes another substrate (9-hydroperoxy-derivatives of linolenic acid) (Itoh et al. 2002). In order to experimentally determine the copy number of *StAOS2* in the potato genome, the Southern analysis was performed. Genomic DNA of potato (cv. Desirée) was used and the probe was designed within the 5´ portion of *StAOS2*. Due to high structural homology between *StAOS1* and *StAOS2*, the probe hybridized to both these genes. The hybridization pattern clearly showed presence of only single copies of both genes (Fig. 12).

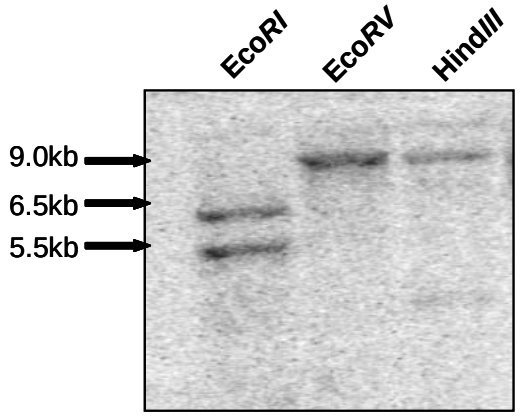


Fig. 12. Southern blot analysis of *StAOS2* copy number. The probe used for the hybridization was designed to 5′ portion of *StAOS2*, but due to high sequence homology also recognized *StAOS1*. Both *StAOS* genes are single-copy in the potato genome.

This is in agreement with the mapping data described above; only a singular segregation pattern for each of the *StAOS* genes was detected and scored in the potato mapping populations.

3.4.2. Allene Oxide Synthase as a plant cytochrome P450 enzyme

P450 enzymes constitute a superfamily of haem-thiolate proteins, widely distributed in bacteria, fungi, plants and animals. The enzymes are involved in metabolism of a plethora of both exogenous and endogenous compounds. Usually, they act as terminal oxidases in multi-component electron transfer chains, called P450-containing monooxygenase systems.

Current P450 nomenclature, based on divergent evolution of the P450 superfamily, was proposed and developed by Nebert and Russell (2002). On the basis of sequence similarity, all P450s can be categorised into 2 main classes, the so-called B- and E-classes. P450 proteins of prokaryotic 3-component systems and fungal P450 (CYP55) belong to the B-class; all other known P450s from distinct systems are of the E-class. E-class P450 may be further divided into 5 subclasses (groups) according to protein

sequence similarities. *St*AOS2, similarly to *At*AOS, is predicted to belong to the subclass IV. An 8-element fingerprint, EP450II, provides a signature for group IV E-class P450s. The fingerprint was derived from an initial alignment of several available member sequences: the motifs were drawn from conserved regions spanning virtually the full alignment length (Nebert and Russell 2002). Although the E-class is the most abundant one among plant P450s (e.g. 355 members in *A. thaliana* and 362 in *O. sativa*), still there is no 3-dimensional structure available for a group IV E-class P450 representative. The preliminary assignment of secondary structure elements for a cytochrome P450 from *Candida albicans* is adopted from a modelling study of Boscott and Grant (1994).

3.4.3. The octadecanoid pathway

Jasmonic acid is a ubiquitously occurring lipid-derived octadecanoid compound found first in the oil of *Jasminum grandiflorum*. Upon wounding or pathogen attack, the octadecanoid pathway (Fig. 13) is activated by the polypeptide systemin and by oligouronides. Then a lipase generates α-linolenic acid, the first precursor in the pathway. α-linolenic acid is then converted by a lipoxygenase (LOX) to give rise to 13-hydroperoxylinolenic acid via incorporation of O_2. Several examples of wound-inducible LOX genes have been identified (Royo et al. 1996), suggesting that regulation of the level of this enzyme, and of others downstream, may serve to control the production of JA. Allene oxide synthase (AOS) is of a particular importance in biosynthesis of JA. AOS, a cytochrome P450 enzyme, represents the first committed step on the JA branch of the octadecanoid pathway. AOS catalyses the conversion of 13-hydroperoxylinolenic acid to 12, 13-epoxy-linolenic acid which, in turn, is cyclized by allene oxide cyclase (AOC) giving rise to 12-oxo-phytodienoic acid (OPDA). AOC is the key enzyme that establishes the stereospecificity in the JA branch of the octadecanoid pathway specifically leading to the (-)-JA stereoisomer (Ziegler et al. 2000). OPDA is converted to JA through the action of a reductase and three rounds of β-oxidation (Müller 1997).

OPDA, JA and its derivatives (amino acid conjugates, methyl and sugar esters) are all effective as signals in the plant defense responses (Ziegler et al. 2000).

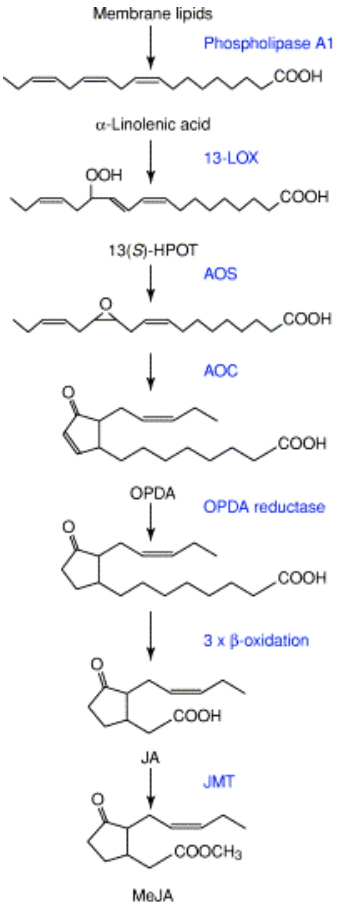


Fig. 13. Main pathway of jasmonate biosynthesis. A phospholipase A1 releases a-linolenic acid from membrane lipids. The α-linolenic acid is oxygenated by lipoxygenase (LOX) to form 13(S)-hydroxy linolenic acid (13-HPOT), which is then converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). Jasmonic acid (JA) is synthesized from OPDA through reduction and three steps of β-oxidation, and is further converted to methyl jasmonate (MeJA) by JA carboxyl methyltransferase (JMT) (Ziegler et al. 2000).

3.4.4. Subcellular localization of *St*AOS2

For the potato AOS2, *in silico* subcellular localization prediction program "iPSORT" deduced a clear 30-31 amino acid long chloroplast signal, confirmed by a highly significant certainty value (0.00). Other online tools for prediction of chloroplast localization, ChloroP, TargetP and Predotar supported this result with 82-88% probability.

Subcellular localization of *StAOS2* was determined experimentally by means of transient expression of fluorescently tagged protein. The coding sequence of green fluorescent protein (*GFP*) was fused in frame to the 3' end of the full-length cDNA of

StAOS2, and the construct was driven by constitutively expressed double cauliflower mosaic virus (CaMV) *35S* promoter (Odell et al. 1985). The fusion construct *2x35S::StAOS2-GFP* (Fig. 14D) was transformed into *Agrobacterium tumefaciens* and infiltrated into *Nicotiana benthamiana* leaves, together with another *A. tumefaciens* strain, carrying the *35S*-driven viral suppressor of silencing (*p19*). Confocal laser scanning microscopic observations were performed 72 hours after transformation. Using the chlorophyll and GFP excitation wavelength (488 nm), characteristic red fluorescence in the chloroplasts of tobacco epidermis guard cells (Fig. 14B) was detected, with emission spectrum in range between 650 and 700 nm, corresponding to chlorophyll focal attributes. Using the GFP emission channel (490-560 nm), *St*AOS2-GFP was visualized in the subcellular structures corresponding by shape and location to chloroplasts (Fig. 14A).

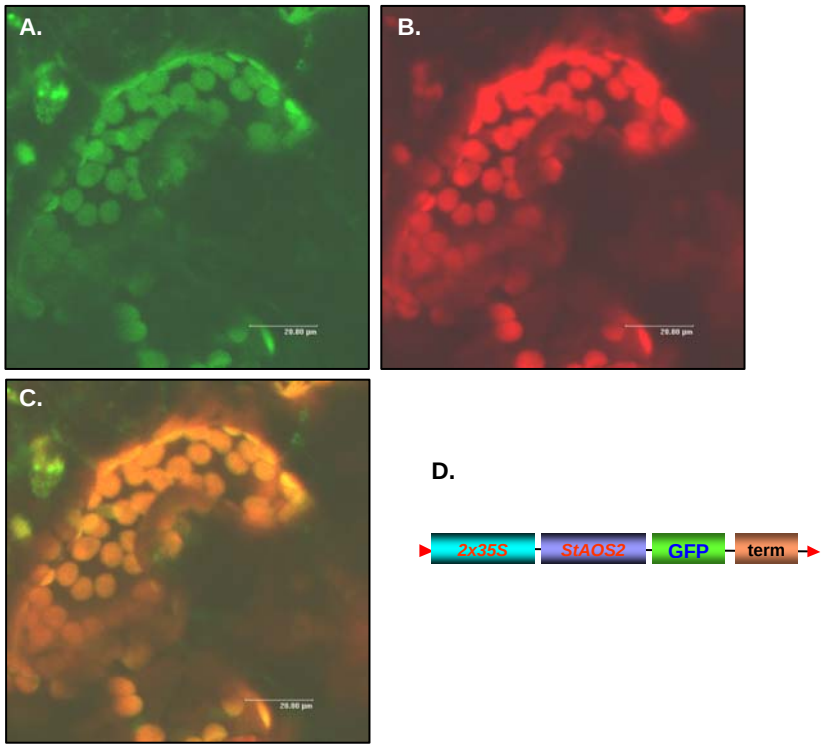


Fig. 14. Confocal laser scanning microscope photographs of a *N. benthamiana* leaf epidermal guard cell transformed with a construct ectopically expressing (from double *CaMV 35S* promoter) the full-length *StAOS2* cDNA translationally fused with Green Fluorescent Protein (GFP) (D). A: fluorescence in GFP channel, B: autofluorescente of chlorophyll, and C: overlay demonstrating perfect co-localization of *St*AOS2-GFP with chloroplasts.

After computational processing of the images, the autofluorescence in the GFP channel was removed and in overlay, *St*AOS2-GFP and chlorophyll can still be observed in the same guard cell, perfectly overlapping by position (Fig. 14C). These data clearly indicate that *St*AOS2-GFP, while overexpressed in a closely related Solanaceous plant, is targeted to chloroplasts. Correct subcellular localization is therefore fulfilled as a pre-requisite for the functionality of the potato AOS orthologue.

3.4.5. Complementation of *Arabidopsis thaliana aos* mutant by ectopic overexpression of *StAOS2*

In *Arabidopsis*, *AOS* is a single-copy gene, and no other enzyme has been reported to replace AOS activity. *aos* mutant has a visible phenotype, showing severe male sterility, due to a complete block in JA biosynthesis. The flowers of *aos* fail to develop siliques, as a result of various defects in anthers and pollen formation. Importantly, this drastic phenotype can be fully compromised by exogenous application of JA or its derivatives (Park et al. 2002).

Transformation

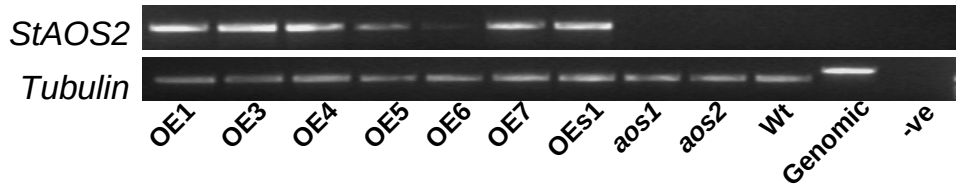
The Arabidopsis *aos* knock-out mutant plants were transformed by the inflorescence dipping method. The *Agrobacterium tumefaciens* strain used carried a fusion construct *2x35S::StAOS2-terminator* (Fig. 15A), together with the marker gene *PAT*, conferring resistance to the herbicide Basta. *PAT* encodes phosphinotricin acetyl transferase, a protein that inactivates phosphinotricin, the active component of the herbicides Bialaphos and Basta (Thompson and Seto 1995).

Male-sterile *aos* plants were sprayed with 1.5 mM jasmonic acid during the entire flowering period, before and after the transformation event, in order to allow formation of viable pollen grains and proper seed set. The seeds obtained were grown in soil under greenhouse conditions. Seedlings at cotyledon stage were repeatedly sprayed

with 0.1% Basta solution. Recovered Basta-resistant plants were subjected to further, molecular analyses.

A.

B.



C.

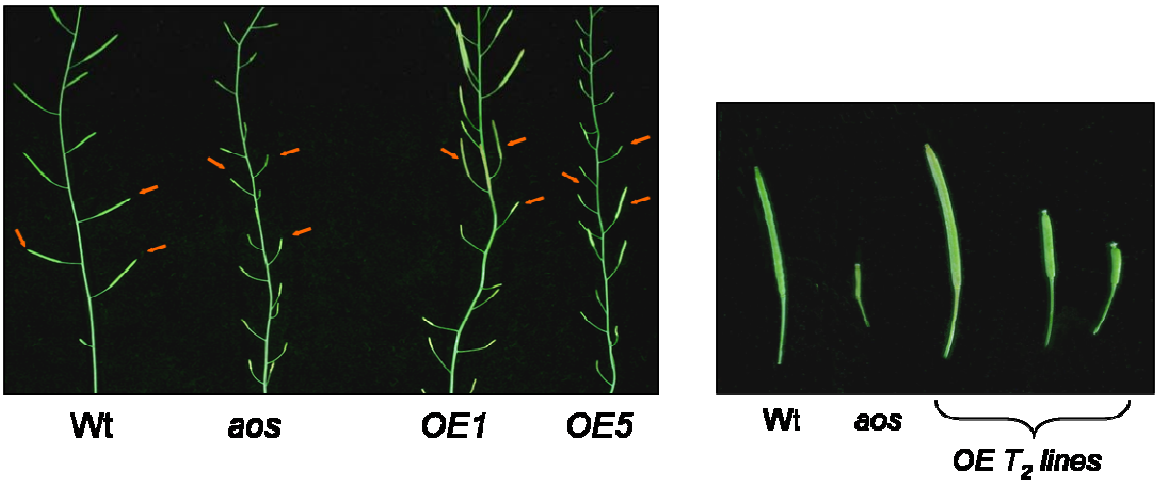


Fig. 15. Overexpression of *StAOS2* in the *Arabidopsis thaliana aos* mutant. A full-length coding sequence of *StAOS2* was 3´-terminally fused with a synthetic terminator and the entire construct was driven by a double CaMV *35S* promoter (A).

Recovered transgenic lines displayed differential levels of *StAOS2* - specific transcript, while no endogenous *AtAOS* expression could be detected by RT-PCR (B). OE1-OE7 and OEs1: various independent *StAOS2* overexpressor lines, *aos1* and *aos2*: the *A. thaliana aos* mutant, Wt: *A. thaliana* wild type Col-*6*, genomic: gDNA of *A. thaliana* Col-*6*, -ve: negative (water) control. Phenotypical analysis of independent transformants in T_1 and T_2 generations revealed that *StAOS2* can partially complement the male sterility, restoring the seed production at later stages of flowering (C).

Detection of *StAOS2* transcript by RT-PCR

RNA was extracted from rosette leaves of 4-week-old plants. After purification steps, the first strand of complementary DNA was synthesized on poly(A)$^+$ RNA template. The obtained cDNA was used for RT-PCR analyses. The potato *AOS2* transcript was detected in Arabidopsis transgenic lines using *StAOS2* - specific primers, while no endogenous *AtAOS* was found. Tubulin β-subunit specific primers were used as internal control for the experiment, in order to adjust the loading volumes and to exclude the possibility of genomic DNA contamination in the template used. A number of lines showed differential levels of *StAOS2* transcript (Fig. 15B).

Phenotypic analysis of complementation lines – siliques, pollen

Over-expression of *StAOS2* in the *A. thaliana aos* mutant resulted in partial complementation of the severe male-sterile phenotype. Initially, the siliques of the complementation plants were clearly short and contained no seeds, like in the case of mutant (Fig. 15C). Later on in the flowering period, plants were developing longer siliques, but still not all of them displayed the length observed in wild type (Fig. 15C). In order to find out what was the actual reason for this incomplete complementation, the structure of flowers was examined in detail. The Arabidopsis *aos* mutant has a very characteristic defect in anther formation: the structures are drastically retarded in development and do not reach the height required to get in touch with the stigma, which is the mechanism preventing self-pollination. However, in flowers of the complementation lines, no more abnormalities could be detected (Fig. 16, left panel).

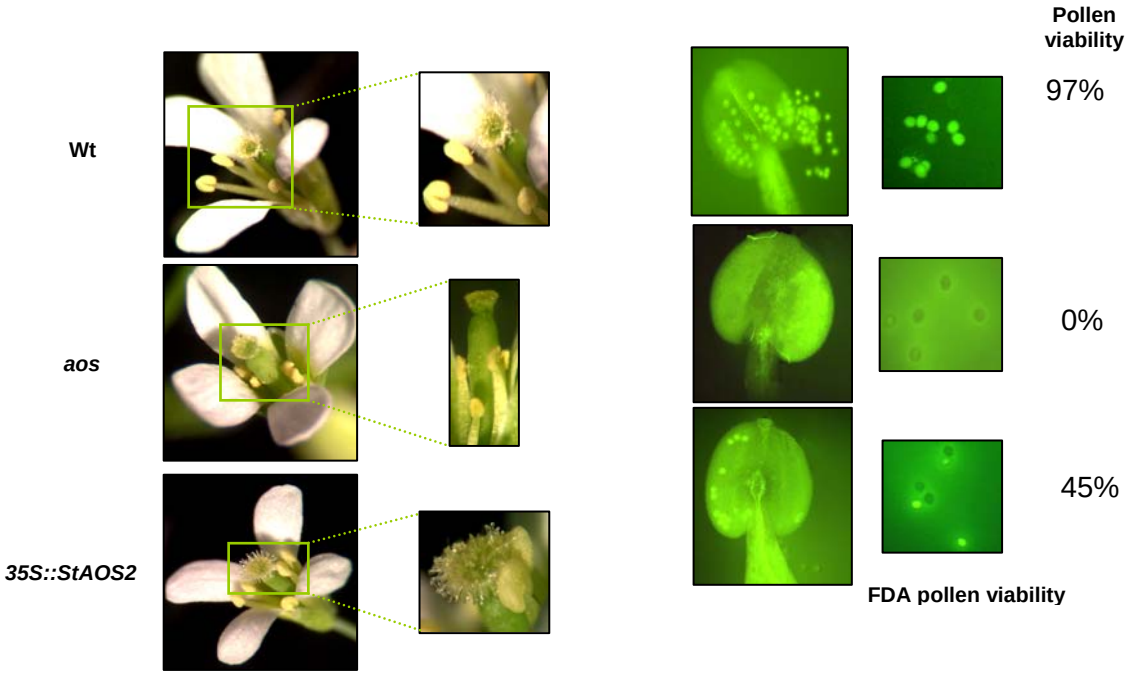


Fig. 16. *Arabidopsis thaliana* floral organ structure in wild type (Wt), *aos* mutant and complementation plants (a typical flower was chosen for each of the lines)

Left panel: anthers of wild type plants are fully developed and long enough to get in touch with stigmatic papillae. Anthers of *aos* mutant are much shorter, which prevents the self-pollination. Fifty flowers of various *35S::StAOS2* complementation lines display fully restored wild-type like phenotype of anthers.

Right panel: Pollen viability was examined using the FDA staining test. While 97% of wild type pollens are fully viable, *aos* mutant produces no viable pollen grains at all. However, the *35S::StAOS2* complementation lines display only partial restoration of pollen fertility (~45%).

In order to find the explanation for the partial sterility, viability of pollen produced by the complementation lines was investigated. Pollen grains were stained with the FDA (fluoresceine diacetate), which is a substrate processed by an esterase in a living cell. Released fluoresceine results in a bright fluorescence of a viable pollen grain and proves the intactness of the plasma membrane. The pollen was taken from five independent *35S::StAOS2* complementation lines and stained. The viability of stained pollen grains was inspected under a fluorescence microscope. The count of viable

pollen grains revealed that on average 45% of the pollen coming from complementation lines had restored fertility (Fig. 16, right panel).

3.4.6. Analysis of *StAOS2* linkage to quantitative resistance against *P. infestans* and *E. carotovora* ssp. *atroseptica*

Sequencing of 13 potato genotypes and deduced alleles of *StAOS2*

A panel of 13 unrelated potato genotypes (representing a population of 38 homologous chromosomes) was assembled in order to gain an initial insight into *StAOS2* polymorphism among different *S. tuberosum* cultivars. The panel contained the diploid genotypes G87, PX, P3, P38, I88, DG83, DG81, and the tetraploid genotypes Desirée, Leyla, Nikita, Escort, NK5 and NK6. This set of accessions was used for amplification and sequencing a fragment of *StAOS2*, located in the central part of the gene (between 490 and 1062 bp).

Remarkably, a 1.5-fold higher than reported by Rickert et al. (2003) variability of this locus (1/14 bp) was detected while analyzing the single nucleotide polymorphisms within the selected fragment, even though the affected region is entirely coding sequence. Altogether, 22 different SNP positions were found within the amplicon. Interestingly, some of them result in non-conserved amino acid exchanges. Haplotype models were deduced using this sequence information. Solution of the haplotype models has been attempted, and a model of 13 putative alleles of *AOS2* was designed (Fig. 17). Sequences of alleles 1, 6, 7, 8 and 12 were subsequently confirmed by cloning of full-length *StAOS2* gene variants from corresponding potato genotypes.

		***StAOS2* SNP positions**								
Genotype	Allele	SNP670	SNP682/683	SNP781	SNP799	SNP857	SNP866	SNP916	SNP949	SNP973
I88	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
I88	5	Thr	Thr	Thr	Phe	Ser	Ala	Ile	Leu	Leu
G87	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
G87	7	Ala	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
PX	12	Thr	Thr	Thr	Phe	Ser	Ala	Ile	Met	Leu
PX	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
Leyla	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Leyla	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Leyla	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
Leyla	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
Nikita	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Nikita	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Nikita	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
Nikita	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
NK6	9	Thr	Ser	Thr	Phe	Asn	Val	Ile	Met	Met
NK6	3	Thr	Ser	Thr	Phe	Ser	Ala	Leu	Met	Leu
NK6	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
NK6	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
NK5	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
NK5	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
NK5	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
NK5	2	Thr	Gly	Thr	Phe	Ser	Ala	Leu	Met	Leu
Escort	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Escort	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
Escort	13	Thr	Thr	Thr	Phe	Asn	Ala	Ile	Met	Leu
Escort	11	Thr	Gly	Thr	Phe	Asn	Val	Leu	Met	Met
DG81	10	Thr	Gly	Thr	Phe	Asn	Val	Leu	Met	Met
DG81	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
DG83	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
DG83	8	Thr	Ile	Ala	Leu	Asn	Val	Leu	Met	Leu
P3	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
P3	4	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Val
P38	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
P38	4	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Val
Desirèe	1	Thr	Thr	Thr	Phe	Ser	Ala	Leu	Met	Leu
Desirèe	2	Thr	Gly	Thr	Phe	Ser	Ala	Ile	Met	Leu
Desirèe	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met
Desirèe	6	Thr	Thr	Thr	Phe	Asn	Val	Leu	Met	Met

Fig. 17. Deduced amino acid *StAOS2* haplotypes found in a panel of 7 diploid and 6 tetraploid potato genotypes. Every row represents a chromosome (2 or 4 per potato cultivar, depending on the ploidy level), and each color symbolizes a different haplotype.

SNP polymorphism analyses in 2 diploid potato populations

In order to test the linkage of *StAOS2* to disease resistance factors, 2 unrelated potato mapping populations were selected, on the basis of resistance QTL present on chromosome XI positionally overlapping with the *StAOS2* locus, namely the 'GC' and 'Erwinia' (2.1.1.).

Linkage of selected *StAOS2* alleles to pathogen resistance

Single Nucleotide Polymorphisms, segregating in the hybrid progeny and originating from either the female, or the male parent were scored as present or absent. The phenotypic data evaluated in the 'GC' and 'Erwinia' populations were in a ranking order (scores from 1 to 9 and from 1 to 5, respectively). By definition, in a F_1 progeny of non-inbred diploid parents up to four alleles may segregate at each genetic locus. If the female parent has alleles *a* and *b* and the male parent has alleles *c* and *d*, four genotype classes segregate in F_1 with allele combinations *a*c, *ad*, *bc* and *bd* in the expected ratio of 1:1:1:1. The four genotype classes can be distinguished by scoring at least two polymorphic SNPs for presence or absence, one *(a* or *b*) of the female parent and another one *(c* or *d*) of the male parent.

The non-parametric Kruskal-Wallis and one-way ANOVA tests using SPSS software release 10.0 for Windows (SSPS Inc., Chicago, IL, USA) were performed to analyze differences between means of the four F_1 genotype classes *ac*, *ad*, *bc* and *bd*.

The linkage of *StAOS2* polymorphism to pathogen resistance could be reliably tested for 79 out of 80 individuals for the 'GC' population and 138 out of 158 individuals for the 'Erwinia' population. The missing scores were caused by problems with PCR reactions and sequencing, or incomplete phenotypic data. However, for both populations the number of individuals available was sufficient to reliably perform the statistical analyses.

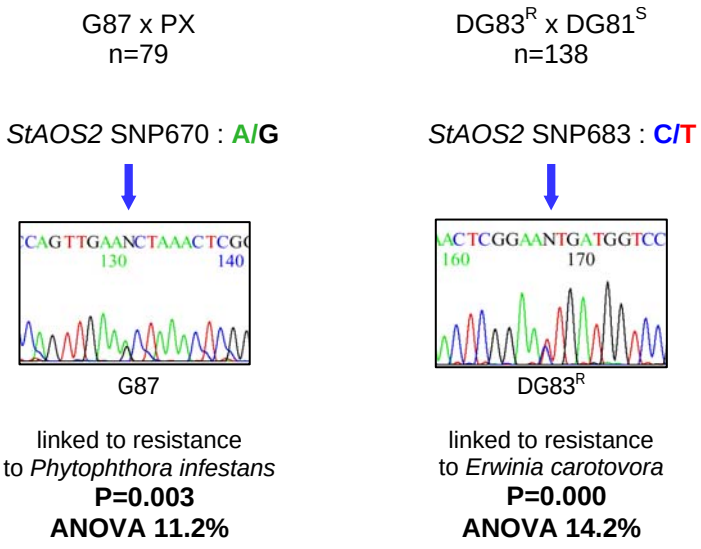


Fig. 18. Single Nucleotide Polymorphisms were scored in the entire 'GC' (G87 x PX) and 'Erwinia' (DG83 x DG81) population. SNP670 in the 'GC' population and SNP683 in the 'Erwinia' population were tightly linked to *P. infestans* and *E. carotovora* ssp. *atroseptica* resistance, respectively. Analysis of variance (ANOVA) showed the percentage of explained disease resistance.

Diagnostic SNPs for each deduced haplotype were scored in the 'GC' and 'Erwinia' populations. In both cases a very strong linkage of *StAOS2* to the resistance was detected, with *P*-values 0.003 for 'GC' population and 0.000 for 'Erwinia'. The analysis of variance (ANOVA) revealed that polymorphism of *StAOS2* explains 11.2 and 14.2 % of the total disease resistance to *Phytophthora infestans* and *Erwinia catotovora* ssp. *atroseptica*, respectively (Fig. 18). The Kruskal-Wallis statistical analysis showed that presence of haplotype "1" versus "8" (both coming from the resistant seed parent) results in increased resistance to *Erwinia*, with the mean ranks of phenotypic scores for genotype groups containing haplotype "1" being 1.4-fold higher than for haplotype "8". Haplotypes 12 and 13, inherited from the susceptible pollen parent DG81, had no significant effect on the resistance levels (Table 8). Similarly in the 'GC' population, occurrence of maternally inherited haplotype "6" was linked to 1.4-fold higher levels of foliage resistance to late blight, than presence of the

counterpart haplotype "7". Again, the paternal haplotypes "15" and "16" didn't display any differential effect in terms of resistance (Table 9).

SNP	SNP670 (GC pop -♀)	SNP670 (GC pop -♀)	SNP973 (GC pop -♂)	SNP973 (GC pop -♂)
Phenotypic dataset	PIF_96	PIF	PIF_96	PIF
T-Test	0.003	0.004	n.s.	n.s.
ANOVA	0.004	0.005	n.s.	n.s.

Table 8. Statistical significances for two SNP markers scored in the 'GC' population: SNP670 (maternally inherited) and SNP973 (paternally inherited), calculated using the T-Test and one-way ANOVA (SPSS Release 10.0).

SNP	SNP683 (Erwinia pop -♀)	SNP683 (Erwinia pop -♀)	SNP683 (Erwinia pop -♀)	SNP714 (Erwinia pop -♂)	SNP714 (Erwinia pop -♂)	SNP714 (Erwinia pop -♂)
Phenotypic dataset	L96	L95_96	L94_96	L96	L95_96	L94_96
T-Test	0.025	0.040	0.000	n.s.	n.s.	n.s.
K-W	n.s.	n.s.	0.002	n.s.	n.s.	n.s.

Table 9. Statistical significances for two SNP markers scored in the 'Erwinia' population: SNP683 (maternally inherited) and SNP714 (paternally inherited), calculated using the T-Test and non-parametric Kruskal-Wallis test (SPSS, Release 10.0).

3.4.7. Molecular cloning of selected *StAOS2* alleles

Basing on the statistical data described above, it became clear that presence of haplotypes "6" and "1" increases resistance to *P. infestans* and *E. carotovora* ssp. *atroseptica*, respectively. The haplotype is defined here as "pattern of co-occurrence of

variant sites on the same chromosome" (and therefore within each particular gene). One could assume that polymorphism within the functional gene, which *StAOS2* was proven to be, can lead to the generation of functional variants (= alleles). Therefore, efforts were made to clone the full length sequences of these "quantitatively resistant" alleles, as well as their "quantitatively susceptible" counterparts. This would be a pre-requisite for further functional analyses of *StAOS2* variants, in order to demonstrate whether the differences in the coding sequence are indeed underlying the postulated QTL effect.

Cloning strategy

Five alleles of *StAOS2* were selected to be included into the experiment: "1" and "6", being the "quantitatively resistant" variants with respect to *E. carotovora* ssp. *atroseptica* and *P. infestans* resistance, "8" and "7" – their "quantitatively susceptible" counterparts, and allele "12", which displayed no significant effect on resistance in 'Erwinia' population, but was chosen as an internal control for the subsequent experiments. The alleles were cloned using the PCR-based approach. Gateway Technology-compatible primers were designed to flanking regions of the full-length *StAOS2* sequence, available in the GenBank (Acc. No AJ457081). With this set of primers, alleles "1", "6" and "7" were amplified, on templates of genomic DNAs of DG83 and G87 potato genotypes, respectively. PCRs were carried out using stringent reaction conditions (low $MgCl_2$ and dNTP concentration, low cycles number) and a proof-reading high fidelity *Taq*-polymerase. However, despite multiple attempts, this primer set failed to amplify the remaining alleles "8" and "12". A likely explanation of this fact would be presence of natural polymorphism within the primer annealing sites, which results in creation of mismatch(es) and subsequent failure of the amplification. Therefore, primers were designed, that were anchored within 5´and 3´ UTRs of the gene. Two types of amplicons were generated, covering either the neighborhood of the START codon or the STOP codon (Fig. 19).

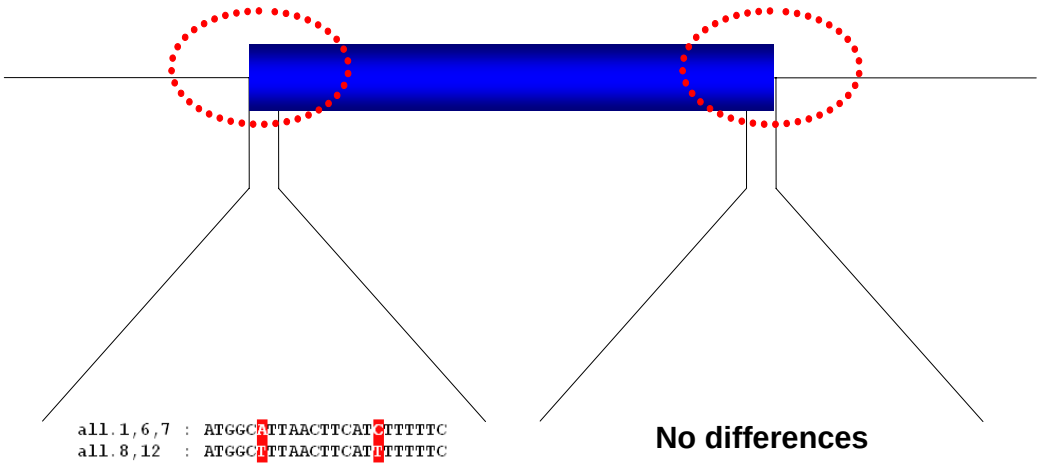


Fig. 19. A schematic drawing of *StAOS2* gene structure, including the 5´ and 3´ untranslated regions. Two Single Nucleotide Polymorphisms were found within 20 base pairs downstream from the ATG Start-codon.

The amplicons were generated on templates of genomic DNAs of three independent 'Erwinia' progeny genotypes, carrying the combination of alleles "8" and "12" ('Erwinia' F_1 10, 14 and 22). Subsequently, the amplicons were cloned and sequenced. While the 3´portion of the gene did not show any polymorphic residue among all the alleles analyzed, the 5´ region was demonstrated to contain two nucleotide exchanges within the forward primer annealing site, being specific to both "8" and "12" alleles. Another, specific forward primer was then designed and used for successful PCR amplification of alleles "8" and "12". For this purpose the "Erwinia" progeny genotype "14" was used.

All the alleles obtained were cloned into the Gateway *pDONR201* vector. Ten independent clones per construct were sequenced in order to obtain a consensus. In each case, the cloned fragment represented an uninterrupted open reading frame and supported the predicted haplotype structure. All the alleles display a high structural homology to the *StAOS2* gene, but are clearly distinct from each other.

Sequences alignment and discussion of the amino acid substitutions and possible influence on enzymatic activity

The five *StAOS2* alleles were further sequence-characterized. Deduced amino acid sequences were subjected to various prediction tools, available on-line. A summary of the sequence comparisons between the five alleles and their Arabidopsis counterpart is presented in the Table 10.

Feature	*StAOS2-1*	*StAOS2-6*	*StAOS2-7*	*StAOS2-8*	*StAOS2-12*	*AtAOS*
Number of amino acids	509	509	510	510	510	518
Molecular weight (kDa)	57.2912	57.2812	57.3674	57.5326	57.4654	58.1969
Theoretical pI	8.91	8.91	8.82	8.91	8.83	8.75
Instability index	35.43 (stable)	35.85 (stable)	37.0 (stable)	35.82 (stable)	36.99 (stable)	39.64 (stable)
Aliphatic index	82.32	81.36	83.69	83.29	81.76	85.83
GRAVY[3]	-0.177	-0.181	-0.162	-0.157	-0.165	-0.232
% similarity to *At*AOS	78	78	79	78	79	100
No. of putative phospho-sites[1]	24	24	21	23	23	20
No. of put. N-Glc sites[1]	11	11	12	12	12	11
No. of put. O-ß-GlcNAc sites[1]	7	7	8	5	6	7
No. of put. Tyr sulfation sites[1]	2	2	0	0	0	3
No. of put. sumoylation sites[2]	5	5	5	5	4	5

Table 10. Comparison of sequence features between the deduced amino acid sequences of five *St*AOS2 alleles and the *Arabidopsis thaliana* AOS.

[1] – cut-off value: 0.5

[2] – cut-off value: 0.65

[3] – **gr**and **av**erage of h**y**dropathicity

A deduced protein sequences multialignment was generated (Fig. 19). Analysis of sequence polymorphisms revealed that 22 amino acid residues are subject to natural variation. Of these, 16 are non-conserved substitutions. Interestingly, an insertion/deletion of a Glutamate was detected within the cyt. P450 domain (position 440).

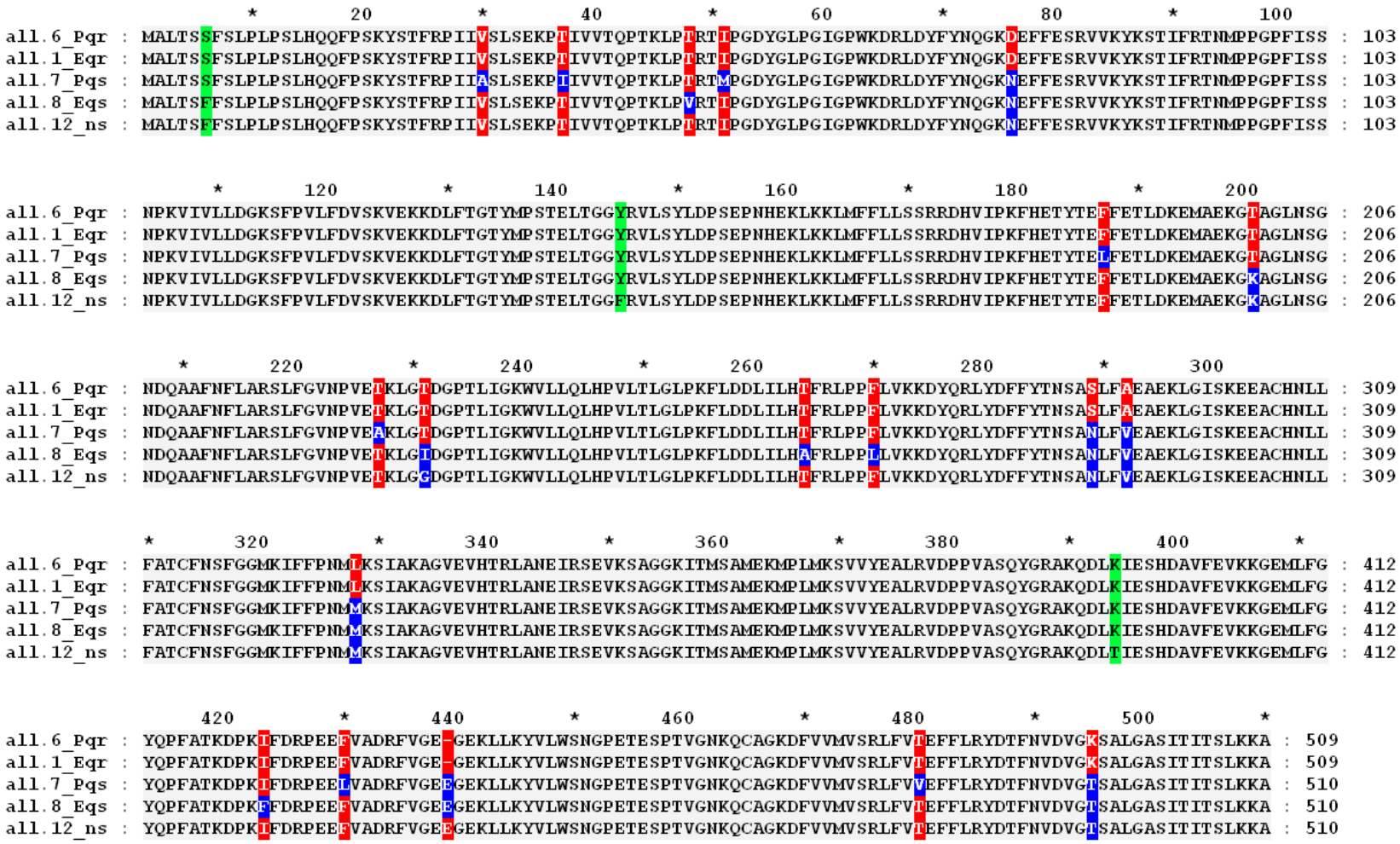


Fig. 19. Alignment of deduced amino acid sequences of five cloned *StAOS2* alleles. Residues characteristic of quantitatively resistant alleles are highlighted in red, residues characteristic of quantitatively susceptible alleles are marked in blue, other amino acid substitutions are shown in green.

	Amino acid position																								
Allele	**23**	**31**	**33**	**37**	**44**	**123**	**149**	**154**	**170**	**171**	**183**	**184**	**190**	**205**	**227**	**276**	**284**	**300**	**348**	**352**	**360**	**418**	**454/455**	**487/488**	**505/506**
StAOS2-1	+	+	+	+	-	+	+	+	+	+	+	±	+	+	+	±	±	+	+	+	+	+	+	+	+
StAOS2-6	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	±	±	+	+	+	-	+	+	+	+
StAOS2-7	+	+	+	-	-	+	+	+	+	+	+	±	-	+	-	±	±	+	+	+	+	+	+	+	+
StAOS2-8	+	+	+	+	-	+	+	+	+	+	+	±	+	+	-	±	±	+	+	+	+	+	+	+	+
StAOS2-12	+	+	+	+	-	+	+	+	+	+	+	±	+	+	-	±	±	+	+	+	+	+	+	+	+

Fig. 20. Putative phosphorylation sites in *StAOS2* alleles sequences: ± **- Ser,** + **- Thr,** ± **- Tyr**

	Amino acid position											
Allele	**72**	**157**	**204**	**207**	**213**	**286**	**289**	**307**	**326**	**344**	**450/451**	**489/490**
StAOS2-1	+	+	+	+	+	+	-	+	+	+	+	+
StAOS2-6	+	+	+	+	+	+	-	+	+	+	+	+
StAOS2-7	+	+	+	+	+	+	+	+	+	+	+	+
StAOS2-8	+	+	+	+	+	+	+	+	+	+	+	+
StAOS2-12	+	+	+	+	+	+	+	+	+	+	+	+

Fig. 21. Putative N-Glycosylation sites in *StAOS2* alleles sequences: + **- Asn**

	Amino acid position				
Allele	**75**	**273**	**390**	**394**	**405**
StAOS2-1	+	+	+	+	+
StAOS2-6	+	+	+	+	+
StAOS2-7	+	+	+	+	+
StAOS2-8	+	+	+	+	+
StAOS2-12	+	+	+	-	+

Fig. 22. Putative sumoylation sites in *StAOS2* alleles sequences: + **- Lys**

	Amino acid position	
Allele	**69**	**71**
StAOS2-1	+	+
StAOS2-6	+	+
StAOS2-7	-	-
StAOS2-8	-	-
StAOS2-12	-	-

Fig. 23. Putative Tyrosine sulfation sites in *StAOS2* alleles sequences: + **- Tyr**

	Amino acid position							
Allele	**4**	**33**	**41**	**44**	**48**	**93**	**139**	**454/455**
StAOS2-1	+	+	+	+	-	+	+	+
StAOS2-6	+	+	+	+	-	+	+	+
StAOS2-7	+	+	+	+	+	+	+	+
StAOS2-8	-	+	-	+	-	+	+	+
StAOS2-12	-	+	+	+	-	+	+	+

Fig. 24. Putative O-ß-GlcNAc sites in *StAOS2* alleles sequences: + **- Ser,** + **- Thr**

Moreover, the prediction of various post-translational modifications shows differences in putative phosphorylation sites (Fig. 20), N-glycosylation sites (Fig. 21), so called Yin-Yang (potential phosphorylation and O-β-glycosylation) sites (Fig. 24), as well as Tyrosine sulfation (Fig. 23) and sumoylation sites (Fig. 22). All these alterations can obviously affect protein solubility, stability, local structure etc., and as a consequence, result in differential levels of enzymatic activity.

3.4.8. Complementation of *Arabidopsis thaliana aos* knock-out by different alleles of *StAOS2* under *AtAOS* promoter

In order to reveal whether the allelic variation encoded at *StAOS2* locus can indeed result in altered enzymatic activity of *AOS2*, subsequent experiments were necessary.
A. thaliana contains only a single copy *AOS* gene, and no other enzyme is capable of replacing the *At*AOS *in planta*. This makes Arabidopsis a perfect biological system to perform a quantitative complementation assay of the *aos* mutant.
The optimal experimental approach for assessing the mechanism underlying the possible QTL effect, would be to complement the Arabidopsis *aos* mutant with the five potato *StAOS2* alleles, driven by the same *AtAOS* promoter, and in parallel test the complementation using the *AtAOS* gene driven by the five potato *StAOS2* allele-specific promoters. Basing on the data on *StAOS2* expression, available at the TIGR database (TIGR SGEdb, study ID 62, probe STMCR05), no significant up-regulation of the gene was detected upon *P. infestans* treatment in potato. Therefore, an assumption has been made that the postulated QTL effect is likely due to differences within the coding part of the *StAOS2* gene.
For this purpose, each allele of *StAOS2* was to be independently transformed into the *aos* knock-out background, in order to quantitatively assess the level of complementation. Use of the *CaMV 35S* promoter is not recommended in such cases, as numerous artefacts can occur, caused by ectopic over-expression, and the quantitative effect could be overwritten by high dosages of protein. Instead, the native regulatory sequences of *AtAOS* constitute an ideal system, allowing proper spatial and temporal regulation of gene expression, so that the plant could react to all stimuli known to induce jasmonate responses in an appropriate manner. Hence, cloning of *AtAOS* promoter region was necessary as a pre-requisite for this experiment.
The *AtAOS* promoter from Col-*0* has been cloned and initially characterized (Kubigsteltig et al. 1998) (Fig. 25). In this study, a full-length 5´ regulatory region of *AtAOS*, 2590 base pairs in length, was cloned from *A. thaliana* Col-*6*, which is the background of the *aos* mutation. The promoter sequences of *AtAOS* share ~99.5%

homology between the two closely related accessions Col-*0* and Col-*6*. The Col-*6* *AtAOS* promoter was cloned to the Three-Fragment MultiSite Gateway – compatible vector *pDONR™P4-P1R*, in order to facilitate generation of the following constructs.

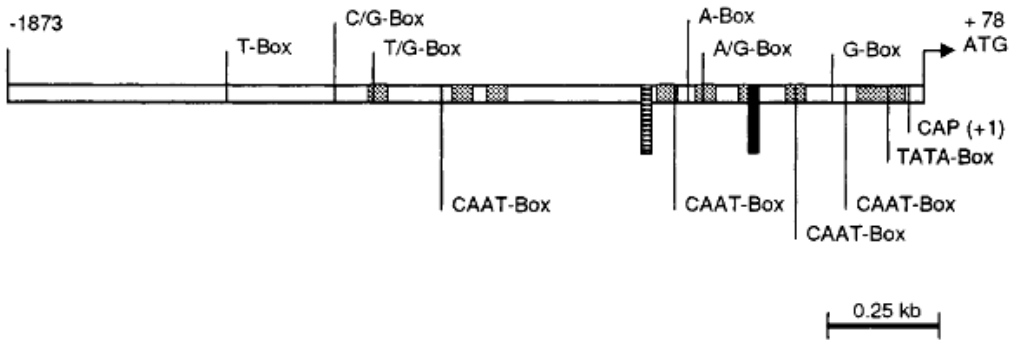


Fig. 25. Schematic representation of the *Arabidopsis thaliana* Col-*0* *AOS* upstream regulatory region (after Kubigsteltig et al. 1998).

Subsequently, a set of MultiSite Gateway constructs was generated, covering the five selected alleles of *StAOS2*, all driven by the native Arabidopsis AOS promoter. As a positive control, native Arabidopsis *AOS* gene was cloned behind the *AtAOS* promoter. The non-significant *StAOS2* allele "12" served as an internal control. Finally, the negative control was generated using the *smGFP* gene (small modified Green Fluorescent Protein), fused downstream of the *AtAOS* promoter (Fig. 26).

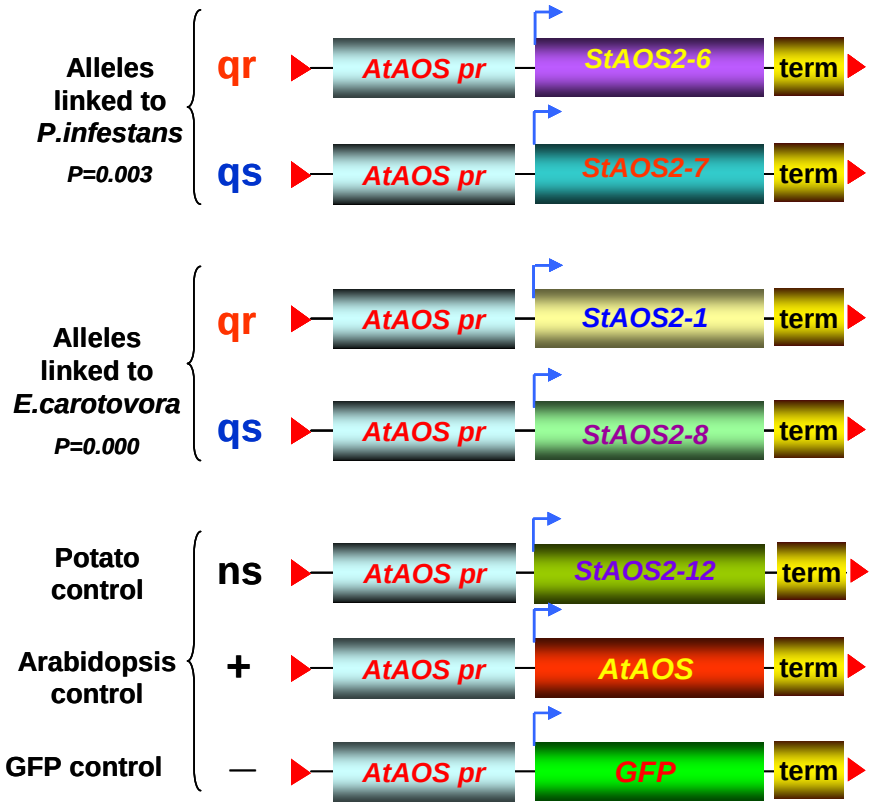


Fig. 26. Scheme of Gateway MultiSite constructs, generated for the purpose of allele-specific complementation of *A. thaliana aos* mutant with series of *StAOS2* variants, including appropriate controls.

This set of Arabidopsis *StAOS2* - expressing lines can be used to evaluate the complementation effect in a quantitative manner, to detect even slight differences between the alleles, which can be assessed by analysis of levels of OPDA and jasmonic acid levels in these plants.

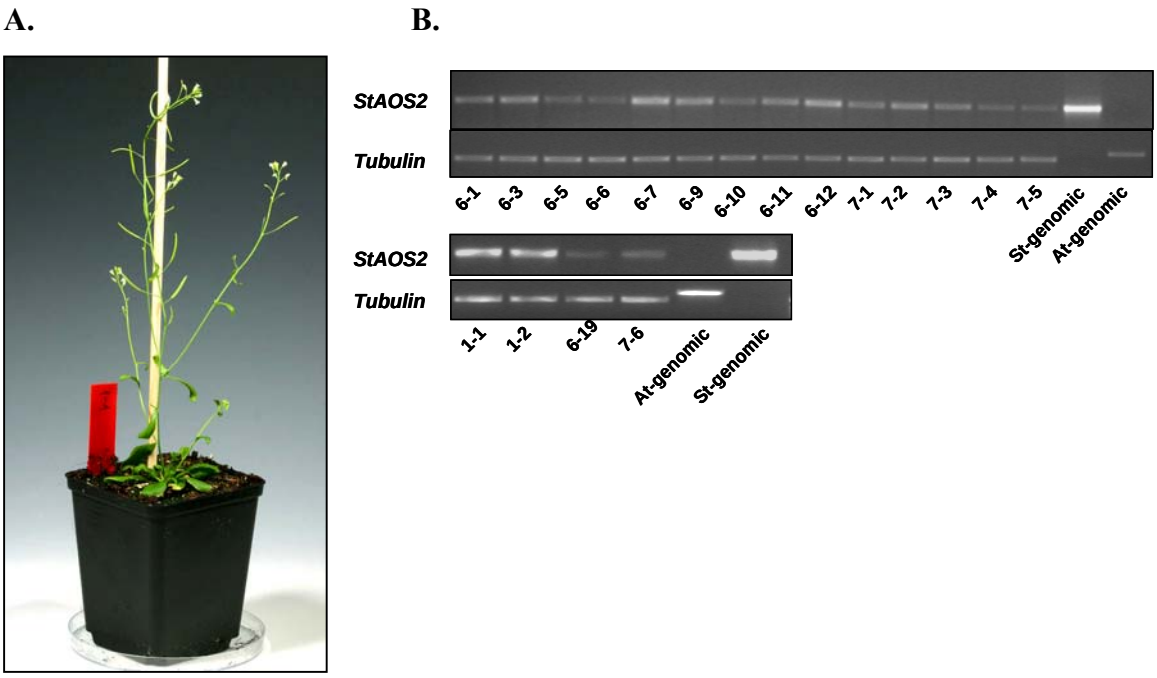


Fig. 27. Allele-specific complementation of Arabidopsis *aos* mutant. (A) All the T_1 and T_2 lines display proper complementation on morphological level, i.e. no anomalies in flower and silique development or in the seed content. Line 1-1 is presented on the picture. (B) *StAOS2* transcripts were detected in recovered transgenic lines by RT-PCR. Levels of *StAOS2* expression differ between lines, which could be explained by positional effects of T-DNA integration in the Arabidopsis genome.

Transformants were selected on the herbicide Basta and molecularly characterized for the presence and quantity of potato-specific *AOS2* transcript (Fig. 27B).

The Basta-survived plants containing the transgene showed differential complementation in terms of male fertility restoration and seed production, ranging from full (Fig. 27A) to partial fertility. The reason for this might be differential functionality of the *StAOS2* alleles. This remains to be tested experimentally. The investigation of transgenic lines performance upon stimuli known to induce JA-related responses (e.g. *P. syringae* pv. *tomato* strain DC3000 *avr Rpm1* or wounding) is beyond the scope of this thesis and will be a subject of future experiments.

3.4.9. Silencing of *StAOS2* in potato genotype G87

In plants, RNA interference [RNAi, also known as post-transcriptional gene silencing (PTGS) or co-suppression] is thought to be a key defense against viruses (reviewed in Waterhouse et al. 2001). Gene silencing acts as an adaptive defense against viruses, as well as a way of regulating endogenous genes. One key feature of RNAi is the production of double-stranded RNA (dsRNA) homologous to the gene being targeted for silencing. This dsRNA is degraded into approximately 21-nucleotide RNAs, known as 'small interfering RNAs' (siRNAs), by the enzyme Dicer. These siRNAs then provide specificity to the endonuclease-containing, RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation (Fig. 28).

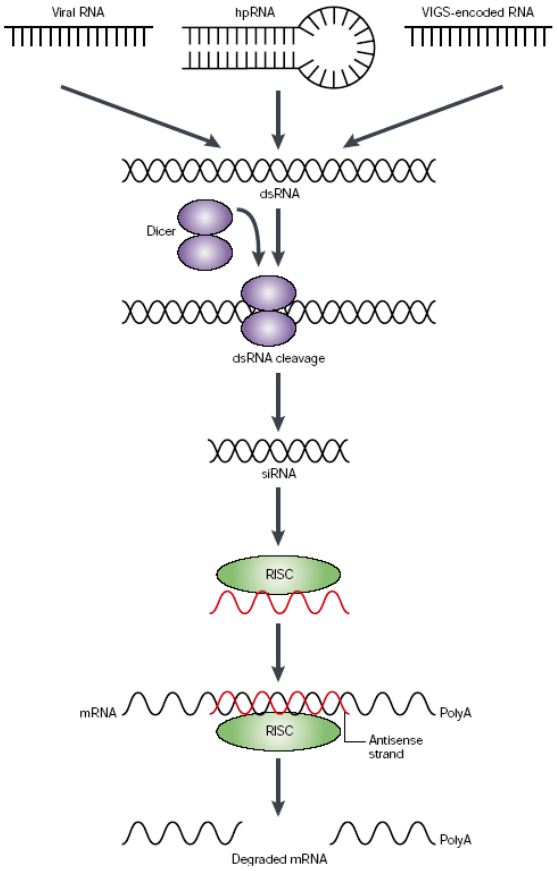


Fig. 28. The current model for RNA-mediated gene silencing in plants. This model is based on the results of in vitro studies of RNA-induced gene silencing, or RNA interference (RNAi) in animals. Double-stranded RNA (dsRNA) from replicating viral RNA, viral-vector-derived (VIGS, or virus-induced gene silencing) RNA or hairpin RNA (hpRNA) transcribed from a transgene, is processed by a Dicer-containing complex to generate siRNAs. An endonuclease-containing complex (called the RNAi-silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs, so promoting their degradation (Waterhouse et al. 2001).

For the silencing experiments, the potato genotype G87 was selected. As described above (3.4.6.), G87 is a quantitatively resistant genotype, that showed a highly significant QTL effect on late blight resistance in the region of linkage group XI where *StAOS2* maps. Therefore, one can speculate on the importance that *StAOS2* might have for *P. infestans* resistance in this particular potato background. If *StAOS2* indeed is required for (partial) resistance to late blight of this genotype, then silencing of this gene should result in increased susceptibility to the oomycete infection.

Generation of RNAi constructs and regeneration of potato transformants

For the purpose of silencing *StAOS2*, a dsRNAi Gateway technology – compatible vector *pJawohl17-RNAi* was selected. In this vector, a short fragment of gene-specific sequence can be cloned in sense and antisense orientations, with an intron present in-between (Fig. 29).

The resulting panhandle silencing constructs can be bound in the cell by the RISC complex and cleaved by Dicer, which results in generation of short interfering RNAs. The silencing constructs were designed in a way to knock-out the whole *StAOS* genes family (construct 1), only *AOS1* and *AOS2*, which have likely redundant functions (construct 2), and finally, to silence specifically only the gene of interest, *StAOS2* (construct 3) (Fig. 30).

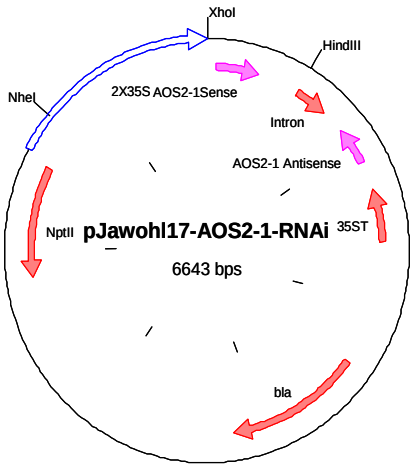


Fig. 29. The *pJawohl17* vector was used for the generation of RNAi constructs. A *StAOS2* – specific fragment is cloned in sense and antisense orientations (pink arrows), with an intron in-between (red arrow).

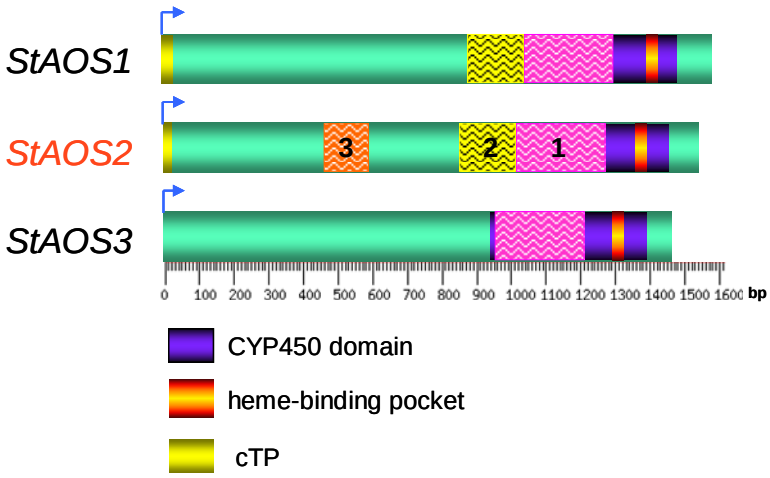


Fig. 30. Schematic representation of structural homology between three members of the potato *AOS* gene family, and regions of the gene (1, 2, 3) to which RNAi silencing was targeted.

A number of putative transgenic lines have been recovered on an antibiotic selection (Fig. 31). Plants didn't display any obvious morphological phenotype, while cultivated on a selection medium under tissue culture conditions or in a phytochamber in sterile soil. 11 months after the transformation, sufficient amount of plant material was available to perform comparative molecular analyses of *StAOS2* transcript level.

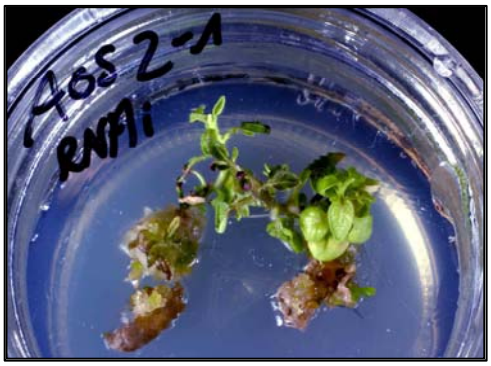
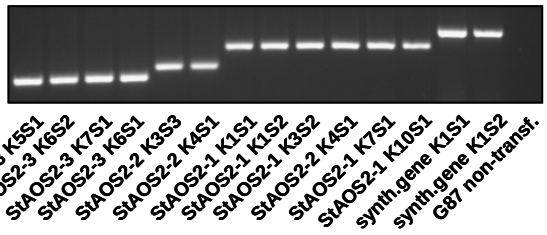


Fig. 31. Calli emerging from the transformed G87 leaves – approximately 6 months after the transformation event.

Evaluation of the silencing effect – semi-quantitative RT-PCR

46 putative transformants were evaluated for the presence of the RNAi vector sequences in the plant DNA (Fig. 32A) and the silencing effect by semi-quantitative RT-PCR (Fig. 32B).

A.



B.

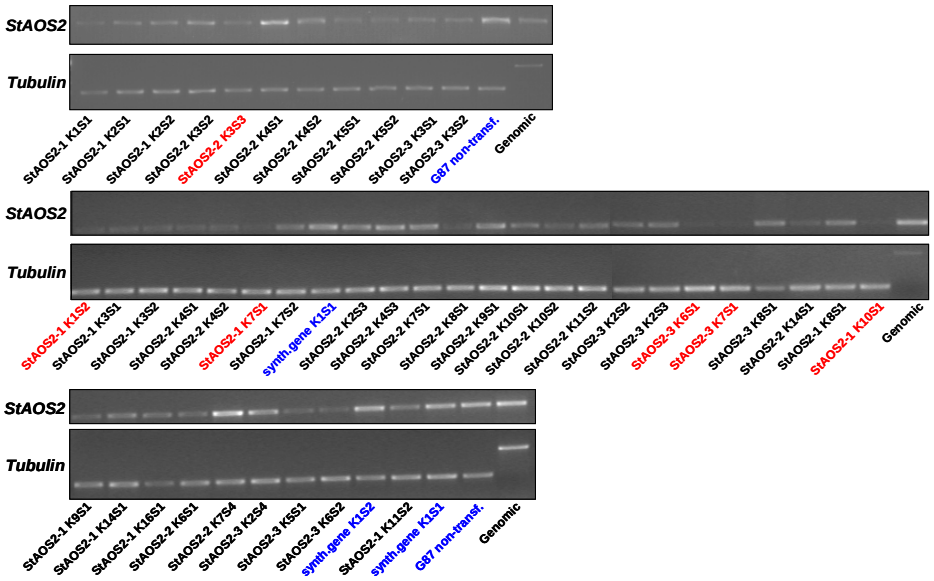


Fig. 32. (A) PCR analysis of genomic DNA isolated from selected *StAOS2*-RNAi lines, confirming presence of the construct in each plant. (B) RT-PCR analysis of 46 potato *StAOS2*-RNAi lines.

A number of tested lines showed differential levels of *StAOS2* transcript. Quantification revealed that the strongest silencing effect (below 10% of the wild-type transcript levels) has occurred in 12 independent lines. As expected, transcript levels in the synthetic gene-RNAi plants were comparable to non-transformed G87, grown

under the same conditions (Fig. 33). The six best lines were selected for further evaluation of silencing effect, in terms of possible depletion of jasmonic acid levels after the *Phytophthora*-derived elicitor Pep-13 and wounding treatments.

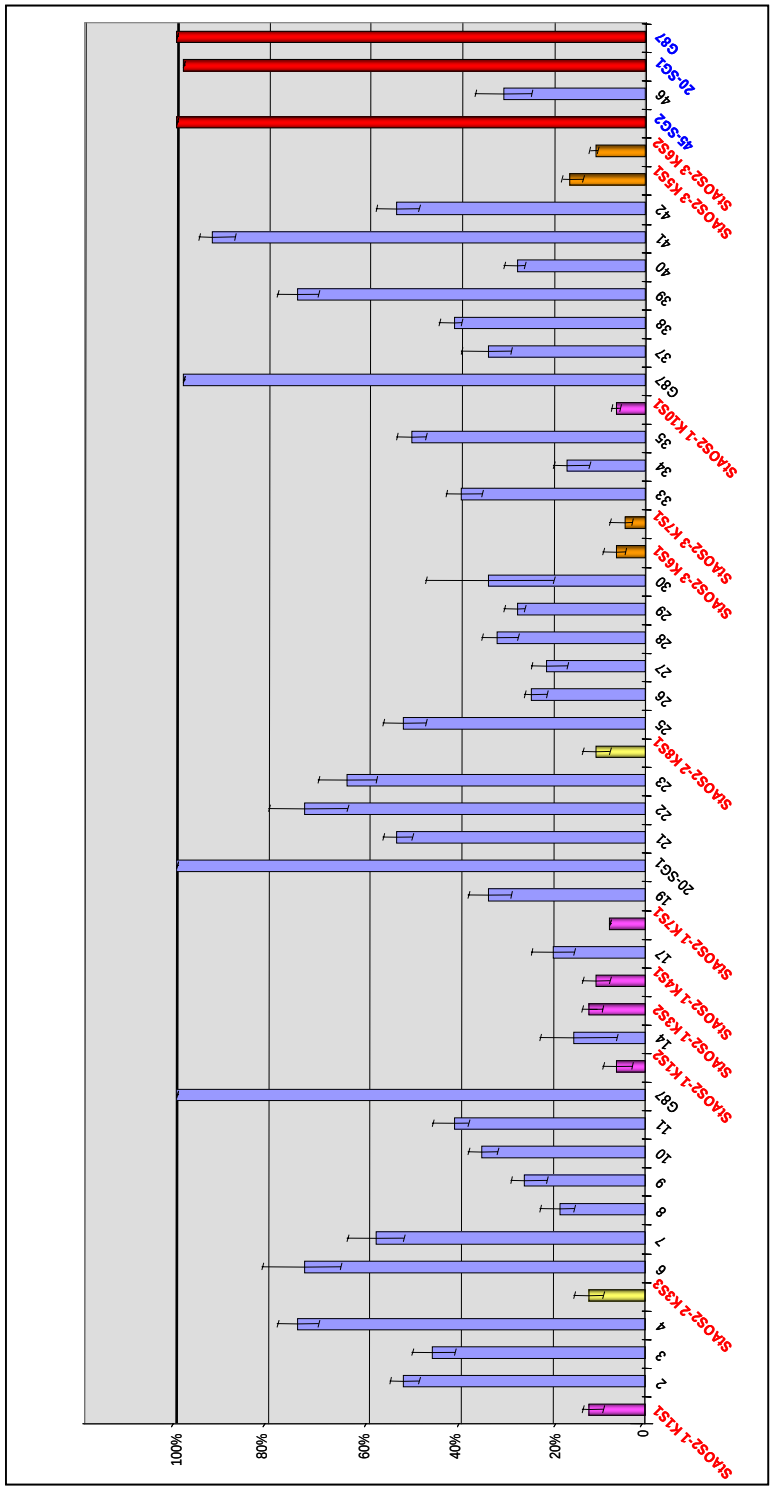


Fig. 33. Quantification of *StAOS2* transcript amounts in 46 potato RNAi lines, including the controls: the two RNAi – synthetic gene lines (SG1 and SG2), as well as non-transformed G87 plants.

Evaluation of the silencing effect – monitoring of OPDA and JA levels upon Pep-13 elicitor treatment

Potato explants were grown on MS medium under sterile conditions in a phytochamber with 16 h of light (200 microeinsteins) at 22ºC for 3 weeks. After transfer to soil, plants were kept in a phytochamber with 16 h of light (200 microeinsteins), 18ºC, and 60% humidity for 4 weeks (Fig. 34).

Fig. 34. Overwiev of the *StAOS2* – dsRNAi growth conditions (phytochamber).

6 independent dsRNAi lines in G87 background were included into the experiment. Two lines carrying the synthetic gene construct were incorporated as negative controls. In addition, non-transformed G87 and Desirée plants were also treated in an identical manner. Lower leaves were infiltrated with distilled water, while upper leaves were treated with 100µM solution of Pep-13, the pathogen-associated molecular pattern (PAMP) present in various *Phytophthora* species (Brunner et al. 2002) (Fig. 35).

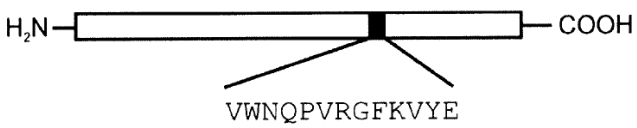


Fig. 35. Schematic representation of the position of Pep-13 sequence, isolated from *P.sojae* GP42 TGase (Brunner et al. 2002).

Pep-13 causes rapid development of lesions, associated with HR (hypersensitive response) on potato Desirée plants (Halim et al. 2004).

Plants were incubated for 48 h under the same growth conditions. While characteristic lesions were developed on Desirée plants after 18 h of treatment, no necroses could be observed either on non-transformed G87 plants, or on the *StAOS2* RNAi and synthetic gene-RNAi leaves infiltrated with the elicitor. After 48 hours, the area of necrotic spots on Desirée leaves increased, while the RNAi lines as well as the control plants displayed no symptoms of hypersensitive response (Fig. 36).

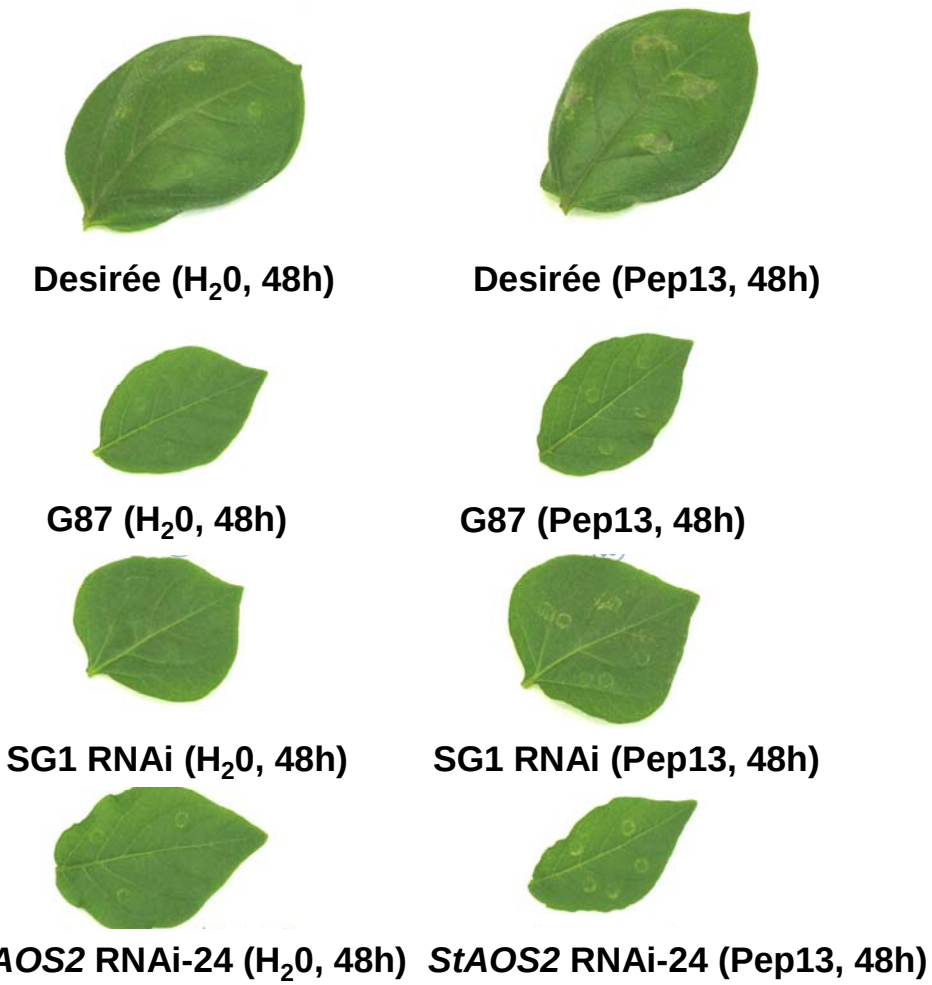


Fig. 36. Leaves of potato genotypes Desirée, G87, Synthetic Gene – RNAi and a selected *StAOS2* – RNAi line. Lesions were developed after Pep-13 infiltration only on Desirée leaves.

Leaf material was independently taken for samples treated with Pep-13 and water 48 h post infiltration. Moreover, upper leaves of plants (50% of leaf area) were wounded, and material was taken 30 min after the treatment. Jasmonic acid and OPDA-enriched fractions were purified and analysed using the Gas Chromatography – Mass Spectroscopy (GC-MS) in order to determine the content of both compounds in the plant material examined.

The measurements of OPDA and JA contents show no elevated levels of these compounds in response to Pep-13, consistently with lack of visible lesions on infiltrated leaves. Neither G87 plants, nor the Synthetic Gene controls and *StAOS2*-RNAi lines display any differential reaction to the elicitor treatment (Fig. 37).

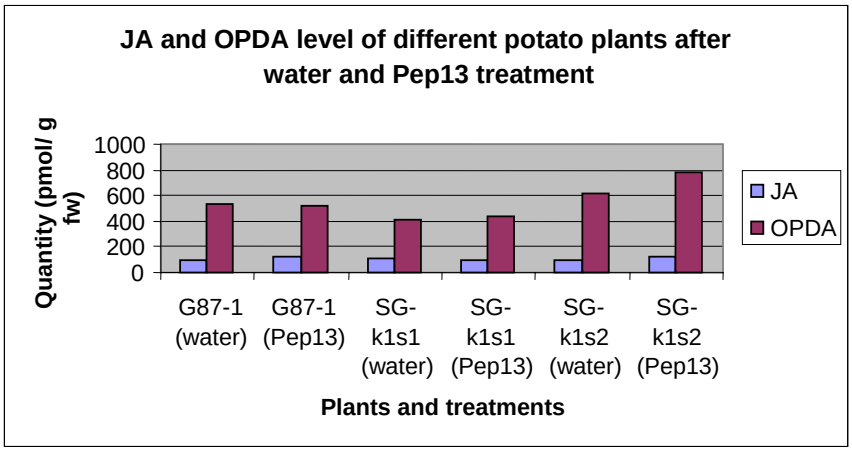


Fig. 37. Diagram showing levels of OPDA and JA in control potato plants (non-transformed G87 and Synthetic Gene – RNAi) upon water and Pep-13 treatment. Contents of both compounds remain unaltered.

Evaluation of the silencing effect – monitoring of OPDA and JA levels upon wounding treatment

Uppermost leaves of plants were wounded (approximately 50% of the leaf area) using laboratory forceps. After 30 min plant material was taken, flash-frozen and subjected to OPDA/JA extraction procedure. This time window was shown to be optimal for

monitoring of octadecanoid pathway products in a number of plant species, including Arabidopsis, tomato and tobacco. GC-MS measurements revealed that while OPDA and JA levels have rapidly increased in wounded G87 and Synthetic Gene control lines (comparing to basal levels), the set of *StAOS2*-RNAi lines show no accumulation of relevant compounds (Fig. 38), confirming the silencing efficiency, and pointing towards *StAOS2* being the major functional allene oxide synthase involved in immediate jasmonate-related stress responses in potato.

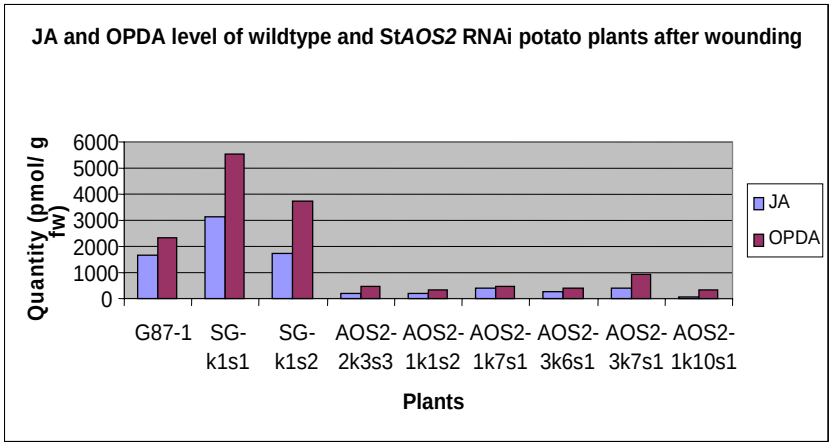


Fig. 38. Diagram showing levels of OPDA and JA in control (non-transformed G87 and Synthetic Gene – RNAi) and *StAOS2* down-regulated potato plants (lines AOS2-2 k3s3, AOS2-1 k1s2, AOS2-1 k7s1, AOS2-3 k6s1, AOS2-3 k7s1, AOS2-1 k10s1) upon wounding treatment (30 min). Contents of both compounds are significantly (down to 25 folds) decreased in *StAOS2* – RNAi plants.

3.4.10. Polymorphism of *StAOS2* among *Solanaceae*

A panel of 82 genotypes (Table 2) selected for the evaluation of *AOS2* polymorphism among *Solanaceae* family encompassed a number of both tuber-bearing and non-tuber bearing *Solanum* species as well as representatives of other genera, e.g. *Lycopersicon*, *Nicotiana*, *Capsicum*, *Petunia*. Clear singular PCR products could be obtained for each genotype tested, using the primer set described above. PCR products were sequenced and the data obtained confirmed presence of *AOS2*-like genes in the genomes of all

species included in the experiment. The sequences maintained high levels of conservation both in terms of length and nucleotide composition, and all seemed to be composed of a single exon.

However, a high number of polymorphic residues could be found. Single Nucleotide Polymorphisms within the central part of the gene were scored across the genotypes. In total, 32 SNP positions were scorable. Among 82 genotypes, 36 were homozygous for all the SNP positions analyzed. For those, partial sequences of *AOS2* alleles could be deduced (Fig. 39). Polymorphic residues are highlighted in different colors. For remaining genotypes, cloning and re-sequencing of individual cloned PCR products would be required in order to solve the haplotype structures. This stays to be examined.

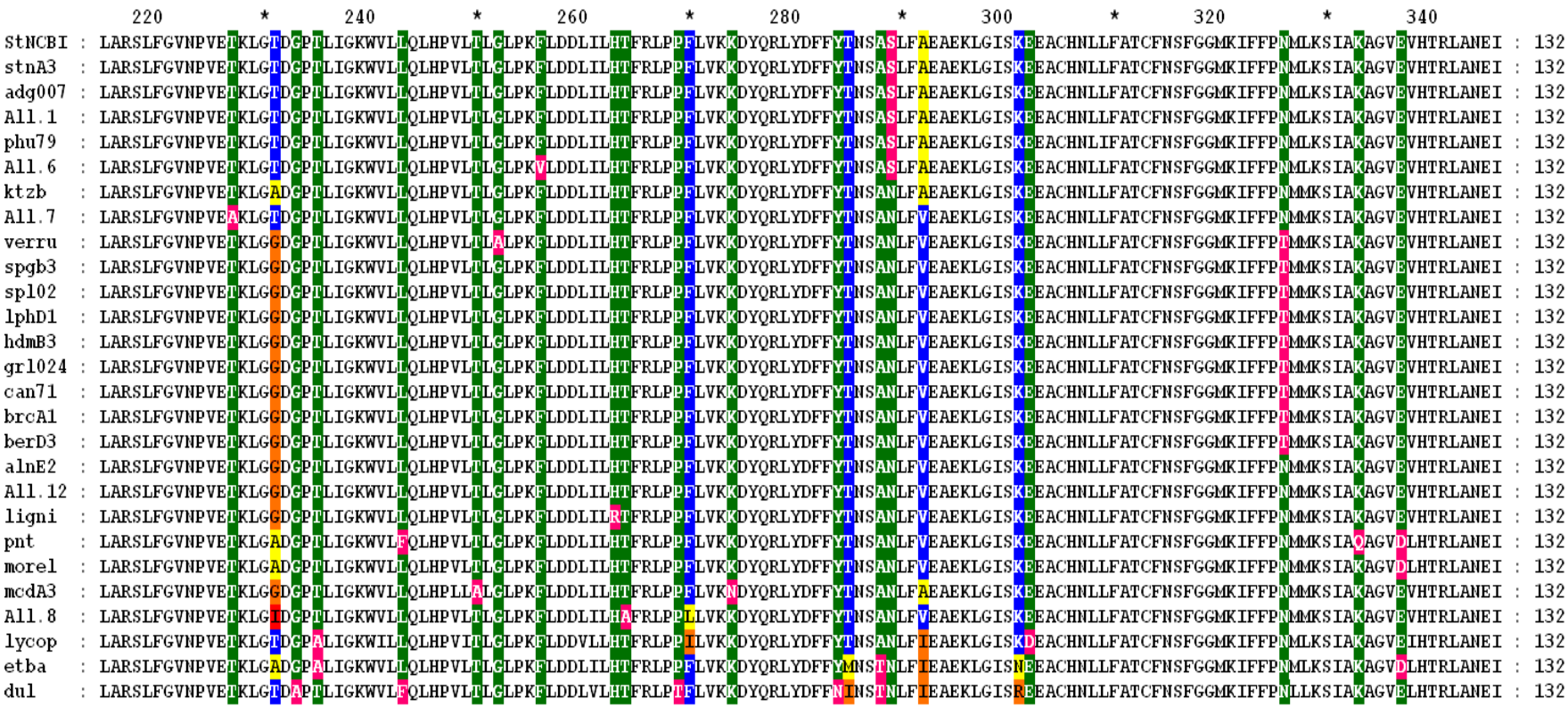


Fig. 39. Alignment of deduced amino acid sequences of *St*AOS2 across 21 *Solanaceae* species homozygous at this locus, comparing to *St*AOS2 sequence available in GenBank (Acc. AJ457081=StNCBI) and the five alleles cloned in this study.

4. DISCUSSION

The development of the *Arabidopsis thaliana* model system is aimed at providing a powerful tool for a better understanding of flowering plants' basic physiology, with a special emphasis on crops. The importance of further improvement of food production is indisputable; nevertheless, the field is not yet much advanced. Recently, a number of attempts have been made in order to exploit the information gained from Arabidopsis for generation of improved crop yield and quality.

4.1. The candidate gene approach as a tool to search for novel plant resistance components

4.1.1. *R*-gene homologs

Initially, a PCR-based approach for isolation of potato genes involved in resistance using degenerated primers designed to conserved motifs was reported by Leister et al. (1996). Using oligonucleotides derived from two consensus motifs of the tobacco *N* and *A. thaliana RPS2* genes, it was feasible to obtain amplification products, highly homologous to the source gene sequences and linked without recombination to known potato *R* genes when used as RFLP probes.

Another attempt to identify new resistance gene candidates was made in lettuce (*Lactuca sativa* L.) (Shen et al. 1998). Using polymerase chain reaction with degenerate oligonucleotides, also designed to NBS motifs of *AtRPS2* and *N* of tobacco, four different families of Resistance Gene Candidates (RGCs) were found. Two of them mapped to known clusters of resistance genes. Further sequence analyses revealed presence of LRR regions adjoining to NBSs in some of the identified genes.

A similar approach was carried out in order to find resistance gene homologs in apple (*Malus* spp.). Candidate ESTs in a database were mined on the basis of homology to five resistance genes classes and screened as RLFP probes on Southern blots of DNA

from seedling populations segregating for a number of resistances to pest or pathogen infection. Linkage analysis suggested that map positions of these ESTs often clustered around apple *R* genes identified so far (Gardiner et al. 2003).

More evidence supporting the efficiency of the NBS-LRR-based approach for identification of novel resistance gene candidates in other crops, like maize, wheat and barley, is provided by many other groups (Collins et al. 1998, Seah et al. 1998).

These pioneer experiments indicate great usefulness of candidate gene–PCR approaches for tagging resistance genes homologs in crop plants. However, *R* gene-based breeding programs, although originally believed to provide durable resistance, turned out to have serious limitations (Rommens and Kishore 2000). A clear example offers the potato late blight control breeding program, which had to be abandoned, because *Phytophthora infestans* overcame the resistance provided by all eleven *R* genes that had been introgressed from wild species *Solanum demissum* into cultivated potato. Only exceptional broad-spectrum *R* genes, like *Bs2* in pepper and *Xa21* in rice, have proven so far to provide durable disease control (Kearney and Staskawicz 1990, Wang et al. 1996).

4.1.2. Defense signaling homologs

Nowadays, in parallel to *R* gene identification and characterization, another field of resistance engineering emerges. The downstream effectors of resistance, particularly genes involved in defense signal transduction, appear to be powerful modulators of pathogen attack responses. Importantly, even a single effector molecule can be placed under a wide range of diverse *R* genes, consequently conferring a broad spectrum resistance (Hammond-Kosack and Parker 2003).

Numerous examples of Arabidopsis mutants, impaired in resistance, pointed out crucial roles of genes *SGT1b*, *EDS1*, *PAD4*, *NDR1*, *NPR1* and others in defense against various pathogens (Austin et al. 2002, Cao et al. 1997, Century et al. 1995, Falk et al. 1999, Glazebrook 1997). A necessity for identification of similar resistance components in crops, especially in the economically important *Solanaceae* family, is

unquestionable. Precise manipulation of only certain defense pathways may help to avoid all kinds of agronomic problems associated with inappropriate activation of *R* genes such as those leading to HR, and could enable better understanding of the contributions of specific pathways to disease resistance (Martin 1999). Moreover, these downstream signaling genes might potentially act as candidates for QTL controlling quantitative resistance to pathogens in crop plants.

One of the first attempts to identify genes involved in signal transduction during defense responses in a QTL context was conducted in wheat (Faris et al. 1999). This candidate gene approach resulted in the discovery of over 50 loci, representing several classes of defense response genes, like oxalate oxidase, peroxidase, superoxide dismutase, chitinase and traumatin. Mapping of these loci revealed positions within previously identified resistance QTL and explained a significant amount of phenotypic variation.

Geffroy and associates (2000), employing the candidate gene approach, were able to find novel QTL candidates for anthracnose, one of the most important diseases of common bean (*Phaseolus vulgaris* L.), caused by the fungus *Colletotrichum lindemuthianum*. Selected candidates included both pathogen recognition (resistance genes and resistance gene analogs) and plant defense response genes. The application of this strategy yielded in identification of 10 novel QTL regions for anthracnose resistance in the bean genome, out of which eight displayed isolate specificity, two were co-localized with known defense genes (phenylalanine ammonia-lyase and hydroxyproline-rich glycoprotein) and three with anthracnose-specific resistance genes and/or RGAs.

Similarly, Pflieger et al. (2001b) carried out a candidate gene-based approach to search for defense-related genes, possibly explaining quantitative resistance in pepper (*Capsicum annuum* L.). Degenerate oligonucleotide primers were designed for conserved regions of two defense response gene families: pathogenesis-related proteins (PR) of class 2 (β-1,3-glucanase) and PR proteins of class 5 (osmotin-like; antifungal activity). Some of the identified *PR* genes co-localized with QTL regions

controlling resistance to *Phytophthora capsici*, *Potato virus Y* and *Potyvirus E*. Again, large effect (35%) of the resistance to *Potyvirus E* could be explained by statistical linkage analysis.

In this study, the recently available information comprising a detailed and extensive characterization of numerous Arabidopsis defense signaling genes was utilized. The picture emerging from the studies on Arabidopsis defense systems clearly proves that impaired signal transduction mechanisms can lead to drastically increased pathogen susceptibility, even in presence of corresponding major *R* genes.
Comprehensive EST databases available for several Solanaceous species provide access to sequence information covering a significant proportion of the gene content in potato. At least one EST with high sequence similarity was found *in silico* for each of the nineteen targeted defense signaling genes from *Arabidopsis thaliana*. Based on potato, tomato or tobacco EST sequences, PCR-based marker assays were developed that allowed detection and mapping of 16 novel loci on the potato molecular linkage maps. These loci encode genes that may have important functions in defense signaling in potato. They represent a third class of candidate loci for controlling quantitative resistance to pathogens, besides loci coding for genes with similarity to *R* genes or defense response genes that have been detected and mapped previously (reviewed in Gebhardt and Valkonen 2001). Due to their potential functional relevance, the PCR-based markers identifying these loci have added value as anchors for resistance QTL mapping in potato and other Solanaceous species and may be useful in marker-assisted selection experiments.

4.2. Map-based selection of candidate genes for QRL

Of all genes that function in defense signaling, only a subset may be relevant for natural variation of pathogen resistance, when selective constraints reduce or prevent allelic variation of functionally essential genes. A positional criterion was therefore

used to identify candidate genes that are most promising for further functional and structural characterization in the context of quantitative pathogen resistance in potato. Five of 16 putative "defense signaling" (DS) genes were located in the same genome segments as known potato QRL. These five positional candidate genes were *StSGT1-1, StSGT1-2*, *StPAD4-1*, *StPAD4-2* and *StAOS2*. The eleven DS loci that were not positional candidates for known QRL are still the most closely related potato homologues of Arabidopsis defense genes, and we anticipate that they have conserved functions in potato. Overlapping positions of QRL and candidate genes are observed either by chance or because there is a causal relationship between allelic variation of the candidate gene and the observed QRL. Two DS genes, *StSGT1-2* and *StAOS2*, were also closely linked to *R* gene-like loci, indicating that there are several candidates for the same QRL. Similarly, *StSGT1-1* was also linked to the defense response locus *StKI* encoding Kunitz-type proteinase inhibitor (Fig. 8). Whether these loci are, in fact, responsible for the QRL cannot be resolved in the populations used for linkage mapping. Further studies such as association mapping and quantitative complementation analysis are necessary.

4.3. Structural and functional relationships of selected candidate genes with pathogen resistance

4.3.1. *St*SGT1

To further analyze the structure and function of the potato candidate genes, full-length cDNA and genomic fragments of potato *SGT1*, *PAD4* and *EDS1* were cloned by PCR-based approaches and sequenced. For *StAOS2*, this information was available in the GenBank database (accession AJ457081). Although not a positional candidate itself, *EDS1* was included because *At*EDS1 and *At*PAD4 are known to interact directly and cooperate in expression of basal and *R* gene-mediated resistance (Feys et al. 2001). In the case that *St*PAD4 and *St*EDS1 proteins also interact in potato, allelic variation of the interacting proteins could be the molecular basis of an interaction QRL that was

detected by markers linked to the *StPAD4* and *StEDS1* loci in progeny derived from crossing the potato varieties Escort and Leyla (Bormann et al. 2004). This possibility requires further investigation.

The potato genes *StSGT1-1* and *StSGT1-2* are both highly sequence related to *AtSGT1b*, a functional ortholog of yeast SGT1 (Austin et al. 2002; Azevedo et al. 2002). In yeast, SGT1 was originally described as a regulator of centromere and kinetochore function in cell cycle progression as well as in ubiquitin-mediated proteolysis (Kitagawa et al. 1999). Current data reveal multiple sites of action of plant, yeast and human SGT1 as a co-chaperone of Hsp90 in assembly and activation of protein complexes (Muskett and Parker 2003; Schulze-Lefert 2004). These include plant R protein complexes governing resistance to bacterial, viral and fungal pathogens. So far, only one full-length cDNA for *SGT1* has been cloned from another Solanaceous species, *Nicotiana benthamiana* (Liu Y et al. 2002), and no information has been reported regarding its copy number and chromosomal position in tobacco. *SGT1* is present in at least two copies in the potato genome which are located on chromosomes III and VI. Interestingly, both copies of *StSGT1* mapped to segments of the potato genome, where QRL were previously identified. The RACE-PCR-based approach identified only one *StSGT1* transcript in uninfected leaf tissue. The second gene might be expressed at different developmental stages, in other tissues or under environmental conditions, which were not tested.

SGT1 proteins have a remarkably conserved structure across distantly related plant species such as barley, rice and Arabidopsis, and even across kingdoms, consistent with an ancient evolutionary origin and conserved function (Shirasu and Schulze-Lefert 2003). The potato equivalent described in this thesis is no exception. *StSGT1-1* shares with Arabidopsis *AtSGT1a* and *AtSGT1b* the ten exons and the exon-intron boundaries, protein molecular mass, amino acid composition and domain architecture.

A slight (two-fold) up-regulation of transcript levels upon infection of potato leaves with a compatible strain of *Phytophthora infestans* has been recorded in expression arrays, which included the *StSGT1-1* and *StSGT1-2* homologous EST probe STMEP46 (TIGR Solanaceae Gene Expression Database, study ID 62). Similarly, increased

levels of *SGT1* homologous transcripts were detected by the same probe in a late blight field infection experiment of a population, which segregated for quantitative resistance to late blight and in a defense signaling experiment (study IDs 50 and 64, respectively).

4.3.2. *St*PAD4

The two *StPAD4* genes found in this study are homologous to *AtPAD4*, a gene originally identified as a necessary component of basal resistance to the oomycete pathogen, *Peronospora parasitica* (Glazebrook et al. 1997b). *AtPAD4* is also required for resistance conditioned by TIR-type NB-LRR proteins (Feys et al. 2001). Although Arabidopsis *pad4* was first identified in a screen for phytoalexin deficient mutants, *At*PAD4 is not involved in the biosynthesis of camalexin, the phytoalexin of Arabidopsis, as the *pad4* mutant accumulated camalexin in response to infection by the fungus *Cochliobolus carbonum*, non-pathogenic on Arabidopsis (Glazebrook et al. 1997b). The production of the potato phytoalexins rishitin and lubimin could be controlled in analogous way, indirectly promoted by *PAD4* homologous genes upon challenge with an appropriate pathogen.

Structurally, the *At*PAD4 protein shows high similarity to class 3 triacyl glycerol lipases, although lipase enzymatic activity has not been demonstrated (Jirage et al. 1999). The three catalytic residues, a serine, an aspartate and a histidine, embedded within the N-terminal lipase domain, were found in both potato homologues. *StPAD4-1* and *StPAD4-2* are the first *AtPAD4* homologous genes of Solanaceous plants to be mapped and cloned. Comparison of the genomic sequences revealed structural differences between potato and Arabidopsis *PAD4*. The potato *PAD4* homologues have 4 exons whereas Arabidopsis *PAD4* is composed of two exons.

The potato EST for *PAD4* that was used as template for primer design in this study, originates from a library generated from potato leaves challenged with an incompatible strain of *P. infestans* (TIGR Solanaceae Gene Expression Database, SGEdb). Moreover, an up to four-fold increase in *StPAD4* transcript was detected in leaf tissue after infection with a compatible strain of *P. infestans*, in the QTL study for late blight

disease development in the field (natural infection) and in the defense signaling experiment (SGEdb, study IDs 50, 62 and 64, probes STMER19 and STMEN29). Furthermore, in the field infection experiment (study ID 50), *StPAD4* transcript levels remained elevated even 21 days after infection. The predicted function of both *StPAD4* genes, expression profiles and localization within QRL for *P. infestans* resistance all support a role of *StPAD4* genes in the late blight defense responses. *StPAD4-1* and *StPAD4-2* share substantial sequence homology at the transcript level and are genetically tightly linked. At this stage it cannot be resolved which of the two *StPAD4* transcripts is up-regulated upon pathogen attack or which is involved in defense signaling by another mechanism. Both genes might be required for the establishment of defense responses. The possibility of duplicated loci underlying a QTL was postulated by Szalma et al. (2002) for maize *whp1* and *c2*, both encoding chalcone synthase. A plausible hypothesis would be the existence of *StPAD4-1* and *StPAD4-2* functional alleles that vary in spatial or/and temporal regulation of expression, in enzymatic activity or protein stability.

4.3.3. *St*EDS1

Yeast two hybrid and *in planta* co-immunoprecipitation experiments revealed a direct physical interaction between *At*PAD4 and *At*EDS1, another lipase-like protein (Feys et al. 2001, Wiermer et al. 2005). At their C-termini, EDS1 and PAD4 from diverse plant species share an EP (EDS1 and PAD4 specific) domain, that is also present in the potato PAD4 and EDS1 homologues. *St*EDS1, like *St*PAD4, has all the features of a class 3 lipase. EST probes corresponding to *StEDS1* identified in this study, detected a two-fold up-regulation of the transcript in the compatible interaction with *P. infestans* and in the defense signaling experiment (SGEdb, study IDs 62 and 64, probes STMIX37, STMCD71, STMDT13), without any clear trend in the time course, though. Recently, the orthologous *EDS1* gene of the closely related tomato (*Solanum lycopersicum* L.) was mapped to a corresponding segment of chromosome VI and was shown to be required for resistance to pathogens mediated by certain types of *R* genes as well as for the basal defense (Hu et al. 2005). It is likely, therefore, that *St*PAD4-1,

*St*PAD4-2 and *St*EDS1 are the functional potato equivalents of these two Arabidopsis disease signaling components.

4.4. Subcellular localization of *St*AOS2

First attempts to characterize the subcellular localization of allene oxide synthase were carried out by Vick and Zimmerman (1987) who demonstrated the activity of the enzyme in spinach (*Spinachia oleracea* L.) leaves confined to the chloroplast fraction. Flax (*Linum usitatissimum* L.) allene oxide synthase was the first plant AOS to be cloned and preliminarily characterized (Song et al. 1993). Presence of a putative chloroplast target peptide was detected in the N-terminus of predicted peptide sequence. Typically, chloroplast target peptides have an overall positive charge and contain a high percentage of hydroxylated amino acids serine and threonine. These peptides tend to be long (up to 40 aa) and amphiphilic. The predicted target peptide of *Lu*AOS displayed all the key features of a chloroplastic protein, which was further confirmed by overexpression of flax AOS protein in potato (Harms et al. 1995): the protein accumulated in the chloroplasts of the transgenic plants. Subsequently, *AtAOS* was cloned and, similarly to flax AOS, displayed presence of a chloroplast target peptide, which was validated by its co-purification with chloroplasts (Laudert et al. 1996).

Barley (*Hordeum vulgare* L.) contains two members of the AOS family. Their subcellular localization was equally supported by co-purification and immunocytochemical localization of *Hv*AOS1 and *Hv*AOS2 to chloroplasts (Maucher et al. 2000).

Similarly, *in vitro* import assays revealed that the tomato *Le*AOS is peripherally associated with the inner plastidic envelope, with the bulk of the protein facing stroma (Froehlich et al. 2001).

In this study, the *St*AOS2-GFP fusion protein was detected to be specifically targeted into chloroplasts of the closely related Solanaceous species *N. benthamiana*. This is

also the first report on the subcellular localization of the allene oxide synthase, using the fluorescent protein reporter system and the confocal laser scanning microscopy.

4.5. The plant Allene Oxide Synthases

AOS is a single copy gene in Arabidopsis, while in other plants more than one enzyme with allene oxide synthase activity can be found. Both monocots and dicots appear to contain small families of *AOS* genes: tobacco and barley – two, tomato – three, rice - four (Howe et al. 2000; Itoh et al. 2002; Maucher et al. 2001). In potato, the *AOS* gene family has at least three members, located on three different chromosomes (*StAOS1* – LG IV, *StAOS2* – LG XI, *StAOS3* – LG X) (TIGR db, mapping data reported in this study). The potato AOS1 and AOS2 share high structural homology, with respect to both, amino acid similarities and domain architecture. Studies on AOS1 and AOS2 (typical CYP74A enzymes) substrate affinities in tomato point towards 13-hydroperoxylinolenic acid as substrate, in contrast to *Le*AOS3 (belonging to the CYP74C subgroup), which preferentially processes 9-hydroperoxylinolenic acid (Howe et al. 2000, Itoh et al. 2002).

4.6. Complementation experiments in heterologous systems

Complementation analyses are belong to key approaches in molecular biology, providing answers to the questions of gene functionality, allowing determination of spatial and temporal gene expression regulation, and supporting the evolutionary studies on the conserved structural motifs required for proper biological functions of proteins. The complementation experiments can be categorized into two major types: homologous and heterologous.

The former offers the opportunity to directly assess the gene function in the native species (e.g., complementation of an Arabidopsis mutant plant with a construct carrying the relevant Arabidopsis gene).

Heterologous complementation is advantageous, when the experimental setup for the species of interest is technically highly demanding, or, due to functional redundancy, wouldn't give the ultimate answer on the biological role of the gene. Often, model systems offer easier and more informative opportunities for investigating gene function. The roots of heterologous complementation attempts reach into late 1980's, when the pioneering experiments on cell cycle regulators were performed, encompassing organisms as diverse as yeasts and man (reviewed in McKinney and Heintz 1991).

Indeed, historically, the first commonly used model for the characterization of plant genes were yeasts. The Brewer's yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* systems, developed more than two decades ago, were and are still used for a magnitude of functional assays. In plant research, the *S. cerevisiae* system is especially useful for the characterization of various enzymes, having their homologs in yeast (Cardazzo et al. 1998, Hamel et al. 1997, Hsu et al. 1993, Issakidis-Bourguet et al. 2001, Minet et al. 1992).

Complementation in heterologous systems can be performed by means of native regulatory sequences or strong, constitutive promoters, e.g. the *CaMV 35S*. These two tactics have both advantages and disadvantages.

The use of the Cauliflower Mosaic Virus (*CaMV*) *35S* promoter induces a powerful protein expression throughout the plant in various developmental stages. This can well serve a purpose of defining basic gene functionality, but is not suitable for subtle investigations of gene regulation or protein activity.

A heterologous complementation between maize (*Zea mays*) and Arabidopsis was described in 1987 by Schwarz-Sommer et al. The *A1* gene, encoding the DFR (dihydroflavonol 4-reductase) enzyme under the control of the *CaMV 35S* promoter demonstrated that this construct was capable of restoring the pigmentation of Arabidopsis dihydroflavonol 4-reductase *tt3* mutant plants and seeds.

Similar experiments conducted later, in 2001 by Dong et al., also dealt with anthocyanin pigment accumulation. The *Arabidopsis thaliana TT4*, *TT5*, and *TT3* loci encoding chalcone synthase, chalcone isomerase, and dihydroflavonol 4-reductase,

respectively, essential for proper anthocyanin accumulation, were now studied in more detail. The three genes were ectopically overexpressed in corresponding Arabidopsis knock-out plants, using the *CaMV 35S* promoter. The products of the maize *C2*, *CHI1*, and *A1* genes complement Arabidopsis *tt4*, *tt5*, and *tt3* mutants, restoring the ability of these mutants to accumulate pigments in seed coats and seedlings.

Recently, the heterologous complementations of Arabidopsis using Solanaceous genes have been also reported.

Fernández et al. (2005) described the functional characterization of the recently cloned gene encoding phytochrome B (*NtPHYB*) from tobacco (*N. tabacum*). Two phytochrome genes, *NtPHYB* and *AtPHYB* were fused with the coding sequence of green fluorescent protein (*GFP*) and overexpressed under the *CaMV 35S* promoter in *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*, in order to investigate the mechanism of phytochrome action *in vivo*. The expression of *35S::NtPHYB-GFP* and *35S::AtPHYB-GFP* cross-complemented the tobacco phytochrome B-deficient *hgl2* and Arabidopsis *phyB-9* mutations (hypocotyls of the seedlings attained to the length comparable to wild-type values). Therefore, a Solanaceous gene introduced into Arabidopsis can fulfill its biological function, and vice versa, the Arabidopsis orthologue is active in the tobacco system.

Leclercq et al. (2001) reported the heterologous complementation of the Arabidopsis *ctr1-1* (*constitutive triple response 1*) mutant with the construct carrying the tomato orthologue *LeCTR1* under the direction of the *35S* promoter. Their data clearly indicate that ectopic expression of *LeCTR1* in the Arabidopsis *ctr1-1* mutant can restore normal ethylene signaling. Interestingly, the light-grown transgenic lines displayed variable degrees of complementation of different aspects of the mutant phenotype. For instance, all the transformed lines displayed a wild-type phenotype in terms of rosette size and inflorescence development, the cotyledon shape, color, and timing of development. However, root length of transgenic plants was highly variable, ranging from the wild-type to *ctr1-1* mutant phenotypes. Moreover, etiolated transgenic seedlings expressing *35S::LeCTR1* displayed a gradual recovery of the hypocotyl elongation rate compared

with the *ctr1-1* mutant. This experiment provides therefore another line of evidence that a Solanaceous gene is capable of cross-complementing the heterologous species *Arabidopsis thaliana*, yet the application of *CaMV35S* system for tomato gene expression resulted in partial complementation of the Arabidopsis mutant.

Recently, the heterologous complementations with the use of native upstream regulatory sequences are more frequently reported in the literature. The tropical pine (*Pinus caribaea* var. *caribaea*) equivalent of *Arabidopsis thaliana LEAFY*, a meristem identity gene, controlling flower formation, was isolated and used for a heterologous complementation of the *Atlfy-26* mutant, using the Arabidopsis *LFY* native promoter fragment. In all the *AtLFYp::PcLFY* transformants complete restoration of defects in male reproductive development was observed (Carnier Dornelas et al. 2005). Similarly, the *LFY* homologue cloned from *Eucalyptus grandis*, while expressed in *Atlfy-26* knock-out background from the native Arabidopsis promoter, fully restores the wild-type situation (Carnier Dornelas 2004).

In this study, both *CaMV 35S*- and native promoter-mediated complementation approaches were undertaken. In *Arabidopsis*, it was shown that JA is involved in pollen maturation and release (Ishiguro et al. 2001, McConn and Browse 1996, Sanders et al. 2000). The results indicated that the ectopic overexpression of *StAOS2-2* (allele 2) in *Arabidopsis thaliana aos* knock-out background indeed does compensate the effect of mutation on the male fertility; however, the complementation effect is not complete. On the other hand, chimeric constructs, carrying the five *StAOS2* alleles under the control of native full length 5´ regulatory region of the *AtAOS* gene resulted in differential levels of fertility restitution, both in terms of siliques length and seed content. Possible explanations for these results would be either an improper and unspecific regulation of *StAOS2* expression by the *35S* promoter, or sequence polymorphisms of the different *StAOS2* alleles, which might be stronger and weaker enzymatic isoforms of the allene oxide synthase. These possibilities require further

investigations, especially *in vitro* measurements of the maximal initial velocity and the Michaelis constant independently for each allele.

4.7. Plant upstream regulatory sequences

The core promoter is a minimum promoter region that is capable of initiating basal transcription. It contains the transcription start site (TSS) and typically spans from −60bp to +40bp relative to the TSS. Approximately 30–50% of all known promoters contain a TATA-box (a sequence of only 7 bases: TATAAAA) located from 45 to 25 bp upstream of the TSS. In the same time, the entire upstream regulatory sequences of *Arabidopsis thaliana* genes can reach up to as much as 38,861 bp in length (*AtTT1*, Dr.Heiko Schoof, personal communication). Additionally, evidence accumulates that some plant gene regulatory elements (enhancers, silencers and insulators) are located in large physical distances from the gene, expression of which they modulate. Some transcription factors (enhancer-binding proteins) bind to regions of DNA that are thousands of base pairs away, upstream, downstream, or even within the gene they control (Qin et al. 2002).

Having to deal with such large and scattered DNA fragments, makes the identification of a promoter for practical work, e.g. cloning, more demanding and complicated. Therefore, numerous efforts have been made in order to develop reliable prediction tools for defining functional promoters.

Currently, promoter identification is one of the most challenging issues in computational biology. A number of on-line tools offer such predictions. The Genomatix-ElDorado database considers 300-1,000 bp of upstream sequence as a potential promoter (http://www.genomatix.de/products/ElDorado/index.html). The PlantCare database (http://intra.psb.ugent.be:8080/PlantCARE/) predicts the transcriptional start sites within 2,000 bp upstream from the ATG codon, and so does the DRAGON Plant Promoter Database (http://research.i2r.a-star.edu.sg/DRAGON/ppdb/). The TAIR Arabidopsis Information Resource (http://www.arabidopsis.org) can handle up to 3,000 bp of upstream sequences as

assumed promoter regions. These tools, however, usually detect the presence of all the elements and motifs necessary for the transcriptional regulation already within -500 – -1,000 bp upstream.

The majority of literature reports state that the usage of ~2 kb of native upstream regulatory sequences is sufficient for the complementation analyses, which is especially helpful in cases when the full length of the putative promoter, defined from the translational STOP codon of the previous gene down to the first ATG codon of the gene of interest (the full-length intergenic region), exceeds this size.

The full length of the *Arabidopsis thaliana AOS* promoter (with respect to the translational start) is 2,850 bp. In the previous study on *AOS* transcriptional regulation (Kubigsteltig et al. 1998), a 1,950 bp upstream region of *AtAOS* (Col-*0*) was shown to be sufficient for driving the expression of GUS in the specific *A. thaliana* and *N. tabacum* organs, that is in older leaves and inflorescences, with a strong induction upon wounding. In this study, an almost complete 5′ regulatory region of *AtAOS*, 2,590 base pairs in length, was cloned from *A. thaliana* Col-*6* accession, which is the background of the *aos* mutation. Arabidopsis *aos* mutant plants, expressing a *AtAOSpr::AtAOS-terminator* MultiSite Gateway construct, displayed full male fertility and completely resembled the wild-type phenotype. The performance of the *AtAOS* promoter in response to a biotic or abiotic stress, e.g. wounding of the plant or pathogen attack, measured in terms of *AtAOS* transcript quantities over time, was neither reported by Kubigsteltig et al. (1998), nor investigated in this study. It is possible, therefore, that the difference in native promoter size could be reflected in differential gene expression response. However, this remains to be experimentally assessed.

4.8. The gene silencing technology. Co-suppression vs. RNAi

It has now been more than fifteen years since Napoli et al. (1990) made a surprising observation in petunias. When trying to intensify the purple color of the flowers, a pigment-producing gene was introduced under the control of the *CaMV 35S* promoter.

Instead of the expected deep purple color, many of the flowers appeared variegated or even white. The observed phenomenon was named "co-suppression", since the expression of both the introduced gene and the homologous endogenous gene was suppressed. The post-transcriptional gene silencing (PTGS), which was initially considered a bizarre phenomenon limited to petunias only, is now one of the hottest topics in molecular biology (Waterhouse and Helliwell 2003). In the last few years, it has become clear that PTGS occurs in both plants and animals and has roles in viral defense and transposon silencing mechanisms.

The first Solanaceous plant being a host for gene silencing was *Nicotiana tabacum* (Ingelbrecht et al. 1994). The *nptII* transgene, responsible for resistance to antibiotic kanamycin, was overexpressed from the *CaMV 35S* promoter. Several independent transformant lines showed substantially lower levels of *nptII* steady state mRNA. Following these pioneer experiments, two host genes: nitrate reductase and nitrite reductase, as well as the *uidA* transgene encoding bacterial glucuronidase were co-suppressed in *N. tabacum* (Palauqui et al. 1997). The percentage of plants affected by co-suppression differed among the lines, ranging from 3 to 57%. Silencing of *Nia* and *Nia2* host genes leads to dramatic and visible chloroses, while plants silenced for the *uidA* no longer show GUS activity when exposed to the chromatic reaction substrate. Phenotypical results were also correlated to RNA blot analyses.

The first evidence that dsRNA could lead to gene silencing came from work in the nematode *Caenorhabditis elegans* ten years ago. An attempt to use antisense RNA to shut down expression of the *par-1* gene in order to assess its function resultet in an interesting discovery. As expected, injection of the antisense RNA disrupted expression of *par-1*, but surprisingly, injection of the sense-strand control did, too (Guo et al. 1995). This phenomenon was resolved in 1998 by Fire and co-workers, who discovered that injection of dsRNA in sense and antisense form results in highly efficient gene silencing. Introduction of just a few molecules of dsRNA per cell was sufficient to completely silence the homologous gene's expression (Fire et al. 1998).

A key contribution to the elucidation of dsRNAi silencing mechanism was done by Hamilton and Baulcombe (1999). They identified RNAs of ~25 nucleotides in plants undergoing co-suppression that were absent in non-silenced plants. These RNAs were complementary to both the sense and antisense strands of the gene being silenced. Further work in *Drosophila* shed more light on the subject, confirming presence of similar in length, 21-23 nucleotide mRNA fragments in embryo lysates (Zamore et al. 2000). As a result of these discoveries, specific RNAi silencing vectors have been developed (Brummelkamp et al. 2002), that contained a gene fragment cloned in sense and antisense orientation. When the construct is expressed in the cell, sense and antisense strands recognize each other, based on complementary sequences, and the "intron" in-between them forms a loop. The structure of the resulting RNA resembles a frying pan (intron loop) with the panhandle (complementary sense and antisense strands) or a hairpin. Such constructs were initially used for gene silencing in mammalian systems.

In plant functional genomics, the interruption of gene expression by RNAi has only been utilized relatively recently (in contrast to insertional mutagenesis, e.g. T-DNA and transposon tagging, being still the most popular tool for reverse genetics approaches). dsRNAs can be delivered to plants in several ways (reviewed in Waterhouse and Helliwell 2003): microparticle bombardment with double-stranded RNA (dsRNA) or intron-containing hairpin RNA (ihpRNA)-expressing vectors; infiltration of plant tissue with an *Agrobacterium* strain carrying a T-DNA expressing an ihpRNA transgene; virus induced gene silencing (VIGS), in which the target sequence is integrated into viral sequences which are used to infect the plant, or are expressed from *Agrobacterium*-introduced transgenes, and by stable transformation with ihpRNA expressing transgenes. The various RNAi techniques each have advantages and disadvantages with respect to how persistent their effect is and the range of plants to which they can be applied. For instance, bombardment can be applied to any plant, but produces only transient effects. Alternatively, transformation with ihpRNA-expressing transgenes provides stable and heritable gene silencing, but requires efficient plant transformation techniques. ihpRNA transgenes have been

shown to be very effective for a wide range of target genes in various plant species (reviewed in Waterhouse and Helliwel 2003, and Wesley et al. 2001), indicating that the RNAi mechanism is probably conserved in all plant species. This is supported by a recent report of RNAi in the non-vascular moss *Physcomitrella patens* (Bezanilla et al. 2003).

Currently, there are increasing numbers of literature reports on successful application of the dsRNAi technology in plants. Not surprisingly, major efforts focus on the plant model *Arabidopsis thaliana*. The AGRIKOLA project (**A**rabidopsis **G**enomic **R**NA**i** **K**nock-**o**ut **L**ine **A**nalysis, http://www.agrikola.org/) was established almost three years ago, and aims at generating a large number of gene-specific tags (GSTs, 150-600bp long) cloned into binary hairpin RNA vectors and subsequent usage of the resulting plasmids to create a collection of as many of the 25000+ Arabidopsis genes as possible, silenced lines. These will help to identify the function of Arabidopsis genes for which mutants are currently not available, clean T-DNA knock-outs are lethal, or gene is not accessible to genetic analyses due to functional redundancy within gene families. The sets of plasmids and transgenic seed generated within the scope of AGRIKOLA project are now available to the scientific community via the Nottingham Arabidopsis Stock Centre (NASC).

The RNAi technology became a popular tool for the forward genetic approaches in important crop plants, e.g. tomato, barley, cotton, tobacco, etc. (Lu et al. 2004, Wesley et al. 2001). However, few literature reports on stable RNAi lines in potato are available.

Göbel et al. (2003) have silenced the potato *9-LOX* (9-lipoxygenase), an initial enzyme in the jasmonic acid biosynthetic pathway, crucial for lipid peroxidation during the hypersensitive response. A binary vector carrying a 374 bp fragment of *St9-LOX* in sense and antisense orientations was used for potato Desirée plants Agrobacterium-mediated transformation. Two of the RNAi plants had nearly undetectable levels of 9-LOX activity (below 5%), whereas another two RNAi lines showed only residual enzyme activity (5-10%).

Hofius et al. (2004) reported stable RNAi-mediated silencing of the potato SXD1 (*Suc Export Defective1*) gene, coding for a single copy tocopherol cyclase. They used a binary hairpin-expressing vector carrying a 756 bp long gene-specific fragment and they were able to retrieve three transgenic, kanamycin resistant potato lines with α-tocopherol contents below 5% of the wild type levels.

In this study, three different ihpRNA-carrying constructs were generated that carried fragments of 253, 150 and 128 bp of the *StAOS2* gene. The recovered kanamycin-resistant putative transformants were assessed for *StAOS2* transcript levels, and, subsequently, for OPDA and JA contents. The efficiency of silencing was high: some of the transgenic lines showed drastically reduced transcript levels and similarly decreased contents of the octadecanoid pathway products (OPDA and JA). However, only ~25% of the lines analyzed by semi-quantitative RT-PCR showed a clear decrease in the transcript abundance, regardless of the size and target sequence of the ihpRNA construct used. A reason for such variation may be the number of T-DNA insertions in the potato genome and the integration positional effects. Moreover, independent shoots emerging from the same callus often differ significantly in the strength of the silencing effect. This might be explained by the somaclonal variation phenomenon (Evans 1989). Taken together, the results reported here offer clear evidence that *StAOS2* encodes the major, early-responsive allene oxide synthase, functional in potato plants upon stress-inducing conditions.

4.9. The role for jasmonate signaling in potato defense responses to abiotic and biotic stresses

The wounding treatment, applied to the leaves of *StAOS2*-RNAi lines, is known to be a robust stimulus for jasmonic acid-related stress responses. Mimicking the herbivorous insects attack, wounding results in a very rapid activation of jasmonate biosynthetic pathway in many plant species, e.g. *Arabidopsis thaliana* (Bell and Mullet 1993, Bell

et al. 1995, Park et al. 2002), tomato (*Solanum lycopersicum* L., Sivasankar et al. 2000), soybean (*Glycine max* L., Creelman et al. 1992), common bean (*Phaseolus vulgaris* L., Porta et al. 1999), citrus (*Citrus paradisi,* Gomi et al. 2003), *Nicotiana sylvestris* and *N. attenuata* (Baldwin et al. 1997, Ziegler et al. 2001), potato (*Solanum tuberosum* L., Royo et al. 1996) and flax (*Linum usitatissimum* L., Harms et al. 1998). The only exceptions known so far are species/subspecies of the *Artemisia* and *Jasminum* genera*,* being unusual in a manner that they do not require wounding or other types of induction for constitutively high oxylipin product formation (Hildebrand et al. 2000).

In this study, a time window of 30 min was selected for sampling leaves after wounding treatment. This short time interval was shown by a number of research groups to be sufficient for monitoring the *AOS* transcript accumulation peak (Laudert et al. 1996). Indeed, a rapid accumulation of the intermediate, as well as final products of the octadecanoid pathway was detected in the control G87 potato plants, while six independent *StAOS2*-RNAi lines analyzed didn't react to the treatment with elevating of OPDA and JA levels.

Significant research has been done on the elucidation of jasmonate-mediated plant defenses against herbivorous insects. Halitschke et al. (2004) reported stable silencing lines for allene oxide synthase (*AOS*) and hydroperoxide lyase (*HPL*) in native tobacco (*Nicotiana attenuata* L.). Feeding performance, growth speed and the weight gain of the two closely related lepidopteran larvae, the specialist species *Manduca sexta* and *M. quinquemaculata* and the unrelated generalist species *Spodoptera exigua*, were increased up to 3 fold on the *NaHPL* and *NaAOS* antisense lines, while comparing to the wild type plants. The effect in *NaHPL* plants was comparatively stronger, which could be explained by compensation of silenced *NaAOS* with the subsequently identified second *AOS* in *N. attenuata* (Hui et al. 2003).

An interesting experiment was reported recently by Kessler et al. (2004). Native tobacco (*N. attenuata*) plants, down-regulated for lipoxygenase (*NaLOX3*), hydroperoxide lyase (*NaHPL*), and allene oxide synthase (*NaAOS*) genes, were

planted into the natural habitats. The vulnerability of these antisense lines to both adapted and novel herbivores was assessed. In laboratory studies, plants deficient in the expression or recognition of jasmonates are unable to elicit defense compounds and are more susceptible to herbivore attack. In the repeated field trials, the three silenced tobacco lines showed dramatically increased attractiveness as prays for the herbivorous larvae. Surprisingly, two new herbivores species were found feeding, and even ovipositing on the experimental plants: the leafhopper *Empoasca* sp. and the western cucumber beetle *Diabrotica undecimpunctata tenella* Le Conte. Under natural conditions, these two species never feed on *N. attenuata*, but show preference towards *Solanum americanum*, *Mirabilis multiflora* and *Cucurbita foetidissima*, all abundant in the study area. These results show that the jasmonate-mediated plant defense responses strongly affect the insect feeding and ovipositing behavior, thereby largely contributing to their host specificity.

Similarly, the octadecanoid signaling pathway has been shown to play an important role in tomato defense against various chewing insects, e.g. a cell-content feeding arachnid herbivore, the two-spotted spider mite (*Tetranychus urticae* Koch) (Li et al. 2002). The tomato *def-1* (*defenseless-1*) mutant, deficient in jasmonic acid production, shows enhanced spider mite feeding compared to the wild type plants. This effect was accompanied by a significant increase in the arachnid fecundity: number of mite eggs found on *def-1* leaves was up to six folds higher, compared with WT leaves. These results were well correlated with the decreased transcript levels of Serine Proteinase Inhibitor II, a marker gene for octadecanoid signaling, in the tomato *def-1* plants.

While the link of plant resistance to chewing, piercing and sucking insects to jasmonates is known since long, little was known until recently about the involvement of jasmonic acid signaling in plant defenses against bacterial, fungal and oomycete pathogens.

In the earliest report, Cohen et al. (1993) found that induction of the JA-mediated signaling response using JA or MeJA resulted in increased resistance of potato to *Phytophthora infestans*. Consistent with these observations, recent experiments on the

above mentioned jasmonate-deficient *def-1* tomato mutant revealed that plants lacking jasmonic acid show significantly increased susceptibility to a number of pathogens, among others to *P. infestans* (Thaler et al. 2004).
These reports pave a new path towards understanding the involvement of jasmonic acid-mediated response in potato defense against the late blight.

A recent report by Halim et al. (2004) indicates that jasmonate-induced defense response, associated with rapid lesions formation on leaves of susceptible potato Desirée plants can be triggered by the oomycete elicitor Pep-13, isolated from the cell wall of *Phytophthora sojae* (Brunner et al. 2002), one of the known PAMPs (Pathogen Associated Molecular Patterns). In this study, however, no necroses development was observed on both the non-transformed quantitatively resistant G87 control plants, and the *StAOS2*-RNAi transgenic lines. Following measurements of the OPDA and JA contents illustrated no significant difference in the level of these compounds among all the plants tested. An explanation of the lack of lesion formation might be the absence of the putative Pep-13 receptor, or a downstream component of the signal transduction cascade, that blocks the hypersensitive response progression. Based on the current literature reports, G87 is the first tested potato genotype that displays no reaction to the Pep-13 treatment, therefore behaving similarly to *N. tabacum* or *A. thaliana* plants, insensitive to Pep-13 elicitor (Dr. Sabine Rosahl, personal communication) and therefore could constitute an interesting system for studying of the early Pep-13 recognition events.

A case study in Arabidopsis indicated that the jasmonate-insensitive *coi1* mutant displayed enhanced susceptibility to necrotrophs, including *Erwinia carotovora* ssp. *carotovora* (Thomma et al. 2001, Vijayan et al. 1998), which is a close relative of *Erwinia carotovora* ssp. *atroseptica*, the potato pathogen of interest. A detailed survey, described in 2000 by Norman-Setterblad and colleagues, clearly shows that jasmonate responses play a major role in the defenses against Erwinias on Arabidopsis.

As recently reviewed by Toth and Birch (2005), challenging Arabidopsis with either Ecc (*Erwinia carotovora* ssp. *carotovora*) or CF (Erwinia culture filtrates) elicits the early induction of ET- and JA-dependent genes, whereas the induction of SA-dependent genes, such as *Pathogenesis-Related 1* (*AtPR1*), is considerably delayed. Moreover, the rapid JA-dependent defense is antagonistic to SA-dependent gene induction, suggesting that SA is not the signaling molecule that leads to early plant responses to SRE (Soft Rot Erwiniae). Little is known about the molecular basis of potato – Erwinia interactions. No literature report to date is available, that dissects the soft rot defense signaling in the *Solanaceae*.

4.10. The plant QTL cloning efforts

To our knowledge, this study is the first ever reported case of isolation of numerous, naturally occuring functional allelic variants of a plant gene. To date, the QTL cloning approaches were exclusively dealing with identification of two contrasting alleles, in some cases exhibiting drastic or extreme differences in the functionality, e.g. the rice QTL for heading time *Hd6* identified as a calcium-dependent kinase *αCK2*, tomato *Ovate* influencing the fruit shape and Arabidopsis transcription factor *BRX*, controlling the root architecture, all showing presence of premature stop codons, resulting in formation of truncated, malfunctioned proteins (Table 1) (reviewed in Salvi and Tuberosa 2005). The experimental proof of the QTL effect was frequently performed using the transformation of "strong" and "weak" alleles into the knock-out background, or a direct transformation of the "strong" allele into the "weaker" background, in order to asses the quantitative difference. Less frequently (only twice: for the tomato invertase *Lin5* and for the maize *Tb1*) a complementation approach was used. However, as the molecular identity of the *ZmTb1* locus still remains to be elucidated, factually the *Lin5* is the only example reported so far, where a quantitative complementation approach in a heterologous system (yeast) was successfully attempted.

The high-resolution genetic mapping delimited the tomato sugar content QTL *Brix9-2-5* to a single-nucleotide polymorphism (SNP) – defined region of 484 base pairs spanning part of the third exon and the third intron of the cell-wall invertase (*LIN5*) (Fridman et al. 2004). The Western blot analyses of the LIN5 protein quantities showed no statistical difference between the two parental tomato lines: *S. lycopersicum* and *S. penellii*. However, the invertase activity was statistically different between the genotypes, with values three to five times as high in lines homozygous for the *S. pennellii* allele compared with those of the *S. lycopersicum* lines. The candidate quantitative trait nucleotide (QTN) (SNP^{2878}) responsible for the the $Glu^{348}Asp$ substitution was evaluated in complementation tests using a yeast invertase–deficient strain. This yeast mutant completely lacks the ability to degrade sucrose, and constituted a suitable system to characterize possible differences in the enzymatic activity between the two alleles. Clearly pronounced differences of the Michaelis constant were detected, that couldn't be attributed to changes in the quantity of the yeast-expressed enzyme. The tomato *Lin5* invertase case is unique in the plant QTL cloning efforts, because a QTN causal for the differential effect was identified, and functionally proven using the complementation in a heterologous system.
Interestingly, the *Lin5*-homologous *invGE* invertase gene on the chromosome IX of potato (*S. tuberosum*) was recently found to be associated with the chips color (Li et al. 2005). Moreover, the allelic variation at the adjacent *invGF* invertase locus shows statistically significant impact on both chips color and tuber starch content. Partial sequences of the *invGE* and *invGF* molecular variants were identified, that may underlie quantitative differences in the enzymatic fitness of the tuber invertase. This discovery provides valuable markers, diagnostic for the selection of cultivars with improved chip quality, a major objective in potato breeding.

Among the twelve plant QTL cloned to date, the identification of a QTN (Quantitative Trait Nucleotide) was only possible for nine. In two cases, the polymorphism was affecting the upstream regulatory region of the gene (the maize *Tb1* shaping the plant architecture and the tomato fruit weight QTL *fw2.2*, both of unknown biological

functions). In the deduced protein sequences of *StAOS2* alleles, twenty-two potential non-conserved amino acid substitutions are found, many of which might alter the protein length, molecular mass, stability, folding properties, and post-translational modifications. The five promoters, driving selected *StAOS2* alleles, were not isolated in the scope of this work; thus at this stage it cannot be resolved whether any (functional) nucleotide polymorphism is present within the gene regulatory regions. However, basing on partial information on *StAOS2* expression, transcript levels remained unaffected three days post-inoculation with a compatible strain of *Phytophthora infestans*, when comparing challenged plants to the healthy control (TIGR SGEdb, study ID 62, probe STMCR05). In this case, the postulated QTL effects of *StAOS2* alleles may indeed result from variation in enzyme activity, post-translational modifications or protein stability, rather than transcriptional regulation.

The novelty of this study is, therefore, the identification and isolation of a putative Quantitative Trait Locus, employing the candidate gene approach rather than positional cloning, as well as isolation of more than two (here: five) full-length allelic variants. Moreover, all *StAOS2* alleles described in this work were shown to be functional allene oxide synthase variants, capable of complementing the heterologous system of *Arabidopsis thaliana aos* mutant and restoring fully or at least partially the male fertility in these plants. *StAOS2* is therefore a candidate for the first QTL cloned from potato, and the first plant resistance QTL ever isolated.

5. SUMMARY

5.1. Summary (English)

An increasing number of pathogen-defense related genes are being identified and characterized in *Arabidopsis thaliana*. So far, it is not known whether and which structural and functional homologues of these Arabidopsis genes have any role in natural variation of resistance to pathogens in crops. Using sequence database mining and PCR-based approaches, potato (*Solanum tuberosum* L.) gene fragments with high sequence similarity to 16 Arabidopsis defense signal transduction genes were obtained, sequenced and genetically positioned on potato molecular maps. Of 16 novel loci, five were positional candidates for known potato pathogen resistance QTL. One of the candidate loci, *StAOS2* co-localizing with QTL for resistance to *P. infestans* and *E. carotovora* on linkage group XI, was further characterized in more detail. *StAOS2* encodes a gene for allene oxide synthase, a cytochrome P450-enzyme, acting upstream in the jasmonic acid biosynthesis pathway. A metabolic block at the level of AOS completely abolishes JA production, which affects plant development (e.g. sterile pollen production) and various abiotic and biotic stress responses (e.g. *P. infestans* resistance in tomato, *E. carotovora* resistance in Arabidopsis). The chloroplastic localization of *St*AOS2-GFP was confirmed by confocal microscopy and functionality of the potato protein was proven by complementation of the male-sterile Arabidopsis *aos* mutant. *StAOS2*-RNAi transgenic lines in potato were generated in order to test role of *StAOS2* in *P. infestans* resistance. The measurements of endogenous OPDA and JA in the silenced lines after wounding treatment revealed drastic decrease in the levels of above mentioned compounds (up to 25 folds less than in wild type plants). In addition, natural variation of *StAOS2* locus was characterized. Sequencing of the locus across 38 potato chromosomes revealed high polymorphism. Thirteen distinct alleles were found, and four of them showed highly significant (P=0.000, R^2=14%) linkage to *P. infestans* and *E. carotovora* QTL.

Five alleles of *StAOS2* were cloned. Sequence analyses revealed a substantial polymorphism on amino acid level, including non-conservative substitutions and an insertion/deletion within the cytochrome P450 domain. Currently, an ongoing quantitative complementation of the *Ataos* mutant with the five different *StAOS2* alleles fused to the native *AtAOS* promoter, followed by OPDA and JA levels measurements in the transgenic lines, will possibly provide direct evidence for *StAOS2* being the first plant resistance QTL identified.

5.2. Zusammenfassung (Deutsch)

Eine zunehmende Zahl von Genen der Pathogenabwehr wird in *Arabidopsis thaliana* identifiziert und charakterisiert. Bisher ist nicht bekannt, ob und wenn ja welche strukturellen und funktionellen Homologen dieser Arabidopsis Gene bei der natürlichen Variation von Pathogenresistenz in Nutzpflanzen eine Rolle spielen. Information aus DNA-Sequenz Datenbanken und PCR Methoden wurden genutzt, um Fragmente von Genen der Kartoffel (*Solanum tuberosum*) zu identifizieren, die eine hohe Sequenzähnlichkeit zu 16 Arabidopsis Genen mit einer Funktion in der Abwehr-Signaltransduktion haben. Diese Kartoffel Genfragmente wurden sequenziert und auf der molekularen Genkarte der Kartoffel positioniert. Fünf der 16 neuen Loci waren positionelle Kandidaten für bekannte Kartoffel QTL (quantitative trait loci) für Pathogenresistenz. Einer der positionellen Kandidatenloci, *StAOS2*, hatte eine ähnliche Position wie QTL für Resistenz gegen *P. infestans* und *E. carotovora* auf der Kopplungsgruppe XI. *StAOS2* wurde weiter im Detail strukturell und funktionell charakterisiert. *StAOS2* kodiert ein Gen für Allene Oxid Synthase (AOS), ein Cytochrom P450 Enzym, das am Anfang des Biosyntheseweges für Jasmonsäure (jasmonic acid = JA) steht. Eine Blockierung von *AOS* verhindert die Produktion von JA, wodurch die Pflanzenentwicklung (z. B. die Produktion von fertilem Pollen) und verschiedene abiotische und biotische Stress Antworten beeinflusst werden, in Tomate z. B. die Resistenz gegen *P. infestans* oder in Arabidopsis die Resistenz gegen *E.*

carotovora. Die Lokalisation von *St*AOS2-GFP in Chloroplasten wurde mittels confokaler Mikroskopie bestätigt. Die Funktionalität des Kartoffelproteins wurde durch Komplementation der männlich-sterilen Arabidopsis *aos* Mutante gezeigt. *StAOS2*-RNAi transgene Kartoffellinien wurden hergestellt, um die Rolle von *StAOS2* bei der Resistenz gegen *P. infestans* zu prüfen. Die Bestimmung von endogenem OPDA und JA nach Verwundung von Blättern in Linien mit sehr geringen Mengen von *StAOS2* Transkript ergab eine drastische Verringerung beider Verbindungen (bis zu 25-mal weniger als in Wildtyp-Pflanzen). Die natürliche Variabilität des *StAOS2* Locus wurde zusätzlich charakterisiert. Die Sequenzierung von 38 Kartoffelchromosomen an diesem Locus ergab ein hohes Maß an Polymorphismus. Dreizehn verschiedene Allele wurden identifiziert, von denen vier hoch signifikant ($P = 0.000$, $R^2 = 14\%$) mit QTL für Resistenz gegen *P. infestans* und *E. carotovora* gekoppelt waren. Fünf *StAOS2* Allele wurden kloniert. Die Sequenzanalyse ergab Polymorphismen auf Aminosäure Ebene, einschließlich nicht-konservativer Austausche und einer Insertion/Deletion innerhalb der Cytochrom P450 Domäne. Derzeit wird die quantitative Komplementation der *Ataos* Mutante mit den fünf verschiedenen *StAOS2* Allelen unter der Kontrollen des nativen *AtAOS* Promotors durchgeführt, gefolgt von einer Bestimmung der OPDA und JA Mengen in den transgenen Linien. Dies kann den direkten Nachweis erbringen, dass *StAOS2* das erste in Pflanzen identifizierte Resistenz QTL ist.

6. OUTLOOK

The results available so far require extended and detailed experimental continuation, which is already initiated to a large extent.

The measurements of OPDA and JA levels in the quantitative complementation lines in the *Arabidopsis thaliana aos* knock-out background will provide an answer whether the *StAOS2* harbors a quantitative effect affecting the jasmonic acid production in potato. As the allele-specific complementation lines display differential restoration of the male fertility, it would be interesting to quantify the pollen viability and seed content per silique and compare the obtained values with these of *AtAOSpromoter::AtAOS::terminator* complementation line and wild type plants. Subsequent experiments would be required to assess the performance of the five potato *AOS2* alleles upon stress treatments, like wounding and pathogen infection. Especially interesting pathogens to be tested are necrotrophs (bacterium *Erwinia carotovora* ssp. *carotovora* and fungus *Alternaria alternata*) as well as hemibiotrophic bacterium *Pseudomonas syringae* pv. *tomato* strain DC3000. All these pathogens of Arabidopsis have been shown to partially or fully rely on the host jasmonic acid-mediated defense signaling.

Another important question to be addressed is whether *StAOS2* is associated with the *P. infestans* resistance in a population of unrelated potato individuals.

A computer-based modeling of the 3-dimensional protein structures for the five alleles could be performed, on the template of recently published structure of coral (*Plexaura homomalla*) AOS (Oldham et al. 2005). The prediction of 3D-structures could reveal differential conformation of the enzyme's active center, heme-binding domain or oxygen-binding pocket, thereby helping to sort out which amino acid polymorphism(s) might be functionally relevant in *St*AOS2 variants.

An ultimate proof of the QTL effect harbored at the *StAOS2* locus would be an assay of enzymatic activity (maximal initial velocity, Michaelis constant) for the five allelic isoforms. Detectable differences in the enzymatic activity would lastly validate the

hypothesis of the *StAOS2* involvement in the quantitative pathogen resistance in potato.

Based on promising preliminary results, the *StAOS2*-RNAi lines in potato G87 background will be analyzed further for the accumulation of OPDA and JA upon wounding. Once detailed results of this experiment are available, the lines will be challenged with *Phytophthora infestans*, and their levels of resistance will be assessed compared to non-transformed control plants, using the Real Time RT-PCR assays along with measurements of OPDA and JA levels in the time course of infection.

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8. EIDESSTATLICHE 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.

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9. PUBLICATIONS

Ein Teil dieser Arbeit wurde bereits veröffentlicht:

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