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Molecular analysis of the Arabidopsis-*Phytophthora* pathosystem

THÈSE

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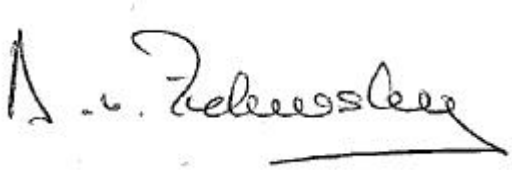

CONTENTS

RESUMÉ

Afin de mieux comprendre l´interaction *Phytophthora*-plante, nous avons développé un nouveau pathosystème: *Arabidopsis thaliana-Phytophthora porri*. Jusqu´à présent, *Phytophthora infestans*, qui a causé famine et désolation en Irlande voilà 150 ans, a été le mieux étudié. Etudier le pathosystème Pomme de terre-*Phytophthora infestans* a, sans aucun doute, des avantages surtout parce que la pomme de terre est très cultivée de par le monde. Toutefois, utiliser Arabidopsis comme plante modèle pour des études à l´échelle moléculaire des réactions incompatibles et compatibles, nous permettra d'élaborer des stratégies pour mieux lutter contre *Phytophthora*. Si différentes lignées ou mutants d´Arabidopsis sont infectés avec *P. porri*, différents phénotypes peuvent être obtenus. En bref, Columbia est résistant, Landsberg erecta est susceptible et le mutant *pad2-1* est hypersusceptible. Ce mutant n´accumule ni l´acide salicylique ni la camalexine. En outre, une étude menée avec différents mutants déficients en production d´acide salicylique, d´acide jasmonique ou de camalexine a montré qu´aucun de ces composé chimiques n´est important pour Arabidopsis pour résister à une infection contre *Phytophthora*. Toutefois, la fonction de la protéine PAD2 est absolument requise (Chapitre II). Il était alors, nécessaire de vérifier si la résistance est réellement indépendante de l´acide salicylique. Pour cela, des inductions par voie chimique ont été réalisées avec l´acide salicylique et son analogue le BTH. L´acide β-aminobutyrique (BABA) a été aussi utilisé dans cette étude car il a été démontré qu´il induit la résistance chez la tomate et la pomme de terre contre *Phytophthora*. Pour estimer le degré de protection *in planta* d´une manière non destructive, nous avons transformé *P. porri et P. infestans* de manière à ce qu´ils expriment une protéine vert fluorescent, la GFP. Cela permet une estimation du degré d´infection assez facile. De cette manière, nous avons estimé la protection que BABA confère à Arabidopsis et la pomme de terre contre *Phytophthora* à respectivement, 100% et 97%. En revanche, le BTH n´induit pas de résistance significative confirmant ainsi nos conclusions antérieures (Chapitre III). Il est clair que cette nouvelle voie de signalisation est contrôlée par PAD2 et que cette voie est indépendante de l´acide salicylique. Nous avons utilisé des "oligonucleotides-based arrays" pour trouver des gènes pouvant servir de marqueurs à cette voie. Cette analyse s´est limitée aux gènes transcrits de importante dans la réaction incompatible mais pas chez le mutant *pad2-1*. En utilisant, le logiciel GeneCluster 1.0, nous avons identifié sept gènes présentant le profil recherché. Quatre d´entre eux ont une fonction inconnue: une putative protéine, une putative "disease resistance" protéine, une putative tyrosine aminotransferase et une protéine inconnue. Les trois autres gènes ont été déjà décrits dans la littérature: une serine/threonine kinase, le géne *AIG1* (avirulence induced gene) et le gène *PAD4*. Une analyse plus poussée a montré que l´induction de ces sept gènes par *P. porri* est dépendante de l´acide salicylique. Donc aucun de ces gènes ne peut être utilisé comme marqueur pour la nouvelle voie de signalisation contrôlée par PAD2 (Chapitre IV). Finalement, pour effectuer des études génétiques avec *P.* porri nous avons déterminé la taille de son génome par les techniques "flow-cytometry" et reconstruction génomique. Nous avons estimé la taille du génome de *P. porri* à 110 ± 10 Mbp ce qui représente à peu prés la moitié de la taille du génome de *P. infestans* (Chapitre V).

ABSTRACT

In order to better understand the *Phytophthora*-plant interaction, we have developed a new pathosystem: *Arabidopsis thaliana-Phytophthora porri*. At present, the best studied *Phytophthora* species is *P. infestans* which caused the dramatic Irish late blight epidemics 150 years ago. Studying the pathosystem *Phytophthora infestans-Solanum tuberosum* has certain advantages mainly because potato is an important crop plant. However, using Arabidopsis as a plant model for molecular studies of both the incompatible interaction and the compatible interaction, will help us to find new strategies to control *Phytophthora*. When different accessions or mutants of Arabidopsis are infected with *P. porri*, different phenotypes can be obtained. For instance, the accession Columbia is resistant, the accession Lansdberg erecta is susceptible and the mutant *pad2-1* is hypersusceptible. The mutant *pad2-1* is deficient in both camalexin and salicylic acid accumulation. Moreover, using different mutants impaired in SA, jasmonate, ethylene or camalexin production showed that neither SA nor jasmonate/ethylene nor camalexin are necessary for resistance towards *Phytophthora* but the function of PAD2 is absolutely required (Chapter II). It was therefore necessary to check if the resistance is completely SA-independent. Induced resistance using the SA analog BTH was performed. ß-aminobutyric (BABA) acid was also used in this study because it was previously shown that this compound can induce resistance toward *P. infestans* in tomato and potato. To estimate the degree of protection *in planta* in a non-destructive manner, we have transformed both *P. porri* and *P. infestans* with the visible marker green fluorescent protein (GFP). This allows an easy scoring of the infection process by measuring the fluorescence emitted by transgenic *Phytophthora in planta*. In this way, we have estimated a protection of 100% in the BABA treated Arabidopsis plants and 97% in the BABA treated potato. In contrast, BTH did not induce significant resistance, confirming our previous conclusions (Chapter III). It is clear that establishment of the resistance is controlled by PAD2 in a SA-independent. We aimed by using oligonucleotide-based arrays to find marker genes for this new pathway. By profiling the transcriptome of both the resistant accession Columbia and the hypersusceptible mutant *pad2-1* we aimed to find those markers. We restricted the analysis to genes which are upregulated in the incompatible interaction with accession Columbia but not in *pad2-1*. Using the GeneCluster 1.0 software seven genes were identified. Four have an unknown function: a putative protein, a putative disease resistance protein, a putative tyrosine aminotransferase and an unknown protein. The three others were already described: a serine/threonine kinase-like protein, an avirulence induced gene *AIG1* and the gene *PAD4*. A closer analysis revealed that the induction of all the seven genes by inoculation with *Phytophthora porri* is SA-dependent. Therefore, none of them is a marker gene for the new PAD2-controlled signaling pathway (Chapter IV). Finally, as a basis for its genetic analysis the genome size of *P. porri* was determined by flow cytometry and genomic reconstruction analysis. We have determined the genome size of *P. porri* to be 110 ± 10 Mbp which is about half of the genome size of *P. infestans* (Chapter V).

CHAPTER I

General introduction

Phytophthora

The name of the genus *Phytophthora* is derived from the Greek: *Phyto* means plant and *phthora* stands for destroyer. *P. infestans* is also known as the Irish potato famine fungus. It totally destroyed staple potato crop during 1845 and 1846. This disease had an immediate sociological and economical impact in that country and led to starvation and emigration of two million inhabitants (Figure 1). After few decades of great debate about the cause of late blight, Anton de Bary affirmed, like Rev. Miles Joseph Berkeley before him, that a fungus could be the actual cause of late blight and in 1876 named it *Phytophthora infestans* (Mont.) de Bary (Bourke, 1991).

Figure 1. Searching for potatoes in a Stubble field

The historical image here portrays the struggle for existence in the aftermath of the famine. *" Illustrated London News" December 22, 1849.*

Phytophthora and other oomycetes have unique features that separate them from fungi. They are included in the Kingdom Stramenopila but no longer in the true fungi Kingdom Mycetae (Dick 1995 and Erwin and Ribeiro, 1996). However, because of its many physiological and morphological similarities to true fungi, *Phytophthora* will undoubtedly continue to be referred as a fungus. The life cyle of *Phytophthora* spp. is well studied and is drawn in figure 2.

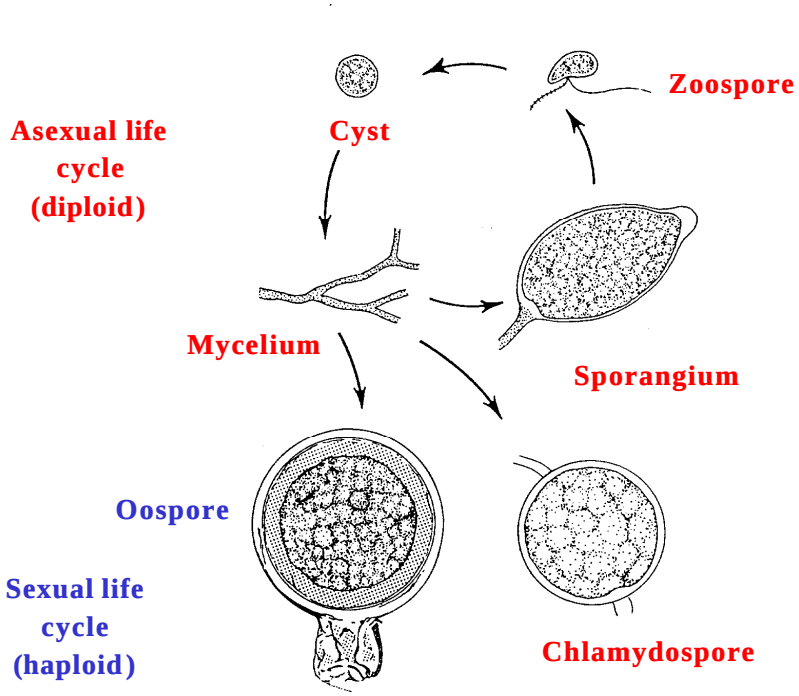

Figure 2. Life cycle of *Phytophthora*.

When *Phytophthora* is cultured on suitable media the mycelium grows rapidly. Under moist conditions it produces asexual spores called sporangia (or zoosporangia). The sporangium germinates in aqueous solutions or if the temperature is reduced, it releases zoospores with heterokont flagella (one whiplash and one tinsel). The zoospores swim for hours and eventually cease to swim, round up (or encyst), and within a minute they develop a cell wall. At this stage the spore is called a cyst. The chlamydospore is spherical to oval. The sexual structure are composed of an antheridium (male component) and an oogonium (egg-containing female component). Meiosis occurs in both antheridium and oogonium. This is the only haploid stage in the life cycle of *Phytophthora*. The major part of the life cycle is diploid. A fertilization tube from the antheridium ruptures the oogonial wall and deposits the antheridial nucleus. A single oospore forms within the oogonium and germinates under suitable conditions by production of single or multiple germ tubes. The genus *Phytophthora* contains some species that are heterothallic (A1 and A2 mating types) including *P. infestans* and also some homothallic species (self-fertile) such as *Phytophthora sojae* or *Phytophthora porri*.

Type of resistance to *Phytophthora*

Contemporary models of resistance of plants towards *Phytophthora* spp. are of two types: host resistance and non-host resistance (Kamoun, 2001). In the host resistance, vertical and horizontal resistance can be distinguished. Horizontal resistance is not race specific and there is no interaction between host and pathogen genotypes whereas vertical resistance is characterized by single-gene interactions (Erwin and Ribeiro, 1996). The race-specific resistance of potato towards *P. infestans* and of soybean towards *P. sojae* has been studied since the 1950s and has been assumed to be a "gene-for-gene" resistance. Flor´s concept (1956) is that for every resistance gene (R) in the host, there is a specific complementary gene

in the pathogen that conditions avirulence (Avr). The subsequent phenotype of such resistance is a hypersensitive response (HR) which is a programmed cell death (PCD). The HR is associated with all known forms of genetic resistance to *Phytophthora* including non-host resistance. The report of Kamoun (2001) questions the traditional view that *Phytophthora* is strictly host-specific and explains that non-host resistance of parsley, *Nicotiana benthamiana* and Arabidopsis to *Phytophthora infestans* is due to a simultaneous expression of multiple Avr genes and an arsenal of R genes present in these hosts leading to HR and thus to resistance.

Modern tools for a historical challenge

Most *Phytophthora* species can be easily grown in the laboratory and numerous biochemical, cytological and physiological studies were performed. However, *Phytophthora* is notorious for being "a fungal geneticist´s nightmare" (Shaw, 1983) and the development of genetic and molecular tools has been difficult. Indeed, it was only in 1991 that the first stable transformation of *P. infestans* was reported by Judelson et al. (1991). This author established a reliable protocol which was applied until now successfully by other groups (van West et al., 1998, 1999, 1999a; Bottin et al., 1999 ; Chapter IV of this thesis). Stable transformants can be obtained by integration of vectors harbouring different reporter genes such as GUS to monitor disease progression *in planta* (Kamoun et al., 1998) or to study temporal and spatial expression patterns of the *in planta* induced genes (Kamoun et al., 1997). More recently, it has been shown that GFP can be expressed as a reporter gene in *Phytophthora parasitica* var. *nicotianae* and in *Phytopthora palmivora* allowing non destructive *in vitro* or *in planta* studies (Bottin et al., 1999; van West et al., 1999a; a; Chapter IV of this thesis). Transformation is a prerequisite for random or directed gene disruption. The use of the insertional mutagenesis in Arabidopsis led to innumerable mutants defective in developmental processes or defence pathways (Koncz et al., 1992) and it is a promising approach in *Phytophthora* research (Kamoun, 2000). Targeted gene knock out has been demonstrated by van West et al. (1999) who showed that a promotorless full length cDNA clone of the elicitin *inf1* gene could be used without modifications to transform *P. infestans* and to generate silenced strains.

The Arabidopsis-*Phytophthora* pathosystem: Is *P. porri* a model for oomycete-plant interaction studies ?

When I started my PhD research four years ago, different *Phytophthora* groups initialized research projects aiming to find *Phytophthora* spp. potentially infecting Arabidopsis. The essential reason was that Arabidopsis is the plant model of choice to study plant-pathogen interaction at the molecular level (Baker et al., 1997). Moreover, powerful genomic methods are available such as the oligonucleotides-based arrays that I used during my thesis (Chapter V) allowing a transcriptional profiling of the genome during the infection. Different Arabidopsis accessions and mutants were infected by mycelium, sporangia or zoopsores of several isolates of *P. infestans*, *P. sojae* or *P. parasitica* but without getting any susceptible phenotype (Kamoun et al., 1999 and Kamoun, 2001). Since it was reported that other oomycetes than the widely used *Peronospora parasitica* can infect Arabidopsis such as *Pythium paroecandrum* (Mauch-Mani et al., 1993), it was assumed, that other non-biotrophic oomycetes could be used as model oomycete pathogens. This allows a parallel genetic analysis of both the host and the pathogen. Interestingly, *Phytophthora porri* was found to infect Arabidopsis. The basis of the pathosystem was established and histological and biochemical studies using different Arabidopsis mutants impaired in defense pathways was achieved during my thesis and described in Chapter II. *Phytophthora porri* is a heterogenous

group and can infect multiple hosts such as carrots, ornamental plants and *Brassicaceae* (Stelfox and Henry, 1978; Kouyeas, 1977; Geeson, 1976 and Semb, 1971). The isolates infectious on *Brassicaceae* appear to represent a different species from *P. porri*, and it was proposed that these be renamed as *Phytophthora brassicae* (De Cock et al., 1992). During my thesis I concentrated my research only on the isolate HH (CBS 782.97). When infected with this isolate, different Arabidopsis accessions showed different symptoms. Briefly, Columbia (Col-0) plants are resistant, Landsberg erecta plants are susceptible and the mutant *pad2-1* which is phytoalexin deficient is hypersusceptible. All these types of interactions using the same isolate facilitated my investigations. According to the molecular phylogeny based on the internal transcribed spacers (ITS) sequences of genomic ribosomal RNA of 50 *Phytophthora* species, the closest species to the isolate HH are *Phytophthora syringae* and *Phytophthora primulae* (Cooke et al., 2000). All these three species are semipapillate (a papilla is a plug composed of hydrated material at the tip of the sporangia), homothallic, have a similar coiled hyphae and were grouped in a clade clearly defined distant from the *P. infestans* and *P. sojae* clade. However, the tree bootsrap values are strong for all the major points (minimum of 59%) suggesting several common characteristics between *P. porri* and the economically more important *P. infestans* and *P. sojae.*

Arabidopsis thaliana

Arabidopsis defense pathways

The recent review of Glazebrook (2001) reporting Arabidopsis pathways and genes controlling them demonstrates how our knowledge about molecular defense mechanisms evolves extremely rapidly. In fact, when I started my thesis in 1997 two separate defense pathways were clearly defined: the salicylic acid (SA) pathway and the jasmonic acid/ethylene pathway. Two marker genes for each pathway *PR-1* and *PDF1.2* respectively were identified. In parallel, mutants impaired in salicylic acid, methyl jasmonate/ethylene and in the phytoalexin camalexin accumulation were isolated. Another class of mutants was isolated and called enhanced disease susceptibility mutants (*eds*) because of their hypersusceptible phenotype towards pathogens. Some of them are deficient in the accumulation of salicylic acid or the phytoalexin camalexin. Four years later, the majority of the mutated genes were cloned and characterized. Recent results have shown that the situation is more complicated. Indeed, several authors reported the existence of a cross-talk between the SA pathway and the jasmonate-ethylene pathway proving a complex interconnection in defense signaling networks. I will describe the ordering of those genes within branches of Arabidopsis defense signaling networks. However, this list is not exhaustive.

R gene signal transduction

It is now clear that gene-for-gene resistance can be mediated through at least three genetically distinguishable pathways (Figure 3). Mutation in NDR1 and EDS1 block gene-for-gene resistance that is mediated by some resistance genes (Warren et al., 1999). Several resistance genes specific *to Peronospora parasitica* or *Pseudomonas syringae* were identified (reviewed in Glazebrook, 2001). Those resistance genes can require either EDS1 or NDR1 but it was recently shown that other *P. parasitica* resistance genes require neither *EDS1* nor *NDR1* (Mc Dowell et al., 2000). NDR1 interacts with the leucine-zipper (LZ) subclass of nucleotide-binding site (NBS)-leucine-rich repeat (LRR) genes. *EDS1* interacts with the subset of the NBS-LRR class of R proteins with an amino-terminal domain similar to the *Drosophila* Toll and mammalian interleukin 2 receptors (TIR-NBS-LRR) (Aarts et al., 1998).

NDR1 encodes a protein with two predicted domains (Century et al., 1997) and probably hold R proteins close to the membrane. As mentioned above, *EDS1* encodes a protein with similarity to triacyl glycerol lipases but its role is not yet known (Falk et al., 1999).

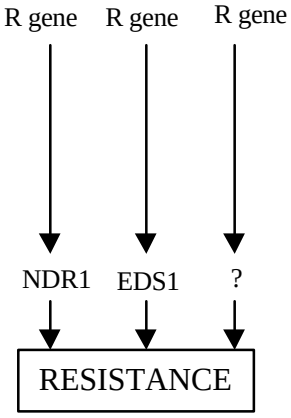

Figure 3. Gene-for-gene resistance

Salicylic acid-dependent resistance

Salicylic acid is a small defense signaling compound which plays a central role in plant disease resistance (Figure 4). There are several mutations that negatively affect SA accumulation such as the *eds1* (Parker et al., 1996) and the *pad4 (phytoalexin deficient4*, Zhou et al, 1998) mutations. Interestingly, both encode proteins that share homology to eukaryotic lipases (Jirage et al., 1994; Falk et al., 1999) suggesting that lipid metabolites are involved in regulating SA accumulation. Another screen performed by Nawrath and Métraux (1999), by measuring SA in mutagenized Arabidopsis population, uncovered two new loci, *SID1* and *SID2* (for salicylic acid-induction deficient) allelic to the previously characterized *eds5* and *eds16* respectively (Rogers and Ausubel, 1997; Dewdney et al., 2000). SA levels in these mutants are low. Unlike *pad4, sid1* and *sid2* are not deficient in camalexin but show the same increased susceptibility to both virulent and avirulent *Pseudomonas syringae* strains *and P. parasitica* isolates. Although *eds1*, *pad4*, *sid1* and *sid2* as well as the transgenic *nahG* (Gaffney et al., 1993) reduce SA levels and the expression of *PR-1*, only *pad4* and *nahG* reduce the production of the phytoalexin camalexin (Zhou et al., 1998; Nawrath and Métraux, 1999). Therefore, it is unlikely that low SA production cause the camalexin defect but rather that *pad4* and *nahG* affect signaling pathways in addition to the SA-dependent pathway. EDS1, PAD4, SID1 and SID2 act upstream of SA. The *SID2*(*EDS16*) gene encodes an isochorismate synthase1 (Wildermuth et al., 2001) indicating that SA might be synthesized not only by the previously pathway proposed by Mauch-Mani and Slusarenko (1996) but also by way of chorismate similar to the pathway used by certain bacteria (Serino et al., 1995). *npr1 (non-expresser of PR genes1)* also known as *nim1* (*non inducible immunity 1*) do not express PR genes in response to SA treatment (Cao et al., 1997; Rate et al., 1999). *NPR1* encodes a protein that contains ankyrin repeats and concentrates in the nucleus in response to SA and nuclear localization is required for the activation of *PR-1* expression by NPR1 (Kinkema et al., 2000).

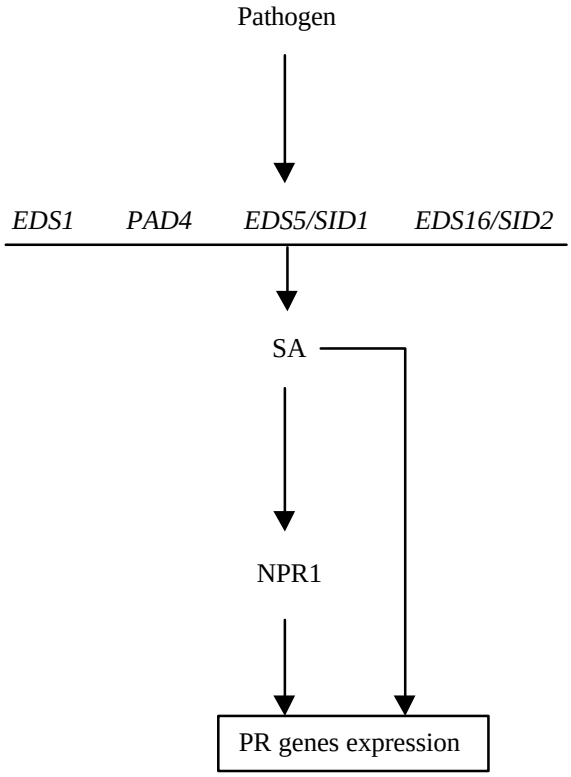

Figure 4. The salicylic acid pathway.

Jasmonic acid/ethylene-dependent resistance

Jasmonic acid (JA) plays an important role in the regulation of defense against several pathogens (figure 5) such as *Alternaria brassicicola* (Penninckx et al., 1996) or *Pythium* species (Vijayan et al., 1998; Staswick et al., 1998). When Arabidopsis plants are infected with these fungal pathogen, the defensin gene *PDF1.2* is strongly induced. This induction does not require SA or NPR1, but it does require JA signaling (Penninckx et al., 1996). Some mutations block the JA signaling and *PDF1.2* expression. The *coi1* mutant (coronatine insensitive, Feys et al., 1994) and the *jar1* mutant (jasmonate response, Staswick et al., 1992) were intensively studied. The *COI1* gene has been cloned and encodes an F-box protein that presumably functions in targeting proteins for ubiquitin-dependent degradation. It was proposed that COI1 has pleiotropic effects beyond its effect on JA signaling by promoting degradation of a factor with a negative regulatory effect on the JA signal transduction (Glazebrook, 2001). Some other mutants block the ethylene signaling such as the ethylene insensitive mutant *ein2* (Guzmán and Ecker, 1990) and *etr1* (ethylene receptor, Hua and Meyerowitz, 1998). Like *jar1* and *coi1*, *etr1* and *ein2* mutants do not express PDF1.2. If *coi1* and *jar1* plants are treated with ethylene or if *ein2* and *etr1* plants are treated with jasmonic acid, PDF1.2 expression is not activated leading to the conclusion that JA and ethylene seem

to be required simultaneously (Penninckx et al., 1998). *ETR1* and *EIN2* have been recently cloned. ETR1 belongs to the ethylene receptor family characterized by an amino-terminal ethylene-binding domain (Chang et al., 1993). *EIN2* was reported to code for a structurally novel protein with an amino-terminal integral domain that has similarity to the Nramp family of metal-ion transporters (Alonso et al., 1999).

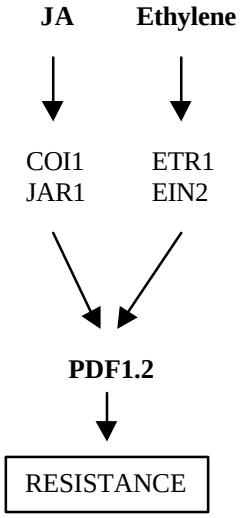

Figure 5. The Jasmonic acid/Ethylene pathway

Chemical activation of systemic acquired resistance in Arabidopsis

Systemic acquired resistance (SAR) is being used extensively as a concept for disease control on commercial scale. SAR is a phenomenon whereby resistance to infectious disease is systemically induced by localized infection or treatment with a pathogen or by organic and inorganic compounds (Sticher et al., 1997). This type of resistance is expressed against a broad spectrum of organisms. Salicylic acid could be a putative endogenous signal for SAR. Indeed, Métraux et al. (1990) early reported an increase of SA in the phloem of infected cucumber before the expression of SAR. Moreover, in the mutant *nahG* SAR is blocked confirming a role of SA as a signal molecule for SAR (Delaney et al., 1994). Arabidopsis plants that were treated with SA react rapidly by expressing PR-1 protein what correlates with the induction of SAR (Uknes et al., 1992). Other chemicals are also able to induce SAR and *PR-1* expression such as INA (2,6-dichloroisonicotinic acid) and BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester). These chemical compounds have almost no *in vitro* antifungal activity and are efficient SAR activators against a wide spectrum of pathogens including fungi, bacteria and viruses (Sticher et al., 1997). BTH can replace SA in the natural SAR signaling pathway, inducing the same spectrum of resistance and the same set of molecular markers. It has found a commercial application under the name Bion® and it protects both monocots and dicots. In monocots, activated resistance is very long lasting, while the lasting effect is less pronounced in dicots (Oostendorp et al., 2001).

More recently, D,L-β-aminobutyric acid (BABA) has been reported to activate disease resistance, especially against downy mildews in various crops (reviewed in Jakab et al., 2001). In Arabidopsis BABA potentiates natural defense mechanisms against biotic and

abiotic stresses and confers resistance to *Peronospora parasitica* in an SA- and JA/ethylene-independent way (Zimmerli et al., 2000).
As reported by Kuæ(2001), different factors are favourable for the development and use of chemical activators of SAR such as the increased popularity of organic crops and sustainable agriculture and moreover, the resistance of the public to genetically modified plants.

Outline of this thesis

The study of a new pathosystem involves the development of several new methods and protocols. In the Arabidopsis-*Phytophthora* pathosystem both partners are amenable to genetic analysis. During my thesis I worked in parallel with both Arabidopsis and *Phytophthora*. In order to characterize the pathosystem, several mutants deficient in salicylic acid accumulation, jasmonate/ethylene production and camalexin accumulation were analysed using *PR-1* and *PDF1.2* as markers. This molecular characterization coupled to the histological studies of the phenotype led us to conclude that neither the SA pathway nor the jasmonate/ethylene pathway are involved in Arabidopsis resistance against *P. porri*. Furthermore, the mutant *pad2-1*, deficient in camalexin and SA accumulation, showed a hypersusceptible phenotype. However, we concluded from our study that camalexin or SA do not play a role in the defense toward *P. porri*. Resistance of Arabidopsis against *P. porri* appears to depend on unknown defence mechanisms that are under the control of PAD2 (Chapter II). In parallel, in order to easily quantify the *Phytophthora* biomass *in planta* and for a further gene knock-out project, a transformation protocol using an improved vector containing the Green Fluorescent Protein (GFP) reporter gene and a selectable marker (nptII) was developed. *P. porri* was successfully transformed and a high rate of transformation was obtained. The same protocol was applied to *P. infestans* and both transgenic *Phytophthora* spp. were used for the chemical induced resistance study in Chapter III. The use of this new method of quantification allows an easy *in planta* scoring of the infection processes in both Arabidopsis and potato plants. Taking advantage of this technology we sprayed Arabidopsis and potato plants with BTH or BABA and found that BTH does not induce any resistance in the susceptible plants thus confirming our statement in Chapter II. In contrast, BABA is a powerful inducer of resistance toward *P. porri* and *P. infestans*. It was clear for us that a new pathway SA-, JA/ethylene-independent is involved in Arabidopsis resistance toward *P. porri*, that this new pathway requires *PAD2* and that BABA might activate this new pathway. In order to quickly identify new marker genes for this pathway, I have profiled 30% of the Arabidopsis genome using oligonucleotides-based arrays (Chapter IV). Among the 8734 Arabidopsis genes contained on the DNA chip, we have identified seven potential candidates which could be used as markers for resistance. Some of them are known such as the *PAD4* gene but the majority are of unknown function. Their putative roles are discussed in Chapter V. As mentioned above, an efficient method of transformation was achieved during my thesis rending a random insertional mutagenesis in *Phytophthora porri* feasible. As mentionned above *P. porri* is homothallic (self-fertile) thus allowing an easy recovery of the progeny homozygous at the mutated locus. It was therefore necessary to estimate the genome size of *P. porri* and its ploidy. A flow cytometry-based measurement was used to successfully estimate the genome size of *Phytophthora* species (Chapter V). Finally, in Chapter VI the relevance of these studies to identify key processes governing Arabidopsis defense against *Phytophthora* and the use of *P. porri* as a model for *Phytophthora* spp. is discussed.

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CHAPTER II

Characterization of an Arabidopsis-*Phytophthora* Pathosystem: resistance requires a functional *PAD2* gene and is independent of salicylic acid, ethylene and jasmonic acid signaling

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ABSTRACT

Arabidopsis accessions were screened with isolates of *Phytophthora porri* originally isolated from other crucifer species. The described Arabidopsis-*Phytophthora* pathosystem shows the characteristics of a facultative biotrophic interaction similar to that seen in agronomically important diseases caused by *Phytophthora* species. In susceptible accessions, extensive colonization of the host tissue occurred and sexual and asexual spores were formed. In incompatible combinations, the plants reacted with a hypersensitive response (HR) and the formation of papillae at the sites of attempted penetration. Defence pathway mutants such as *jar1* (jasmonic acid-insensitive), *etr1* (ethylene receptor mutant) and *ein2* (ethylene-insensitive) remained resistant towards *P. porri*. However, *pad2*, a mutant with reduced production of the phytoalexin camalexin, was hyper-susceptible. The accumulation of salicylic acid (SA) and PR1 protein was strongly reduced in pad2. Surprisingly, this lack of SA accumulation does not appear to be the cause of the hyper-susceptibility because interference with SA signalling in *nahG* plants or *sid2* or *npr1* mutants had only a minor effect on resistance. In addition, the functional SA analogue benzothiadiazol (BTH) did not induce resistance in susceptible plants including *pad2*. Similarly, the complete blockage of camalexin biosynthesis in *pad3* did not cause susceptibility. Resistance of Arabidopsis against *P. porri* appears to depend on unknown defence mechanisms that are under the control of PAD2

Keywords: Arabidopsis, *Phytophthora,* resistance, *pad2*, salicylate, BTH.

II.1. INTRODUCTION

Plant diseases caused by oomycetes are known for their important economical and social impact, the most prominent example being the late blight disease caused by *Phytophthora infestans* (Bourke, 1991; Gregory, 1983). The oomycetes have long been classified as fungi because of their fungus-like life cycle. However, based on their biology and phylogeny, they belong to the separate kingdom *Stramenopila*, and are believed to form a monophyletic group with the *Hyphochytriomycota* and *Labyrinthulomycota* (Barr, 1992; Dick, 1995). The nearest relatives of the oomycetes are not fungi but heterokont algae (Patterson, 1989). The most thoroughly investigated plant-oomycete pathosystems are the interactions between *Bremia lactucae* and lettuce, *Phytophthora infestans* and potato/tomato, and *Phytophthora sojae* and soybean (Judelson, 1996). Many resistance genes have been genetically identified in these pathosystems (Al-Kherb et al., 1995; Anderson and Buzzell, 1992; Buzzell and Anderson, 1992; Crute and Pink, 1996; Illot et al., 1989; Spielman et al., 1989), but none, nor any of the corresponding avirulence genes, have been isolated. Much effort has been put into the investigation of these agronomically important diseases, but rapid progress has been hindered by some intrinsic attributes such as the obligate parasitic nature of the pathogen (*P. parasitica*, *B. lactucae*) or difficulties encountered in efficient genetic transformation of the host (soybean). To overcome these limitations, we have developed an Arabidopsis-*Phytophthora* pathosystem in which both organisms are more accessible to genetic analysis and transformation. The genus *Phytophthora* consists of over 60 different species; all but three species are plant pathogens. As no natural infections of Arabidopsis with *Phytophthora* have been reported in the literature, we decided to test a species, *Phytophthora porri*, that is able to infect plants of the family *Brassicaceae.* P. porri is mainly known as a pathogen of the family of the *Amarillidaceae* (Foister, 1931). Later reports describe infections on carrots (Ho, 1983; Semb, 1971; Stelfox and Henry, 1978), cabbage (Geeson, 1976; Semb, 1971) and various ornamentals (Kouyeas, 1977; Legge, 1951). Similarly to *P. infestans* and *P. sojae*, *P. porri* has only a limited host range. Differences in mtDNA as well as in morphology and physiology suggested that *P. porri* forms a heterogeneous group containing different species (De Cock et al., 1992). Isolates capable of infecting members of the *Brassicaceae* were not infectious on members of the *Amarillidaceae* and vice versa (De Cock et al., 1992). The isolates infectious on *Brassicaceae* appear to represent a different species from *P. porri*, and it was proposed that these be renamed as *P. brassicae* (De Cock et al., 1992). In the present publication, we report on the initial chararacterization of a novel Arabidopsis-*Phytophthora* pathosystem. It is shown that Arabidopsis is a true host of *P. porri* isolates. Susceptible accessions are extensively colonized and the pathogen produces asexual and sexual spores while resistant accessions react with a hypersensitive response and the rapid halt of pathogen ingress. The disease phenotype of various Arabidopsis defence response mutants in the resistant Col-0 background and the fact that neither SA nor its functional analogue benzothiadiazol (BTH; Görlach et al., 1996) are able to induce resistance in susceptible plants suggest that, in Arabidopsis, the establishment of resistance against *Phytophthora* is not based on SA-, ethylene- or jasmonic acid-dependent mechanisms. Thus, the resistance mechanisms effective against *Phytophthora* appear to be different from the ones effective against many other pathogens (Mauch-Mani and Métraux, 1998) and are reminiscent of the situation recently observed for some Arabidopsis/*P. parasitica* interactions (Bittner-Eddy and Beynon, 2001; McDowell et al., 2000). Interestingly, resistance against *Phytophthora* was completely abolished in the previously described *pad2* mutant (Glazebrook and Ausubel, 1994), indicating that PAD2 plays an important role in controlling the expression of resistance responses of Arabidopsis against *P. porri*.

II.2 RESULTS

P. porri has long been considered a pathogen with a narrow host range, infecting plants mainly from the family *Amarillidaceae*, the best known example being leek, after which it was named (Foister, 1931). Later it was also identified as infectious on cabbage, causing root rot (Heimann, 1994). Seven isolates of *P. porri* were tested on wild-type Arabidopsis accessions to determine whether these plants could serve as a host (Table 1). This screening resulted in the identification of susceptible and resistant hosts. The resistant accessions Columbia (Col-0) and Wassilewskija (WS-0) and the susceptible accession Landsberg erecta (Ler) and Mt-0 were chosen for further analysis.

Table 1. Comparison of resistance phenotypes of wild-type accessions and defence mutants of *Arabidopsis* against *Phytophthora porri* isolates

	Resistance phenotype[a] after inoculation with various *P. porri* isolates						
Accessions/mutants	HH	II	CBS 212.82	CBS 180.87	CBS 178.87	CBS 179.89	CBS 686.95
WS-0	R	R	R	nd	nd	S	S
Nd-0	R	R	R	nd	nd	S	S
Wei-0	R	R	R	nd	nd	S	S
RLD	R	R	R	nd	nd	S	S
Mt-0	S	S	S	nd	nd	S	S
C-24	R	R	R	nd	nd	S	S
Ler	S	S	S	S	S	S	S
Col-0	R	R	R	R	R	S	S
nahG	R–	R–	nd	nd	nd	nd	nd
sid2	R	R	R	R	R	S	S
npr1-1	R–	R–	nd	nd	nd	nd	nd
etr1-1	R	R	nd	nd	nd	nd	nd
ein2-1	R	R	nd	nd	nd	nd	nd
jar1-1	R	R	nd	nd	nd	nd	nd
pad2-1	S+	S+	S+	S+	S+	S+	S+
pad3-1	R–	R–	R–	R–	R–	S	S

[a]R, resistant; R–, resistant with a slight shift towards susceptibility, trailing necrosis; S, susceptible; S+, hyper-susceptible; nd, not determined

II.2.1 Incompatible interaction between Arabidopsis and *P. porri*

P. porri can penetrate Arabidopsis plants over the roots (data not shown) as well as over above-ground parts. The mode of penetration is independent of the initial propagule used for infection (zoospores, agar plugs with young mycelium, or suspended hyphal fragments), and the initial steps are the same in resistant and susceptible plants. With both zoospores and mycelium, penetration occurred preferentially over anticlinal walls of epidermal cells (Figure 1 a,b), occasionally via the stomatal opening (data not shown). Zoospores applied on leaves of Arabidopsis encysted, and developed a germ tube reaching up to several spore diameters in length before forming an appressorium over the point of penetration (Figure 1a). A penetration hyphae then started to grow between the anticlinal walls of two epidermis cells. At this point, differences between compatible and incompatible interactions became apparent. In resistant plants, the earliest microscopically visible response was observed starting 6 h after inoculation and consisted of the deposition of dense material, presumably of host origin, around the site of penetration as visualized for an attempted infection of WS-0 by *P. porri* isolate HH (Figure 1b). Staining of the tissue with aniline blue revealed that these depositions contained callose, which is specifically stained by this dye (Figure 1c). Another resistance phenotype frequently encountered was the hypersensitive reaction (HR). One or several

epidermal cells in the case of direct penetration through the epidermis (Figure 1d), or one or several mesophyll cells in the case of indirect penetration through a stomatal opening (data not shown), underwent rapid cell death visualized microscopically by the retention of trypan blue in their cytoplasm. In cells adjacent to the dead ones, a dense deposition of material was observed at the wall directly in contact with the dead cell (Figure 1d). Aniline blue staining revealed that the material encasing the HR cells consisted of callose (Figure 1e). Occasionally, the hyphae were able to penetrate further into the plant tissue but were soon surrounded by necrotic cells (Figure 1f). This trailing necrosis response successfully stopped further infection and became macroscopically visible as small necrotic regions on the leaves (data not shown).

II.2.2 Compatible interaction between Arabidopsis and *P. porri*

In susceptible Arabidopsis accessions, penetration also occurs preferentially at the border of adjacent epidermal cells. In an initial phase, lasting up to 3 days depending on the Arabidopsis accession, the mycelium grew exclusively in the intercellular spaces spreading in all directions away from the penetration site (Figure 2a,b). The hyphae were fairly regular in diameter and often in close contact with the plant cells (Figure 2b). Haustoria-like protuberances into the plant cells were only rarely observed (data not shown). During this first biotrophic phase, no reactions of plant cells were visible microscopically (Figure 2b) or macroscopically (data not shown). In a later phase, the tissue was colonized by a dense network of intra- and extracellular hyphae, and plant cells started retaining the trypan blue stain (Figure 2c). Macroscopically, this phase was characterized by the water-soaked and wilted appearance of the infected tissue. Under conditions of high air humidity, *P. porri* started to grow out of the stomata (Figure 2d), and the emerging sporangiophores gave rise to mostly obpyriform zoosporangia (Figure 2e). Seven days after inoculation, sexual spores, the oospores, started to appear (Figure 2f). Antheridia were either amphigynous as shown in Figure 2(f) or paragynous (data not shown). In the latter case, one to three antheridia per oogonium were observed. The results show that *P. porri* can extensively colonize and reproduce in susceptible accessions of Arabidopsis.

II.2.3 Inheritance of resistance

An attempt to determine the pattern of inheritance of resistance was undertaken using Lister and Dean RI lines of a cross between Col-0 and Ler available from the Nottingham Arabidopsis Stock Centre, UK. Two independent experiments were performed: one with set 1 of 100 RI lines and one with a reduced set of 30 recombinant inbred (RI) lines selected as having the highest frequency of recombination over the five chromosomes. Fifteen plants for each line were inoculated by the agar plug method using *P. porri* isolate II (100 RI lines) or droplets of a suspension of mycelial fragments of *P. porri* isolate HH (30 RI lines). The resistance phenotypes were scored compared to the ones observed in wild-type parents. The Col-0 parental plants were consistently scored as fully resistant and the Ler parental plants as fully susceptible throughout both experiments. In both cases, however, the RI lines frequently showed intermediate phenotypes that differed from the resistant or the susceptible parental phenotypes. It was therefore not possible to assign a map position for the determinant(s) of resistance in the interaction between *A. thaliana* accessions Col-0 and *P. porri* isolates II or HH.

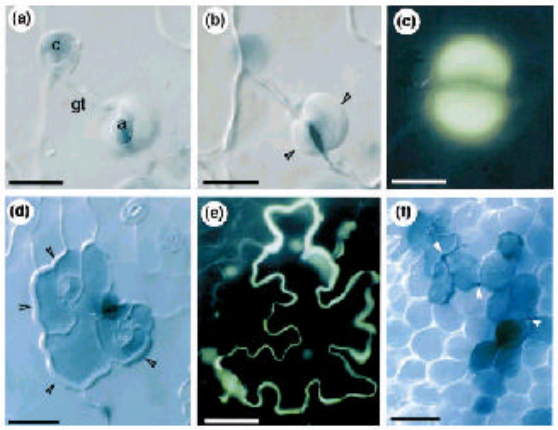

Figure 1.

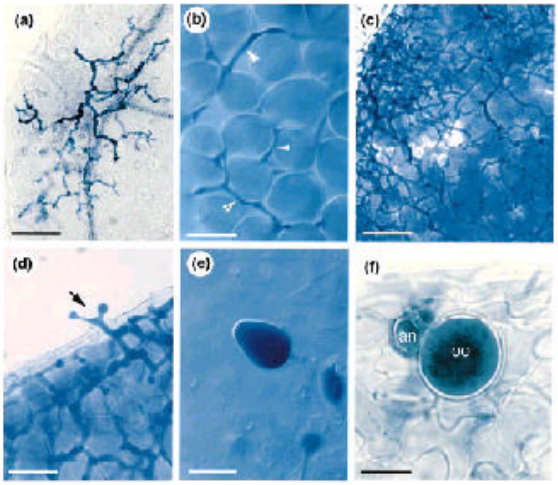

Figure 2.

Figure 1. Cytological characterization of the incompatible interaction of Arabidopsis with *P. porri.*

(a,b,d,f) Differential interference contrast (DIC) micrographs of lactophenol-trypan blue-stained preparations, and (c,e) fluorescence micrographs of decolorized aniline blue-stained preparations as described in Experimental procedures. (a) A cyst (marked "c") of *P. porri* isolate HH has formed a germ tube (marked "gt") and an appressorium (marked "a") on the upper epidermis of a leaf of *A. thaliana* accession WS-0 6 h after inoculation. The faint blue staining inside the cyst and the appressorium indicates the cytoplasm. Bar = 10 µm. (b) Same as (a), focused on the layer immediately below the appressorium. Arrowheads indicate a heavy deposit of material called a papilla surrounding the attempted penetration site at the border of two anticlinal walls of epidermal cells. Bar = 10 µm. (c) Fluorescence of callose in an aniline blue-stained papilla in a leaf of *A. thaliana* accession Col-0 24 h after infection with mycelium of *P. porri* isolate HH. Bar = 15 µm. (d) Hypersensitive reaction of *A. thaliana* accession Col-0 after infection with *P. porri* isolate HH 24 h after inoculation with mycelium. The cells that have undergone an HR are stained a darker blue due to retention of trypan blue. The penetrating hypha is out of the focal plane and only the actual point of penetration can be seen as a dark blue area between the two stomata in the HR region. In the adjacent cells, deposits of material (arrowheads) can be seen on the side where their cell walls are in contact with the HR cells. Bar = 50 µm. (e) Fluorescence of callose showing the limits of an epidermal cell of *A. thaliana* accession Col-0 that has undergone an HR after an attempted penetration by *P. porri* isolate HH. The picture was taken 24 h after inoculation. Bar = 25 µm. (f) Trailing necrosis in a leaf of *A. thaliana* accession Col-0 48 h after inoculation with mycelium of *P. porri* isolate HH. The hypersensitive cells are stained a darker blue; arrowheads point to places where the hypha is visible. Bar = 120 µm.

Figure 2. Cytological characterization of the compatible interaction of Arabidopsis with *P. porri*.

(a,c,d,f) Bright field, and (b,e) differential interference contrast (DIC) micrographs of the compatible interaction. All the preparations were stained with lactophenol-trypan blue as described in Experimental procedures. (a) Young colony of *P. porri* isolate HH in *A. thaliana* accession Mt-0 3 days after inoculation with zoospores. The mycelium is visible as a dark blue network ramifying inside the leaf. Bar = 150 µm. (b) Hyphae (arrowheads) of *P. porri* isolate HH growing intercellularly in the mesophyll of a leaf of *A. thaliana* accession Ler 4 days after inoculation with mycelium. Note the absence of any necrosis in the plant cells. Bar = 60 µm. (c) Heavy colonization as seen in a leaf of *A. thaliana* accession Mt-0 one week after inoculation with zoospores of *P. porri* isolate HH. The hyphae grow inter- and intracellularly and the plant tissue shows macroscopic symptoms of wilting. Bar = 150 µm. (d) Sporangiogenous hyphae of *P. porri* isolate II emerging through the stomatal opening in a leaf of *A. thaliana* accession Mt-0 5 days after inoculation with zoospores. Bar = 40 µm. (e) Tear-shaped zoosporangium on the surface of a leaf of *A. thaliana* accession Ler 4 days after inoculation with mycelium of *P. porri* isolate HH. Bar = 50 µm. (f) Oogonium and amphigynous antheridium of *P. porri* isolate D in a leaf of *A. thaliana* accession WS-0. Bar = 25 µm.

II.2.4 Interaction between *P. porri* and selected Arabidopsis defence pathway mutants

In order to learn more about the basis of resistance towards *Phytophthora*, several Arabidopsis mutants or transgenics with defects in defence signaling were tested for their reaction towards an attempted infection with *P. porri* isolate HH. The tested Arabidopsis mutants included *nahG*, *sid2* and *npr1-1* with defects in SA signaling (Cao et al., 1994; Delaney et al., 1995; Gaffney et al., 1993; Nawrath and Métraux, 1999), the ethylene receptor mutant *etr1-1* (Bleecker et al., 1988), the ethylene-insensitive mutant *ein2-1* (Guzmann and Ecker, 1990), the jasmonate-insensitive mutant *jar1-1* (Staswick et al., 1992), and two mutants with reduced camalexin levels: *pad2-1* and *pad3-1* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). All the mutants were in the background of the resistant accession Col-0. The results of the phenotypical analysis of the mutant collection are summarized in Table 1. Interference with ethylene or jasmonic acid signalling in *etr1*, *ein2* and *jar1* had no effect on the resistant phenotype. Interestingly, the *jar1* mutant showed a much higher incidence of callose-containing papillae (Figure 3e). This, however, had no effect on the already resistant phenotype. Prevention of SA accumulation in *nahG* or SA signaling in npr1 had only a minor effect on the resistance towards *P. porri*. The resistance was slightly shifted towards susceptibility: *P porri* could occasionally colonize small parts of the tissue but was soon stopped by host cell necrosis with the effect that zoosporangia and oospores were never observed in *nahG* or *npr1* plants. The reaction of the SA-deficient mutant *sid2* did not differ from that observed in wild-type plants. A slight shift towards susceptibility was observed in *pad3*, which has a defect in camalexin biosynthesis and as a result is unable to synthesize camalexin (Zhou et al., 1999). Thus, SA signaling and camalexin production appear to contribute to resistance but do not seem to be part of the main defence mechanism. However, the *pad2* mutation appeared to knock-out all mechanisms that are relevant for the establishment of resistance: *pad2* plants proved to be hypersusceptible towards *P. porri*. Figure 3(a,d) shows the results of an inoculation of *pad2* with *P. porri* isolate HH. The pathogen rapidly colonized the leaf tissue. The hyphae ramified in the intercellular spaces, and often the density of colonization was such that several hyphae grew side by side, filling the entire space between two cells (Figure 3a). Characteristic for infections in *pad2* was that *P. porri* was able to colonize host cells intracellularly. Some host cells appeared completely filled with hyphae but there was no apparent reaction of the plant cell to this invasion (Figure 3b). Furthermore, the formation of haustoria happened more frequently compared to a normal compatible infection (Figure 3c). The ring of cells surrounding the base of trichomes seemed especially attractive to *P. porri*. In colonized areas of leaves of *pad2*, these cells were all extensively colonized (Figure 3d). Colonization of *pad2* by *P. porri* was not apparent macroscopically until 3 days after inoculation, when the colonized tissue started to get a water-soaked appearance followed by a total collapse without visible necrosis (data not shown). *pad2* was susceptible to all tested isolates of *P. porri* (Table 1) but remained completely resistant to isolates of *Phytophthora infestans* (data not shown).

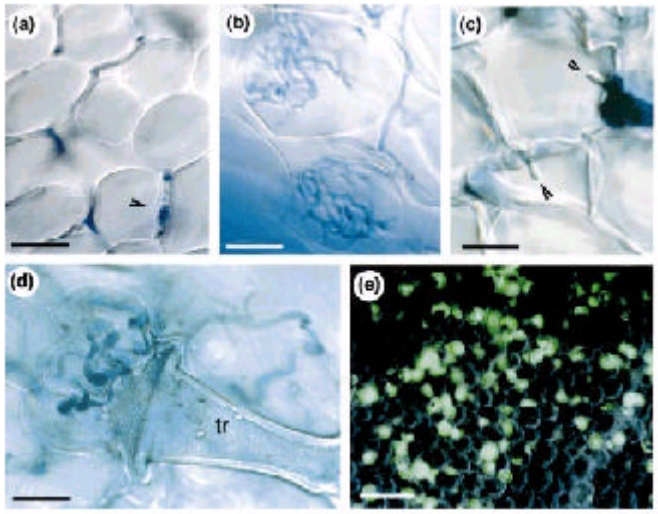

Figure 3. Cytological characterization of the interaction of *P. porri* with the hyper-susceptible *pad2-1* mutant and the jasmonate-insensitive mutant *jar1-1*.

(a,b,c) Differential interference contrast (DIC), (d) bright-field, and (e) fluorescence micrographs; (a-d) show lactophenol-trypan blue-stained preparations and (e) was stained with decolorized aniline blue as described in Experimental procedures. (a) Intercellularly growing mycelium of *P. porri* isolate HH in the mesophyll of the *pad2* mutant. Note the locally high concentration of hyphae (arrowhead) without visible reaction of the host cells. Bar = 60 µm. (b) Extremely dense intracellular colonization of mesophyll cells of the *A. thaliana pad2* mutant with hyphae of *P. porri* isolate HH. Note that no visible reaction of the host cell can be detected. Bar = 40 µm. (c) Intracellular finger-shaped haustoria (arrowheads) of *P. porri* isolate HH in mesophyll cells of the *pad2* mutant. Bar = 20 µm. (d) Preferential colonization of the cells surrounding the base of trichomes (marked "tr") by *P. porri* isolate HH in the *pad2* mutant. Bar = 40 µm. (e) Low-magnification image of part of a leaf of *jar1* after infection with mycelium of *P. porri* isolate HH. All the bright green spots are papillae stained for callose at attempted penetration points of hyphae in the leaf. Bar = 150 µm.

II.2.5 Analysis of marker gene expression in different defence mutants

The expression of PR-protein 1 (PR-1) was used as a marker of SA-dependent defence responses (Ward et al., 1991) and the expression of a plant defensin PDF1.2 served as a marker of ethylene- and jasmonic acid-dependent defence gene induction (Penninckx et al., 1998). As shown in Figure 4, inoculation of the resistant accession Col-0 with *P. porri* isolate HH lead within 24 h to increased expression of *PR-1* and *PDF1.2*. *PR-1* gene expression was completely blocked in *nahG* plants and partially blocked in the *npr1* mutant, while the *etr1* and *jar1* mutations showed no effect on *PR-1* expression compared to wild-type. *PR-1* expression was only slightly down-regulated in the *pad3* mutant but was completely blocked in the *pad2* mutant. *PDF1.2* expression was strongly down-regulated in inoculated *etr1* and *jar1* mutants but remained unaffected in the SA signaling mutants and the two tested *pad* mutants. Despite the lack of *PDF1.2* expression, *etr1* and *jar1* both showed a resistant phenotype, thus suggesting that *PDF1.2* accumulation does not contribute much to resistance against *P. porri*. Figure 4 includes a comparison of the *PR-1* expression pattern in Col-0 and the collection of mutant plants infected with *P. porri* isolate HH or *Peronospora parasitica* isolate EMWA. The profile of *PR-1* expression induced in both pathosystems is nearly identical. *PR-1* expression is at least partially blocked in *nahG*, *npr1* and *pad2*, but remains unaffected in *etr1* and *jar1*. However, the pattern of resistance phenotypes is completely different in the two pathosystems as indicated at the top and bottom lines of Figure 4. *nahG* and *npr1* remain resistant against *P. porri* but become susceptible towards *P. parasitica*. In contrast, *pad2* becomes susceptible towards *P. porri*, but remains resistant against *P. parasitica*. Thus, the resistance mechanisms effective against *P. porri* appear to be fundamentally different from the mechanisms that are effective against *P. parasitica*.

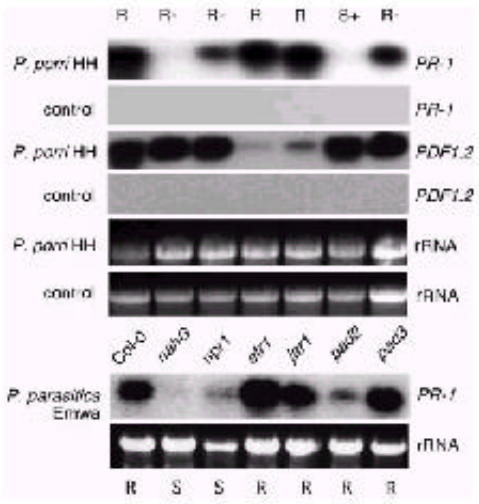

Figure 4. *PR-1* and *PDF1.2* marker gene expression in different Arabidopsis genotypes in response to inoculation with *P. porri.*

PR-1 and *PDF1.2* gene-specific probes were used for RNA gel blot analysis of the indicated genotypes (Col-0, *nahG, npr1, etr1, jar1, pad2, pad3*). Ethidium bromide staining of the gel was used as an estimation of equal sample loading (rRNA). Plants were either uninoculated (control), inoculated with *P. porri* isolate HH or *P. parasitica* isolate EMWA. RNA was extracted 24 h post-inoculation. Resistance phenotypes of the respective interactions are indicated for *P. porri* in the top panel and for *P. parasitica* in the bottom panel. The terms R, R-, S and S+ are explained in the footnote to Table 1.

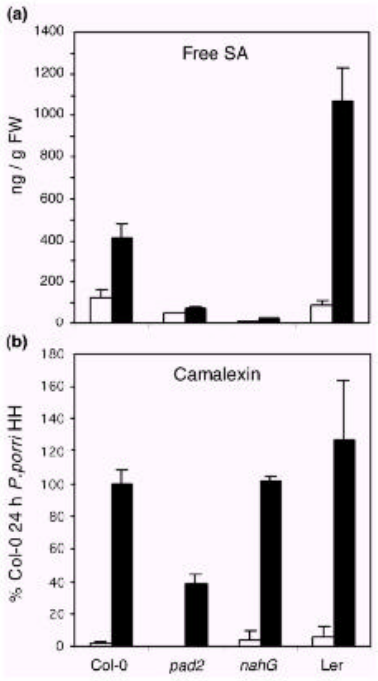

Figure 5. Accumulation of free SA and camalexin in Col-0, *pad2*, *nahG* and Ler after inoculation with *P. porri* isolate HH.

Five-week-old plants were inoculated with *P. porri* isolate HH and leaves were harvested 24 h later. The values represent the average of two independent samples ±SE. (a) Levels of free salicylic acid (SA). (b) Camalexin levels. Because of the lack of a pure standard, values are expressed in relation to the value for Col-0 24 h post-inoculation.

II.2.6 Determination of SA and camalexin levels

PR-1 expression was completely blocked in *pad2*. This *nahG*-like phenotype suggested that the *pad2* mutation might have a negative effect on SA accumulation. To test this hypothesis, the effect of *P. porri* inoculation on SA levels was measured in Col-0, Ler, *nahG* and *pad2*. The results of the SA measurement 24 h post-inoculation are shown in Figure 5(a). Within 24 h following inoculation, the level of free SA increased about threefold in the resistant Col-0 and more than 10-fold in the susceptible Ler. The SA levels of *nahG* plants were very low in controls and hardly increased following inoculation with *P. porri*. A similar SA-minus phenotype was found for *pad2*. Even uninfected *pad2* plants had a threefold lower SA content than Col-0 plants. This value only slightly increased following inoculation and remained lower than the SA content in untreated Col-0. The pattern of SA levels 36 h and 48 h post-inoculation was qualitatively unchanged from that shown in Figure 5 (data not shown). *pad2* clearly shows an *nahG*-like SA-minus phenotype. The values for conjugated SA for Col-0, *nahG* and *pad2* 24 h post-inoculation were in the range of control plants (500-800 ng g^{-1} FW) indicating that the lack of accumulation of free SA was not caused by an increased SA conjugation rate. The level of conjugated SA was increased to 1800 ng g^{-1} FW in the susceptible Ler (data not shown). Because *pad2* was originally described as a camalexin mutant (Glazebrook and Ausubel, 1994), its ability to produce camalexin was tested 24 h post-inoculation (Figure 5b). Inoculation of Col-0 with *P. porri* isolate HH lead to a 60-fold increase in the level of camalexin compared to uninoculated control plants. Very similar results were found for *nahG* plants, while the levels of camalexin in the susceptible accession Ler were slightly higher. The increase in camalexin production appears to be independent of SA accumulation and the occurence of HR. In contrast to Col-0, the level of camalexin in uninoculated *pad2* plants was found to be below the limit of detection. Camalexin accumulation was reduced in inoculated *pad2* plants to about 40% of the values found in Col-0.

II.2.7 Treatment with SA and BTH

In order to further elucidate the role of SA-dependent defence against *P. porri*, the susceptible accession Ler and the hyper-susceptible mutant *pad2* were treated by soil drenching with a solution of the resistance-inducing chemicals SA and BTH. Table 2 shows that neither SA nor BTH were able to induce resistance in the treated plants.

Table 2. Effect of BTH and SA treatment on the resistance phenotypes of susceptible *Arabidopsis* plants towards *Phytophthora porri*

Accession, mutant/treatment	Resistance phenotype[a]
Ler/H_2O	S
Ler/BTH	S
Ler/SA	S
pad2/H_2O	S+
pad2/BTH	S+
pad2/SA	S+

[a]S, susceptible; S+, hyper-susceptible

II.3 DISCUSSION

The Arabidopsis-*Phytophthora* pathosystem

An experimental system for analysis of the interaction of Arabidopsis with the phytopathogenic oomycete *Phytophthora porri* was established. Accessions of Arabidopsis were screened for their reaction to different isolates of *P. porri* known to be pathogenic on family members of the *Brassicaceae.* Accession-isolate combinations were identified that result in either complete resistance or complete susceptibility. Accessions susceptible to a given isolate of *P. porri* are completely colonized by *P. porri* within a few days (Figure 2). In the initial phase, the pathogen grew in the intercellular space and no host reaction was observed. In a later phase, the host cells were macerated, oospores formed inside the colonized tissues, and hyphae grew out of the stomata to give rise to zoosporangia. Thus, *P. porri* can complete its whole life cycle in a susceptible host, and Arabidopsis can therefore be considered a true host of this pathogen. The compatible interaction showed all the characteristics of a facultative biotrophic interaction, very similar to *P. infestans* on potato and other agronomically important diseases caused by *Phytophthora* (Erwin and Ribeiro, 1996). In incompatible host-pathogen combinations, different degrees of resistance were observed (Figure 1). All resistant accessions reacted either with an HR comprising one or a few cells, or the pathogen was able to grow to some extent into the tissue triggering an HR visible macroscopically as a necrotic fleck. The observation of an HR in resistant Arabidopsis accessions is in accordance with observations showing that the HR is associated with all forms of resistance (vertical and horizontal) to agronomically important *Phytophthora* and downy mildews (Kamoun et al., 1999). The formation of callose-containing papillae was frequently observed at the site of penetration. Interestingly, callose production and cell wall appositions were also found in the cells adjacent to cells undergoing HR. These extensive appositions are presumably produced by the neighbouring cells and were restricted to walls in direct contact with the dying cells. It is not known how this directional callose deposition process is regulated. Deposition of callose-containing papillae and wall appositions of cells neighbouring attacked cells has also been observed in other *Phytophthora* plant interactions (Coffey and Wilson, 1983). The advantage of the novel *Phytophthora* pathosystem is its use of Arabidopsis as a host. The availability of complete sequence information, the ease of mutational analysis, the extensive mutant collection and the possibility of using microarrays for gene expression analysis is expected to lead to an acceleration in data generation.

Phytophthora is an agronomically more important pathogen than the obligate biotrophic *Peronospora parasitica* that is frequently used as a model oomycete pathogen of Arabidopsis (Holub et al., 1994; Koch and Slusarenko, 1990). *Phytophthora* has the advantage that it can be cultured in vitro, and both sexual and asexual spores are produced by *P. porri* under these conditions (data not shown). *Phytophthora* is accessible to molecular analysis, and *Phytophthora* species including *P. porri* (Si-Ammour et al., unpublished results) are transformable (Judelson et al., 1991). The genome size of *P. porri* (70-80 Mio bp as determined by flow cytometry; (Si-Ammour et al., unpublished results) is slightly larger than that of *P. sojae* (Mao and Tyler, 1991) and much smaller than that of *P. infestans* (Tooley and Therrien, 1987). The major disadvantage of the novel system is based on an inherent property of the oomycetes compared to fungi. Oomycetes are diploid during most phases of their life cycle (Boccas, 1976; Brasier and Sansome, 1975). The only haploid stages occur in the gametangia formed immediately prior to fertilization. This fact complicates the genetic analysis of *Phytophthora* because the phenotype of recessive mutations can only be discovered after selfing in the F2 generation. In contrast to the heterothallic *P. infestans, P. porri*, like *P. sojae,* is homothallic, forming oospores by selfing (Erwin and Ribeiro, 1996).

This is an advantage for genetic analysis as recessive lethal mutations should be rare and the strains are mostly pure-breeding and therefore homozygous. Crossing of different homothallic strains of oomycetes has been described previously (Bhat and Schmitthenner, 1993; Francis and St. Clair, 1993; Tyler et al., 1995; Whisson et al., 1994), and F1 hybrids are differentiated from selfed progeny by using parental strains carrying single dominant selectable markers conferring resistance to metalaxyl or p-fluorophenylalanine (Bhat and Schmitthenner, 1993).

Inheritance of resistance

The results of the studies on inheritance of resistance in the recombinant inbred lines between Col-0 and Ler did not allow attribution of resistance to a specific single locus in the Col-0 genome. The frequently observed intermediate nature of the phenotypes in the RI lines compared to the parental lines suggests polygenic control of resistance for the accession/isolate combinations tested.

Resistance of Arabidopsis against *P. porri* does not depend on SA-, ethylene- or jasmonate-dependent signaling pathways

Inoculation of Arabidopsis with *P. porri* triggered accumulation of the jasmonic acid- and ethylene-dependent marker gene *PDF1.2* and of the SA-dependent marker gene *PR-1* (Figure 4). Although both of these major defence signaling pathways are activated, they do not seem to be involved in the regulation of the defence mechanisms that are effective against *P. porri* (Table 1). Interference with ethylene or jasmonic acid signaling in the *etr1*, *ein2* and *jar1* mutants had no effect on the resistance phenotype. The *jar1* mutant showed an increased formation of papillae (Figure 3), suggesting a negative correlation between jasmonic acid signaling and the formation of papillae. Increased papillae formation had no effect on the disease phenotype in the resistant genetic background of Col-0. A *jar1* mutant in the susceptible Ler background is not available to test the effect of increased papillae formation on disease susceptibility. In contrast to our results with *Phytophthora*, it was shown that resistance of Arabidopsis towards other oomycete pathogens, *Pythium irregulare* and *Pythium mastophorum*, depends on functional jasmonate signaling (Staswick et al., 1998; Vijayan et al., 1998). Surprisingly, blockage of SA accumulation had only a minor effect on the resistance of Arabidopsis towards *P. porri*. The trailing necrosis observed in these interactions was still effective in preventing colonization. Resistance in *nahG* plants is only slightly shifted towards susceptibility, indicating a minor contribution of the SA signaling pathway to resistance. However, the SA biosynthetic mutant *sid2* remained completely resistant towards isolates of *P. porri* that were unable to cause disease in Col-0. The observed difference in disease phenotype between *nahG* and *sid2* might be caused by the different levels of SA remaining in these plants (Nawrath and Métraux, 1999). The SA level in *sid2* could be just above a critical threshold for efficient induction of HR while in *nahG* plants this critical level is not reached. The prevention of accumulation of SA in *nahG* transgenic plants had a stronger effect on *PR-1* gene expression than in the SA signaling mutant *npr1* (Figure 4). Similar SA-dependent but partially NPR1-independent regulation of PR gene expression has been observed in other pathosystems (Clarke et al., 2000; Rate et al., 1999; Reuber et al., 1998; Shah et al., 1999). A dramatic effect on disease resistance was observed in the *pad2* mutant which was originally isolated as a camalexin mutant (Glazebrook and Ausubel, 1994). *pad2* was found to be hyper-susceptible towards *P. porri*. The pathogen could extensively colonize the plant tissue without causing any of the defence responses observed in the resistant wild-type such as HR and papillae formation (Figure 3). No host response was observed, with the exception an increased *PDF1.2* expression (Figure 4). The results in Figure 5 show that *pad2* behaves as a SA-accumulation mutant similar to *nahG* plants and the *sid*

mutants (Gaffney et al., 1993; Nawrath and Métraux, 1999). It is unclear at what level in the signalling cascade *pad2* is interfering with SA accumulation. The SA-deficient phenotype of *pad2* was also observed in uninoculated plants, indicating that the effect of PAD2 is not limited to *Phytophthora*-specific signalling events. The *pad2* mutant is blocked with respect to SA accumulation and *PR-1* expression, and becomes hyper-susceptible towards *P. porri*. However, the lack of SA accumulation and *PR-1* expression in *pad2* seems not to be the cause of the observed hyper-susceptibility. A similar block in SA accumulation in *nahG* plants has only a limited effect on disease resistance, and in the *sid2* mutant, the lack of SA accumulation has no effect at all on disease resistance against *P. porri*. The susceptibility towards *P. porri* in *pad2* seems not to be caused by the lack of SA-dependent defence responses. In agreement with our conclusion, it was not possible to induce resistance in Ler or *pad2* (Table 2) by prior application of SA or the SA analogue benzothiadiazole (Görlach et al., 1996). Reports on the contribution of SA and PR-1 protein expression towards resistance in other plant-*Phytophthora* pathosystems are controversial (Alexander et al., 1993; Vleeshouwers et al., 2000; Yu et al., 1997). The *pad2* mutation appears to affect SA-, ethylene- and jasmonic acid-independent defence mechanisms which are of crucial importance for the establishment of resistance against *P. porri*. These unknown defence mechanisms only partially include the accumulation of camalexin. The effect of the *pad2* mutation on camalexin production (Figure 5b) is much weaker than in the camalexin biosynthesis mutant *pad3* which is incapable of producing camalexin (Zhou et al., 1999). However, the complete lack of camalexin production in *pad3* has only a marginal effect on disease resistance towards *P. porri* (Table 1). Thus, camalexin production appears to contribute to resistance but does not seem to be part of the main defence mechanisms. It has been shown that camalexin accumulation is not important for defence against avirulent *Pseudomonas syringae* pathovars (Glazebrook and Ausubel, 1994) but appears to play a role in resistance towards *Alternaria brassicicola* (Thomma et al., 1999). In the *Phytophthora* pathosystem, camalexin production seems to be independent of SA content (Figure 5). In contrast, *camalexin* production was strongly reduced in *nahG* plants inoculated with virulent or avirulent bacteria (Nawrath and Métraux, 1999; Zhao and Last, 1996; Zhou et al., 1999). However, it was not reduced in *sid1* and *sid2* mutants which both have a defect in SA accumulation (Nawrath and Métraux, 1999). Both SA-dependent defence responses and camalexin accumulation appear to contribute to the resistance of Arabidopsis towards *Phytophthora*. An alternative explanation to the above-hypothesized PAD2-controlled unknown defence mechanism, which is not excluded by our results, is that the combined effect of the reduced SA and camalexin accumulation causes the hyper-susceptibility of *pad2*. However, it appears unlikely that the weak disease resistance phenotypes of *nahG* and *pad3* in combination would give rise to the hyper-susceptibility of *pad2*. This alternative hypothesis could be tested in *pad3/nahG* and *pad3/sid2* double mutants.

Comparison of the *P. porri* system with the *P. parasitica* system

The *PR-1* and *PDF1.2* gene expression patterns induced by *P. porri* and *P. parasitica* were nearly identical in the different mutants (Figure 4). However, the pattern of resistance phenotypes is different in the two oomycete pathosystems. Interference with SA signaling in *nahG* and *npr1* leads to susceptibility towards the avirulent *P. parasitica* isolate EMWA, but has only a very minor effect on the resistance against *P. porri*. The *pad2* mutant becomes susceptible only towards *P. porri* but remains resistant against *P. parasitica*. The effect of the *pad2* mutation on resistance against several avirulent *P. parasitica* isolates has been tested previously (Glazebrook et al., 1997). In agreement with our results, no significant shift towards susceptibility was observed in plants inoculated with four out of five avirulent isolates of *P. parasitica*. The fifth isolate of *P. parasitica* (Emoy2) was able to colonize *pad2*

to some extent. Thus, resistance against *P. porri* appears to depend on PAD2-controlled defence mechanisms that are different from the mechanisms effective against most *P. parasitica* isolates. Recent evidence suggests that there is some unexpected variety in defence signaling in the *P. parasitica* system. Resistance to some avirulent strains of *P. parasitica* was shown to be SA-independent (Bittner-Eddy and Beynon, 2001) and in one case also independent of jasmonic acid and ethylene signaling (McDowell et al., 2000). A third difference between the two oomycete pathosystems is that the prevention of camalexin biosynthesis in *pad3* had no effect on the resistance against most *P. parasitica* isolates (Glazebrook et al., 1997) but causes a slight shift towards susceptibility against *P. porri*. In conclusion, an Arabidopsis-*Phytophthora* pathosystem was established that allows the simultaneous molecular and genetic analysis of host and oomycete pathogen. The novel pathosystem shows the characteristics of a facultative biotrophic interaction very similar to agronomically important diseases caused by other *Phytophthora* species. Our initial results demonstrate that effective disease resistance of Arabidopsis against *Phytophthora* is dependent on defence mechanisms that are controlled by the *PAD2* gene product. *PAD2* has not yet been cloned and its function in resistance is not well described. In the Arabidopsis-*Phytophthora* system, PAD2 appears to control SA and camalexin production. However, our results demonstrate that, in contrast to most other pathosystems, SA-regulated defence responses play only a minor role in resistance against *Phytophthora*. Resistance of Arabidopsis against *P. porri* appears to depend on unknown SA-independent mechanisms that are under the control of PAD2.

II.4 MATERIAL AND METHODS

II.4.1 *Phytophthora porri* isolates and *in vitro* culture conditions

The *Phytophthora porri* isolates HH and II were kindly supplied by Francine Govers (University of Wageningen, The Netherlands) and isolates CBS 212.82, CBS 180.87, CBS 178.87, CBS 179.87 and CBS 686.95 were purchased from the Centraalbureau voor Schimmelcultures (Baarn & Delft, The Netherlands). They were routinely grown on V8 juice (Campbell Soups) agar (Erwin and Ribeiro, 1996) in the dark at 18°C. Zoospores were produced by placing 15 plugs (5 mm diameter) of mycelium in 10 ml of clarified (by centrifugation, 3000 g 20 min, 4000 rev min^{-1}) V8 juice (10%) in the dark at 16°C for 2-3 days. At that time, the V8 juice was replaced by Schmidthenner solution (Erwin and Ribeiro, 1996). After 3-4 days of incubation, the mineral solution was replaced by cold sterile water and the zoospores were released within 2-4 h into the water. For short-term storage up to several months, the *Phytophthora* strains were cultivated on potato carrot agar (Johnston and Booth, 1968) and kept at 4°C. Long-term storage was accomplished by immersing agar plugs with mycelium in 10% glycerol followed by storage in liquid nitrogen (Smith, 1982).

II.4.2 Plant material

Arabidopsis thaliana seeds of accessions Columbia (Col-0), Wassilewskija (WS-0) and Landsberg erecta (Ler) were purchased from Lehle Seeds (Round Rock, Texas, USA); the other accessions were obtained from the Arabidopsis Information Service (AIS) collection. The mutants *pad2-1* and *pad3-1* were supplied by J. Glazebrook (Novartis, Agricultural Discovery Institute Inc, San Diego, USA) and *jar1-1*, *npr1-1*, *etr1-1* and *ein2-1* seeds and the RI lines were obtained from X. Dong (Duke University, Durham, New York, USA), P.E. Staswick (University of Nebraska, Lincoln, Nebraska, USA) and the Nottingham Arabidopsis

Stock Center, respectively. The *nahG* line was provided by J. Ryals (Novartis, Research Triangle Park, North Carolina, USA) and the *sid2* mutant was provided by C. Nawrath (University of Fribourg, Switzerland). After sowing on a mixture of commercial potting soil and perlite (3:1), the seeds were stratified for 3 days at 4°C in the dark before being transferred to a growth chamber with a 10/14 h day/night photoperiod at 18°C/16°C.

II.4.3 Preparation of inoculum and infection of plants with *P. porri*

Three different methods of inoculation were used. (a) Plugs of young mycelium growing on V8 agar were cut out using a cork borer and placed upside-down on leaves of 3-4 weeks-old plants. (b) For zoospore inoculations, droplets of a zoospore suspension (10^4 spores ml^{-1}) were placed on the leaves. (c) For inoculation of leaves with a suspension of mycelial fragments, plugs of young mycelium growing on V8 agar were cut out using a cork borer, placed into a 10% solution of clarified V8 juice and incubated 4 days at 18°C in the dark. The mycelium was then dissected away from the agar plug, washed twice with tap water and resuspended into tap water (1 ml per plug). The mycelium was homogenized for 5 sec at half maximal speed using a Polytron blender. The resulting suspension was applied as droplets onto the leaves. For the first 24 h the lids of the trays were kept tightly shut in order to ensure 100% relative humidity. Subsequently, a relative humidity of about 70% was kept in the trays. These conditions were maintained for the whole period of the experiments.

II.4.4 Inoculation with *Peronospora parasitica*

Isolate EMWA of *P. parasitica* was transferred weekly onto new Arabidopsis plants of accession Wassilewskija (WS-0) and infections were performed with a spore suspension of 10^4conidia ml^{-1} as described previously (Mauch-Mani and Slusarenko, 1994).

II.4.5 Treatment with SA and BTH

Five-week-old *A. thaliana* plants were treated by soil drench with a solution of SA and BTH (supplied by U. Neuenschwander, Syngenta, Switzerland) to yield a final concentration of 330 mM in the soil. The resistance-inducing treatment was applied 1 day prior to challenge with *P. porri*.

II.4.6 Microscopy

Leaves were harvested at different time-points and stained with lactophenol-trypan blue to visualize fungal structures and dead plant cells in the tissue (Keogh et al., 1980) or with decolorized aniline blue (Smith and McCully, 1978) for visualization of callose. The stained material was viewed using a Leica DMR microscope equipped with bright-field, differential interference contrast (DIC) and UV optics.

II.4.7 RNA gel blot analysis

Plant material was quick-frozen in liquid nitrogen, pulverized and kept at -80°C before further processing. RNA was extracted as described by Zimmerli et al. (2000). RNA aliquots (10 mg) of RNA were separated on a formaldehyde/agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was probed with ^{32}P radiolabelled cDNA (RadPrime DNA Labeling System, Life Technologies, Merelbeke, Belgium) of *PR-1* (Uknes et al., 1992) and *PDF1.2* (Penninckx et al., 1996).

II.4.8 Measurement of salicylic acid and camalexin

The measurement of SA and camalexin was performed as described previously (Meuwly and Métraux, 1993; Nawrath and Métraux, 1999).

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CHAPTER III

In planta quantification of transgenic *Phytophthora infestans* and *Phytophthora porri* expressing Green Fluorescent Protein (GFP) reveals that ß-aminobutyric acid protects *Solanum tuberosum* and *Arabidopsis thaliana* against late blight.

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ABSTRACT

In order to quantify *in planta* the fungal biomass during infection, we have stably transformed both *Phytophthora porri* and *Phytophthora infestans* with a vector carrying both marker gene neomycine phsphotransferase (nptII) and the reporter gene green fluorescent protein (GFP). Drug resistant transformants were obtained and they strongly express GFP during their whole life cycle. They were used to analyze induced resistance towards *Phytophthora* in both Arabidopsis and potato by measuring their emitted fluorescence *in planta.* This method is non-destructive and allows an easy scoring of the infection process and can be coupled to microscopical observations. By this way we have quantified resistance in both Arabidopsis and potato previously treated with BTH and BABA. We evaluated the BABA induced resistance in potato at 97% and 100% in Arabidopsis. In contrast, benzothiadiazoles (BTH or Bion®) did not induce significant resistance in both Arabidopsis and potato.

III.1. INTRODUCTION

With more than fifty species causing destructive diseases of thousands of plant species, *Phytopththora* is one of the major plant pathogens in the world. *Phytophthora infestans*, the causing agent of potato late blight epidemics of the mid-nineteenth century, constrains considerably the world potato production. Worldwide losses in potato production exceed $3 billion annually (Kamoun, 2000). The appearance of new, highly agressive strains of *P. infestans* resistant to the most widely used phenylamide fungicides (PAFs) exacerbate the already alarming situation (Gisi and Cohen, 1996; Judelson, 1997). Projects based on a transgene-induced resistance in potato (Tadege et al.,1998; Abad et al., 1997) were believed to rapidly produce new potato cultivars highly resistant to *P. infestans*. Because of resistance of the public to genetically modified plants, those projects were not followed up by commercial applications limiting a more wide use of the transgenic technology. In fact, the increased popularity of "organic crops" and "sustainable agriculture" place the chemical inducers of systemic resistance (ISR) as the alternative of choice (Kuæ 2001). Those chemicals have no proven antifungal activity and can be divided in three groups: inorganic compounds, natural organic compounds and synthetic compounds (Sticher et al., 1997 and Oostendorp et al., 2001). The synthetic compounds include isonicotinic acids (Métraux et al., 1991), benzothiadiazoles (Kunz et al, 1997), probenazole (Watanabe et al., 1979), cyclopropane carboxylic acid derivatives (Langcake et al., 1983) and ß-aminobutyric acid (Cohen, 1995). This last compound, commonly named BABA, is one of the most promising inducer of resistance in tomato (*Lycopersicon esculentum*) and in potato (*Solanum tuberosum*) toward *Phytopthora infestans*. In tomato, BABA sprayed on leaves at a concentration of 2000 µg/ml provides a protection against *P. infestans* of 90% in tomato and 97% in potato (Cohen et al., 1994; Cohen, 2000). However, the mode action of BABA is still unknown. Symptoms and induction of defense related genes like ß-1,3-glucanase (PR-2) and acidic chitinase (PR-3) by BABA were described in tomato (Cohen et al., 1994). In contrast no studies concerning induction of defense in potato by BABA were reported. In this study, BABA as well as benzothiadiazole (BTH) induced resistance in *Solanum tuberosum-Phytophthora infestans* and in the recently described pathosystem *Arabidopsis thaliana-Phytophthora porri* (Roetschi et al., 2001) were compared. In this new pathosystem resistance is salicylic acid-, jasmonate- and ethylene-independent. The Arabidopsis accession Landsberg erecta is susceptible and the mutant *pad2-1* in the resistant Columbia background is hypersusceptible to *P. porri* and is impaired in SA and camalexin production. Exogeneous applications of SA or its analog BTH does not induce resistance. Assuming that BABA induces resistance toward *P. infestans* in potato and that BABA mode of action in Arabidopsis is not dependent on salicylic acid, jasmonate, ethylene and camalexin (Zimmerli et al., 2000), we tested if BABA could induce protection against *P. porri* in Lansdbserg erecta and *pad2-1*. Several methods can be used to quantify induced resistance in potato toward *P. infestans*. Either leaves or tubers (Dowley et al., 1991) can be inoculated. However, resistance is most easily assessed using detached leaves. Three components of general resistance were reported by Umaerus et al. (1983): 1) resistance to penetration, 2) restriction of growth of the fungus in the host, and 3) reduced sporulation capacity of the fungus on the host. Measurement of lesion size and lesion growth over time (Umareus and Lihnell, 1976) and ELISA-based serological quantification of fungal biomass (Harrison et al., 1991) help to evaluate mainly the second component. Measurement of sporulation (Tadege et al., 1991) evaluates only the third component. In this study we describe a new method to quantify the *Phytophthora* biomass *in-planta.* This method is based on quantification of the fluorescence emitted from transgenic *Phytophthora porri* or *Phytophthora infestans* constituvely expressing the green fluorescent protein GFP. Since the first stable transformation of *Phytophthora infestans* (Judelson et al.,

1991) several *Phytophthora* species were transformed (reviewed in Judeslon, 1996). So far, only *Phytophthora palmivora* (van West et al., 1999a) and *Phytophthora parasitica* var. *nicotianae* (Bottin et al., 1999) were successfully transformed using GFP reporter gene. In this study we report on the transformation of *Phytophthora porri* and moreover, the successfull transformation of *Phytophthora infestans* using GFP as reporter gene.

III.2. RESULTS

III.2.1 Transformation of *Phytophthora porri* and *Phytophthora infestans*

The transformation vector p34GFN in figure 1 was used to transform both *P. porri* and *P. infestans*. This vector is a pBluescript II SK(+) plasmid carrying two cassettes. One cassette contains the selectable marker gene neomycine phosphotransferase (nptII) and the other contains the reporter gene GFP. Both nptII and GFP are fused to the promoter and the terminator of the Ham34 gene (Judelson et al., 1991). Transformation using this construct was preferred to the co-transformation method used until now by other groups (Judelson and Whittaker, 1995; van West et al, 1999a; and Bottin et al., 1999). Indeed, in our preliminary transformation assays we have co-transformed *P. porri* using equal amounts (10µg of each vector DNA) of the vector pHAM34N (Judelson et al., 1991) and the vector p34GF (see material and methods). Few G418 resistant transformants were obtained and only 20% of them showed a weak fluorescence which irreversibly disappeared after 2 weeks. The transformation rate is 3 time more efficient using the double-cassette construct p34GFN and a strong stable fluorescence was observed for 85% of the transformants. One explanation for this is when liposomes and DNA are mixed both GFP and nptII, inserted in the same vector p34GFN, are encapsuled in the same liposome and then subsequently delivered in the same protoplast cytoplasm and further integrated grouped. Using vectors carrying separately GFP and nptII reduce considerably this probability. Stable transformations using p34GFN were obtained from the first assay and the protocol described in material and methods was optimized until a reproducible number of transformants (36 in average) was obtained using 10^4 zoospores as starting material (Table I). This optimized protocol was then applied to *P. infestans*. The difference between transformation protocol of the two *Phytophthora spp.* is the starting material. We used sporangia for *P. infestans* and zoospores for *P. porri* because sporangia of *P. porri* can not be easily detached from mycelia. A minimum of $1x10^4$ zoospores for *P.porri* and $2x10^5$ sporangia for *P. infestans* germinating in V8 10% (v/v) and ALBA medium, respectively, is required to obtain enough young mycelium (~1g) to be digested. One gram of mycelia was always used to obtain the same amount of protoplasts after digestion. The ratio liposomes/protoplats was found to be critical to obtain high transformation efficiencies. Using this protocol, we have, successfully transformed *Phytophthora porri* with an efficiency of 1 to 2.25 transformants per µg DNA. This is close to the *Phytophthora* transformation efficiency published by van West et al. (1998) which was 2 transformants/µg DNA. Our transformation efficiency of *P. infestans* was lower (0.5 transformant/µg DNA in average). However, it is higher than the one published by Judelson et al. (1991). Protoplasts of *P. infestans* isolate CRA208 used in our study regenerated better (35 to 40%) than the one used by Judelson et al. (1991) which had a regeneration rate of 5%.

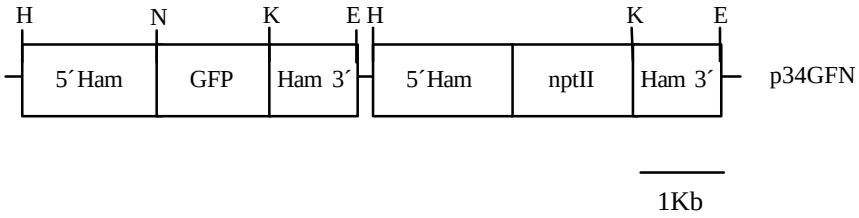

Figure 1 . Transformation vector p34GFN

The reporter gene green fluorescent protein (GFP, 725 bp) and the marker gene neomycine phosphotransferase (nptII, 795 bp) were inserted in two different cassettes both containing the Ham promoter (5´Ham, 920 bp) and the Ham terminator (Ham 3, 550bp). Solid line represents the pBluescript II SK(+) backbone. H: HindIII, N: NcoI, K: KpnI, E: EcoRI.

Table I. Parameters and efficiency of *Phytophthora* transformation.

	***P. porri*[a]**	***P. infestans*[b]**
Starting material[c]	$1x10^4$ zoospores	$2x10^5$ sporangia
Number of protoplats[d]	$1.5x10^6$ to $0.5x10^7$	$2.5x10^6$ to $1x10^7$
Percentage of germination[e]	30-80%	35-40%
Transformants per assay[e]	27-45	3-20
Transformants per µg of vector DNA[e]	1-2.25	0.15-1

a: Isolate HH (CBS782.97).
b: Isolate CRA 208.
c: The indicated numbers are the minimum number of zoospores or sporangia required to produce enough mycelium (~1 g) for one assay.
d: The number of protoplasts should be in this range to ensure an optimum ratio between liposomes and protoplasts.
e: Minimum-Maximum range taken from 20 experiments for *P. porri* and 3 experiments for *P. infestans.*

III.2.2 Analysis of the transformants

Among about 360 *P. porri* G418 resistant transformants analyzed, 85% showed a stable fluorescence during their whole life cycle. The same percentage was also observed for *P. infestans.* This is a much higher yield than the ones reported by Bottin et al. (1999) and van West et al. (1999a). Indeed, only 13% of the hygromycine resistant *Phytophthora parasitica* (*Ppn)* transformants (Bottin et al., 1999) and 40% of the G418 resistant *P. palmivora* transformants (van West et al., 1999a) showed GFP fluorescence. Thus confirming the efficiency of our improved transformation construct compared to the co-transformation protocol used in those studies. We concentrated our further microscopic investigations only on few GFP transformants of both transformed *Phytophthora spp*. Two *P. porri* (35m and 155m) and one *P. infestans* ($208m_2$) transformants were chosen from the collection. They showed a strong and stable bright-green fluorescence when observed under blue light. All the observations were done using a fluorescence microscope with excitation filters at 470/40 nm or 480/40 nm. Sub-culturing these stable transformants twice a month during two years had no influence on the stability of the GFP and the intensity of the fluorescence. We did not observe any difference in the shape of the mycelium, sporangium, zoospore and cyst of *P. porri* 35m and 155m (Figure 2) and *P. infestans* $208m_2$ (Figure 3) compared to their respective wild-types *P. porri* HH and *P. infestans* CRA208. Zoospore and sporangial production were not affected in 35m, 155m and $208m_2$. Temperature variations (from 12°C to 30°C) and elevated humidity (up to 100%) had no effect on the stability of the GFP used in this study. However, subculturing several times *P. porri* GFP transformants had an influence on their growth. Therefore, new cultures were often started from the stock kept in liquid nitrogen. In contrast, no changes in growth were observed in *P. infestans* GFP transformants.
Landsberg erecta and *pad2-1* plants respectively susceptible and hypersusceptible to *P. porri* were infected with *P. porri* 35m or 155m and potato plants with *P. infestans* $208m_2$. Leaf tissue was rapidly colonized by the mycelium growing in the intercellular space in a similar manner in both plants. Six days after infection Arabidopsis and potato leaves were completely colonized and sporangia started to form in the aerial space. However, in potato plants an impressive number of sporangia were formed on hundreds of sporangiophores coming out through the stomata of both the abaxial and adaxial faces. By mechanical action (touching, watering or blowing) thousands of sporangia are released and dispersed on the leaf surface (Figure 3). Zoospore root inoculation was also successful in Arabidopsis (Figure 2, E). Zoospores were attracted by the root within 24h and colonized the tissues. In some cases encystment occur far away from the root and the germinating tubes converged towards the root and penetrated the root tissues. We observed, in the fluorescence microscope, leaves of Arabidopsis and potato infected plants every eight hours during 6 days and we concluded that all the structures of 35m, 155m and $208m_2$ developped *in planta* were uniformly bright-green and no variation in the intensity of the fluorescence occur. Therefore, confirming both the constitutive expression of the GFP and its stability *in planta.*

III.2.3 *In planta* quantification method

Landsberg erecta and the Arabidopsis mutant *pad2-1* (in the resistant Col-0 background) are susceptible to *P. porri.* When infected with 155m the leaf tissue is heavily invaded within 6 days and a strong green fluorescence emitted by numerous intra- and intercellular growing hyphae can be observed in all the cell layers (Figure 2). This is also the case when Bintje potato plants are infected with $208m_2$ but with a more heavy sporulation of *P. infestans* compared to *P. porri*. Whole infected plants can be screened easily and rapidly using a fluorescence dissecting microscope. This is possible because of the strong fluorescence of the synthetic *S65Tpgfp* used in this study (Pang et al., 1996) which has clear advantages

compared to other reporter genes. Observation of the area around the infection site (about 5mm) at the sixth day after inoculation is enough to evaluate the progression of *Phytophthora* in the tissue and conclude whether the plant is susceptible or resistant. The speed of intercellular growth in this area and more importantly the subsequent severity of the symptom is extremely influenced by the density of the innoculum (Hächler and Hohl, 1984). Special care was given to the inoculation step because of the strong fluorescence of 155m and $208m_2$ important variations during quantification of the GFP around the infection site could be expected. Therefore, we preferred the plug method for Arabidopsis and the sporangial inoculation for potato. Six days after inoculation, discs of 5 mm were cut out around the infection site and were carefully deposited on the surface of 200µl sterile distilled water contained in a 96 multiwell-plate. Emitted fluorescence from the GFP transgenic *Phytophthora* growing inside the tissues or in the aerial parts was immediately (within 30 secondes) measured using a fluorescence microplate reader (see material and methods). Basic autofluorescence for both plant spp. were recorded. Potato plants have a basic autofluorescence level of 3386±256 a.u for Bintje cultivar and 2596±100 a.u for Matilda when excited by blue light (485/20 nm). In the same conditions Arabidopsis mutants plants *pad2-1* have an autofluorescence of 1530±200 a.u and their wild-type Col-0 1160±200 a.u. Landsberg plants have an autofluorescence of 1150±200 a.u.

III.2.4 *In planta* quantification of GFP fluorescence emitted by transgenic *Phytophthora porri* infecting BABA and BTH-treated Arabidopsis

Among the known chemical activators of systemic acquired resistance (SAR), BTH (Bion®) and ß-aminobutyric acid (BABA) have no detectable antifungal activity *in vitro* (Sticher et al., 1997). It has previously been shown that neither SA nor BTH were able to induce resistance in Ler and in *pad2-1* against *Phytophthora porri* (Roetschi et al., 2001) but that BABA efficiently protect Arabidopsis against the oomycete *Peronospora parasitica* (Zimmerli et al., 2000). We tested whether BABA could also protect Arabidopsis against *Phytophthora porri*. The hypersusceptible *pad2-1* plants and the susceptible Landsberg (Ler) plants were soil drenched with 30 mg/l BABA and infected 24 hours later with plugs of young growing mycelium of *P. porri* 155m. Plants treated with water, soil drenched with 330 µM BTH and the resistant plants Col-0 were taken as controls and also infected 24 hours after the treatment with 155m. After 6 days visual estimation of the symptoms was performed. *pad2-1* plants treated with BABA showed only a small infected area immediately in the vicinity of the infection site. Ler plants treated with BABA did not show any symptom of infection. Ler and *pad2-1* plants treated with BTH or water presented a much larger area of infected tissue. The resistant interaction (Col-0) is characterized by the apparition of necrotic spots corresponding to several epidermal cells which reacted with a hypersensitive reaction. Discs of 5 mm covering all the area around the infection site were cut out and the fluorescence emitted by the GFP expressing *Phytophthora* mycelia was recorded (Figure 3, A). Highest values corresponded to the water treated *pad2-1* with 6700±1400 a.u. and BTH treated *pad2-1* with 4900±500 a.u. indicating therefore a massive infection but BTH treated *pad2-1* induced about 32% protection. The *pad2-1* BABA treated plants were infected but the mycelium growing inside the tissue did not spread as much as in the water and BTH treated plants. Indeed, a value of 3000±530 a.u. was recorded for *pad2-1* BABA treated plants representing approximatively 67% of protection compared to the water treated plants. In contrast, BABA pretreated Ler plants showed a complete resistance (100%) similar to Col-0 with a recorded value of 1350±200 a.u. which was basically the autofluorescence of the leaf tissues and the fluorescence of small rests of dying glowing hyphae. Ler plants treated with water and with BTH showed highest value with respectively 3580±1200 a.u and 3400±600 a.u. corresponding to a BTH protection of 5%. All the measured discs were then removed from the 96 mutliwell

plate and observed with a fluoresence microscope. Water and BTH *pad2-1* treated plants were inter- and intracellularly invaded (Figure 4, B; Figure 4, C). We could not see any relevant difference under the microscope for BABA treated *pad2-1* plants in term of pattern of infection. However the lower fluorescence value recorded indicate that the plant reacted and that *P. porri* 155m intercellular growth was weaker when Arabidopsis was pretreated with BABA. We observed that the mycelia growing in the BABA treated *pad2-1* plants did not reach all the cell layers in the leaf tissues. Therefore the biomass of *P. porri* is reduced and the GFP emitted not so important as for BTH and water plants.

III.2.5 BABA and BTH induced resistance in potato and quantification of the fluorescence emitted *in situ* by the GFP transgenic *P. infestans*

Some studies reported the efficiency of BABA on induced resistance against *Phytophthora* species (Cohen et al., 1994). We were particularly interested in quantifying the efficiency of protection that BABA can induce in potato against late blight. It was reported that the efficiency of BABA as an inducer of resistance in potato plants is dose-dependent (Cohen, 2000). We applied BABA at a concentration of 1000µg/ml to susceptible potato plants cv Bintje by spraying leaves. Plants sprayed with BTH and water were taken as controls. The same concentration of BTH (1.5 mM) used to protect tomato against *Fusarium oxysporum* (Benhamou and Bélanger, 1998) was used in our study. Thus, both BTH and BABA solutions have concentrations in the same range and were sprayed two days before challenge inoculation with *P. infestans* 208m_2. Analysis was performed 6 days post-inoculation. Interestingly, BABA completely protected potato plants against *P. infestans* in the same manner as the resistant potato cultivar Matilda. In contrast, BTH treated plants were not protected and the severity of the symptoms were similar to water treated plants (Figure 5A). Microscopic observations revealed a complete invasion of the leaf tissues in the BTH and water treated plants and a heavy sporulation (Figure 5, E; Figure5, F). Quantification of the GFP emitted by the mycelia of *P. infestans* 208m_2 revealed that BABA induced 97% protection and a BTH induced only 7% protection (Figure 5, B). Cohen (2000) reported also a protection of 97% when BABA at 2000 µg/ml is applied. However, at a concentration of 1000 µg/ml BABA induces also macroscopically visible necrotic lesions. In order to avoid the side effect of necrosis induced by the application of high concentrations of BABA, we reduced progressively the applied BABA concentration. There was no difference in the recorded *in planta* fluorescences with all the concentrations tested but at 100 µg/ml *P. infestans* 208m_2 started to successfully penetrate the potato tissue but without invading it (Figure 5, C). The recorded fluorescence for treated plants with BABA 100 µg/ml was the same like for plants treated with BABA 1000 µg/ml. Application of 10 time less BABA induced the same degree of protection. To prove that defense pathways are activated by BTH and that the treatment was really efficient, we performed a northern blot analysis using a specific *Solanum tuberosum* pathogenesis related protein, *StPR-1* probe (van´t Klooster et al., 1999). It has been shown that 1,3-ß-glucanases and chitinases are strongly induced by *P. infestans* and BABA (Cohen et al., 1994). *StPR-1* is strongly induced by BTH 4 days after treatment (Figure 6). BABA at a concentration of 100µg/ml also transiently induces *StPR-1* gene expression. At the moment of challenge inoculation, the *StPR-1* is expressed but this induction completely disappeared 2 days after infection. *StPR-1* is induced in both resistant and susceptible cultivars (Figure 6) and therefore unlikely to play a direct role in defense against *P. infestans*.

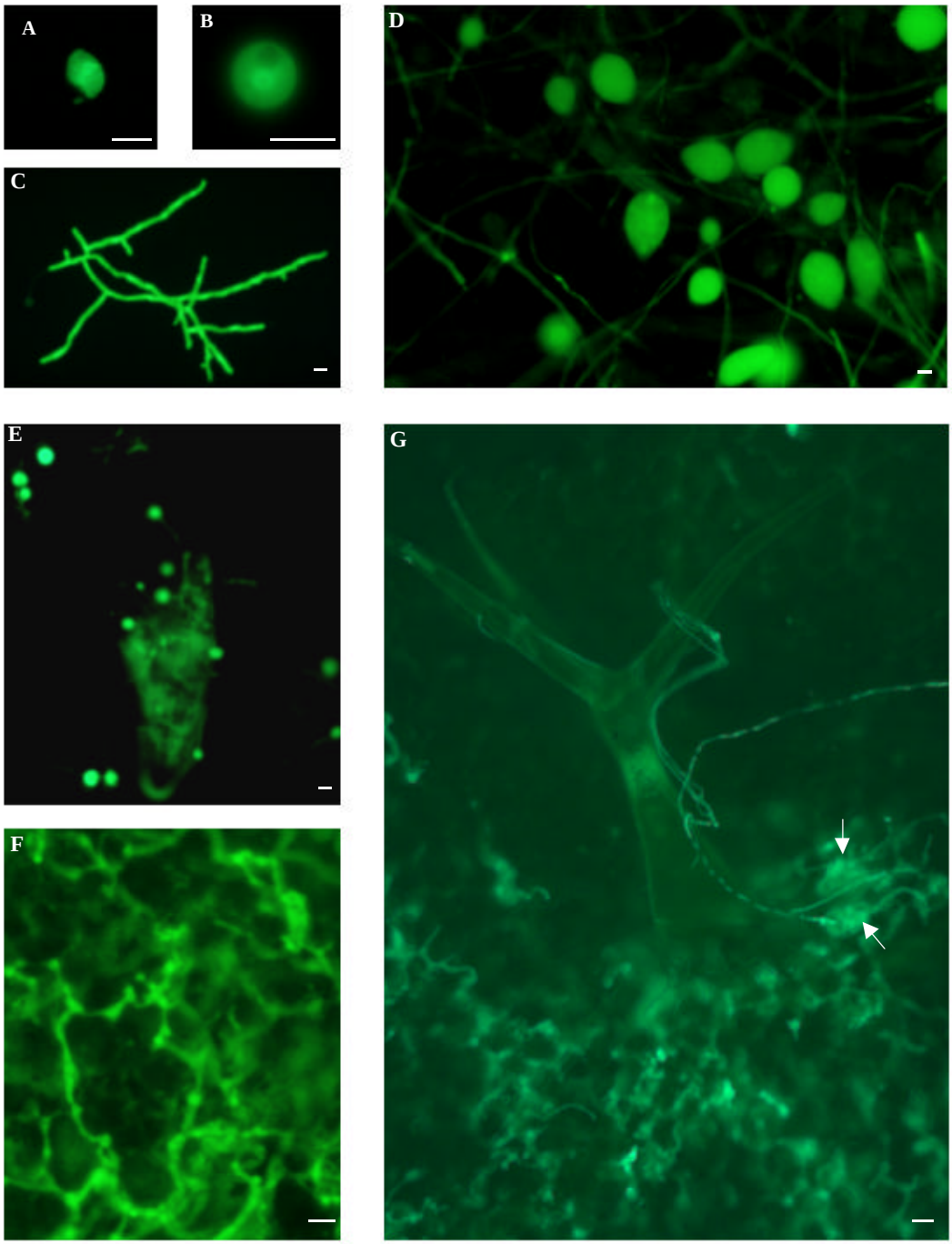

Figure 2. Microscopic analysis of *P. porri* GFP transformants.

P. porri GFP transformants 35m (A to F) and 155m (G) were observed under a fluorescence microscope through their life cycle and *in planta*. All the structures showed a strong bright-green fluorescence through all the asexual life cycle. The zoospores (A) were released and rapidly encysted (B). After few hours the cyst germinate and the mycelium grows rapidly (C). By adding Schmithenner salt solution, mycelia produced sporangia (D). Plants can be infected either by root inoculation (E) or by leaf inoculation (F and G). For root inoculation zoospores were released in 2 ml water and the Arabidopsis root were dipped in the zoopsore suspension and kept at 18°C in the dark. The encystment occured either on the root surface or far away from it. In both cases hyphal tips converged toward the root and penetrated. After 24 h the root was completely invaded and the mycelium was growing toward the aerial part (E). Susceptible plants (Landsberg erecta) were inoculated and after 72h, the leaf tissue was growing mainly in the intercellular space (F). The mutant *pad2-1* is hypersusceptible to *P. porri* and within 48 hours its leaf tissue in completely colonized (G). Intracellular growth occured mainly in the cells surrounding the trichomes (arrow). Scale bars represent 10 µm.

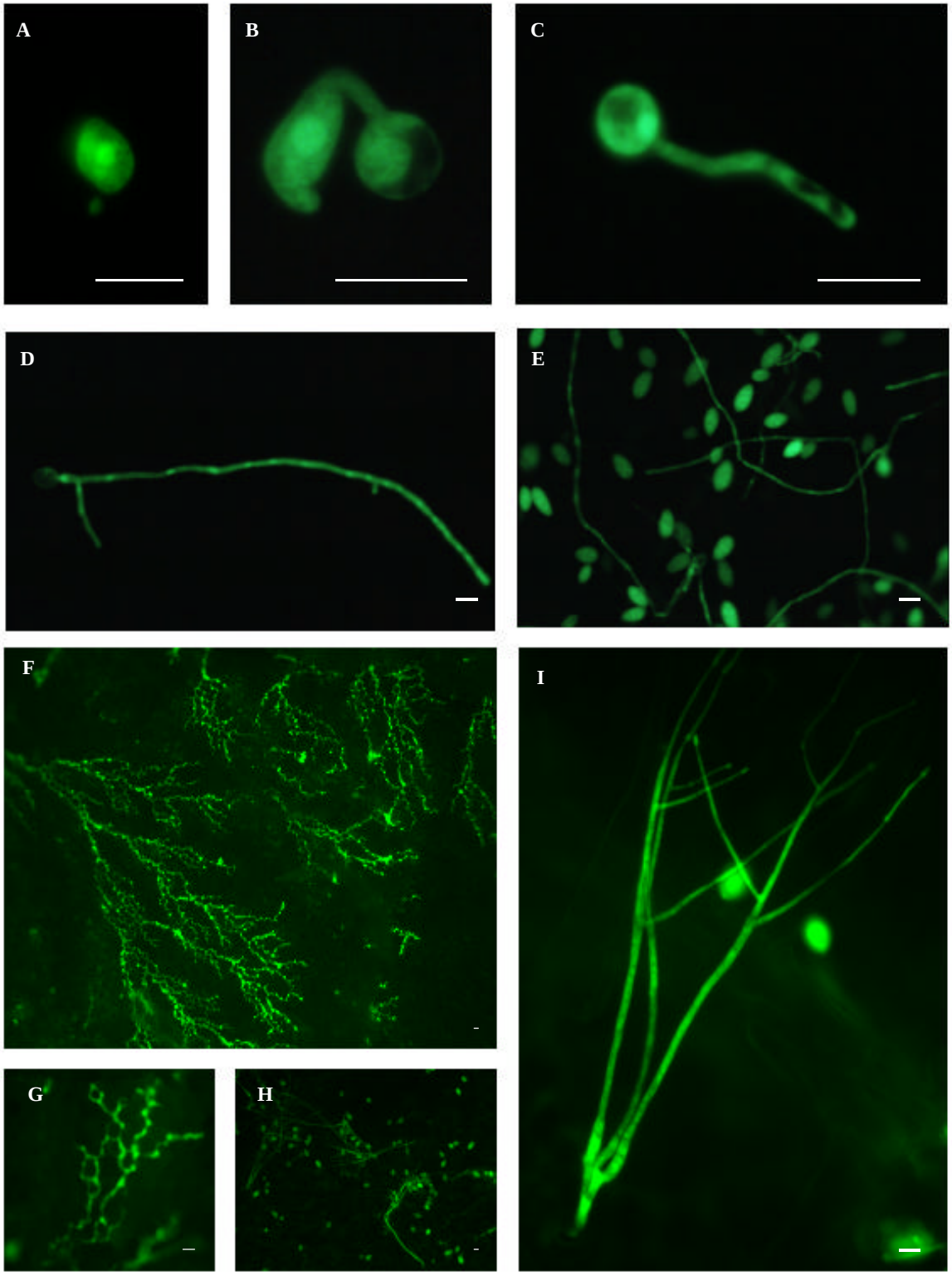

Figure 3. *In vitro* and *in planta* microscopic analysis of GFP *Phytophthora infestans* transformant $208m_2$.

The transformant $208m_2$ showed a strong fluorescence, stable through all its asexual life cycle: zoospores (A), germinating cyst (B and C), mycelium and sporangia (D and E). The cyst germinate and can either produce an appressorium (B) or a germ tube (C). Germination can also occur directly from sporangia (D). Sporangial inoculation was preferred in our study (E). Bintje plant were leaf inoculated and within 48 hours the tissue is impressively invaded (F). The mycelium grows rapidly in the intercellular space (G) and go deeper in the tissue to colonize all the cell layers until reaching the abaxial face where a heavy sporulation occurs within six days. Sporangia can be easily removed from the sporangiophore by mechanical action and sprayed on all the leaf surface and on the neighbouring leaves (H). Sporangiophores come out form the internal tissues through the stomata (I). Scale bars represent 20 µm.

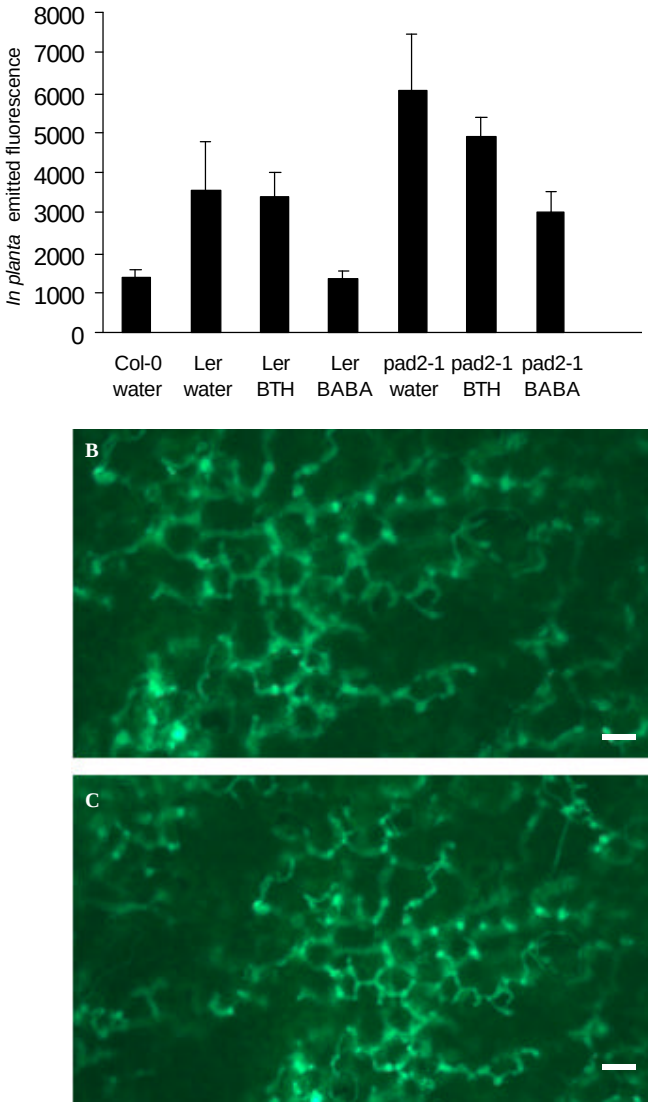

Figure 4. *In planta* quantification of GFP emitted by transgenic *Phytophthora porri* infecting BABA and BTH treated Arabidopsis.

Arabidopsis mutant plants *pad2-1* were treated with BABA, BTH and water and 24 hours later inoculated with *P. porri* transformant 155m. Columbia plants resistant to *P. porri* were taken as control. Six days later, differences in symptoms were clearly visible. Discs leafs were cut out around the infection site and the fluorescence emitted by *P. porri* 155m within the tissue measured using an automatic fluorescence reader. The highest values were recorded for BTH and water treated plants followed by BABA treated plants (A). This experiments was repeated five times with five replicates each time. Microscopic observations correlated with the measured value. Indeed, BTH treated plants were colonized by 155m (B) in the same way like water treated plants (C). Scale bars represent 20 µm.

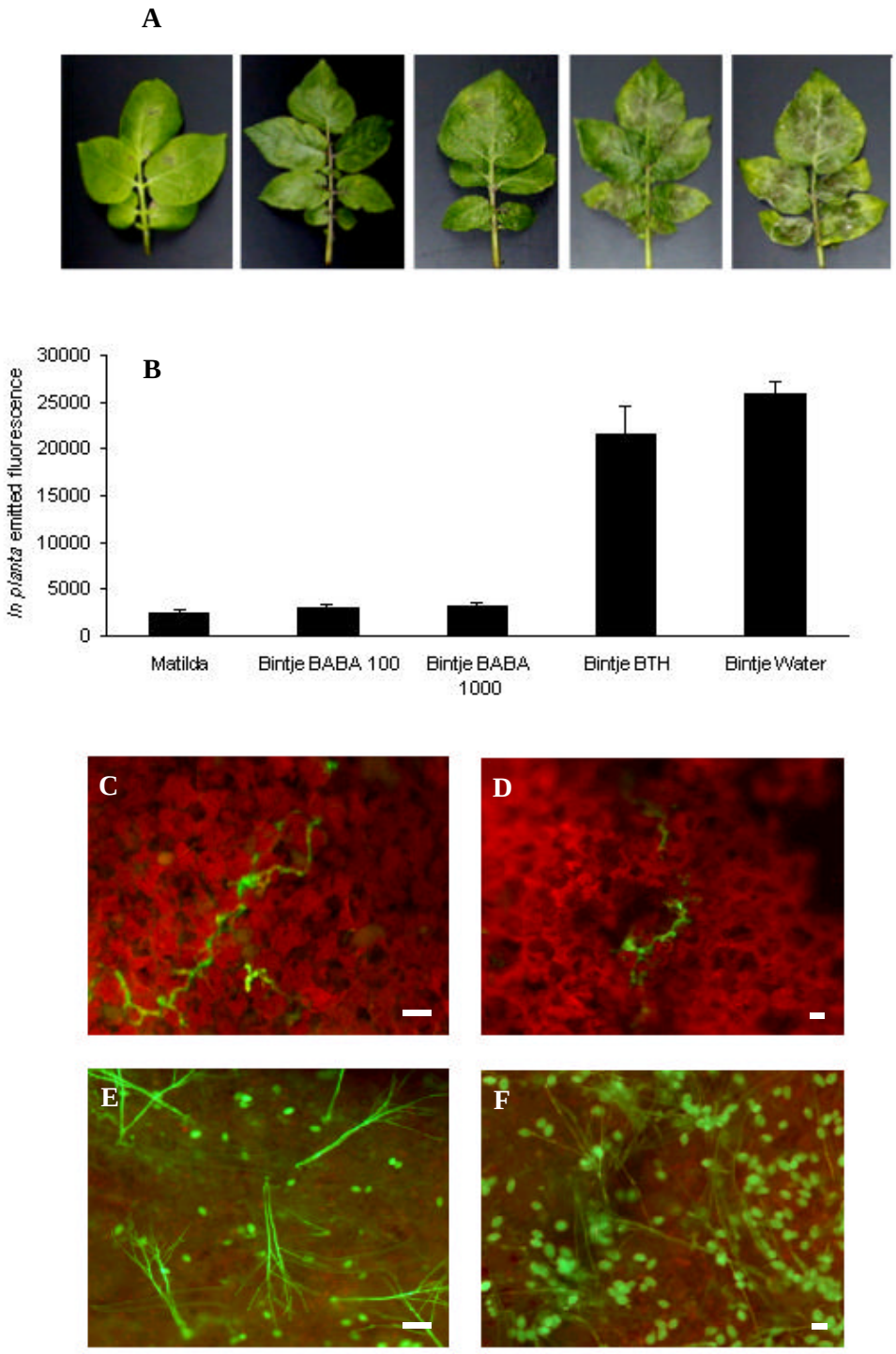

Figure 5. BABA and BTHinduced resistance in potato and quantification of the fluorescence emitted *in situ* by the GFP transgenic *P. infestans.*

BABA, at a concentration of 1000 µg/ml and 100 µg/ml, water and 1.5 mM BTH solution were sprayed on potato leaves two days prior to inoculation. Matilda is a resistant cultivar and Bintje is susceptible. Plants were inoculated with a sporangial suspension of the GFP transformant *P. infestans* $208m_2$. Six days after infection plants treated with BABA were as resistant as Matilda. No necrosis due to BABA was seen using 100 µg/ml. A BABA concentration as low as 100 µg/ml also protected potato plants against *P. infestans*). Recorded values of *in-planta* emitted fluorescence of BABA (100 µg/ml) treated plants were as low as the ones of BABA (1000µg/ml) and Matilda resistant plants. BTH and water treated plants showed the highest values of *in planta* emitted fluorescence (B). These values were correlated with the microscopical observations since Matilda (C) and Bintje treated with BABA 100µg/ml (D) showed a limited growth of $208m_2$ (about 100µm to 200µm). In contrast, BTH (E) and water (F) treated plants were completely invaded and 6 days after inoculation $208m_2$ accomplished its asexual life cycle as shown by the heavy sporulation. Scale bars represent 20 µm.

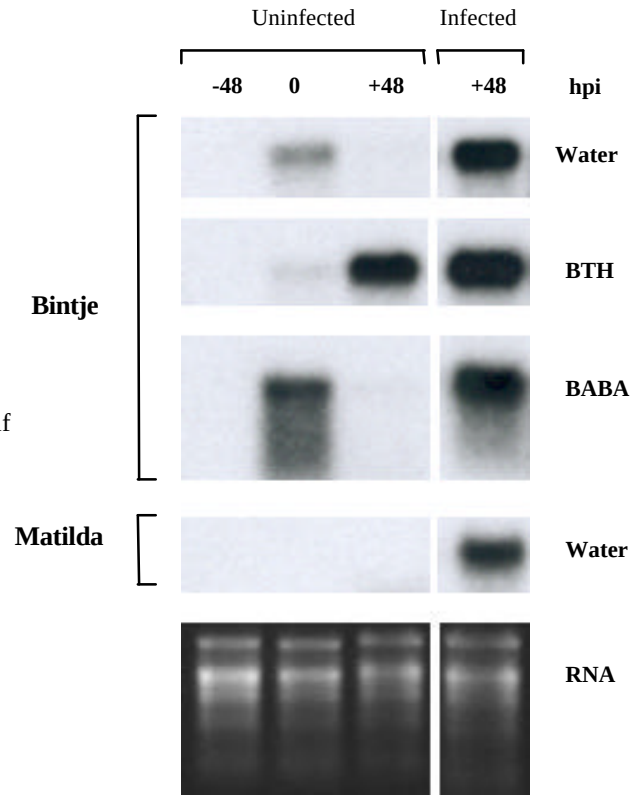

Figure 6. Expression of *StPR-1* mRNA in potato plants treated with BABA and BTH.

Potato plants were sprayed with water, BTH 1.5 mM and BABA at 100 µg/ml two days prior inoculation (time -48). Matilda plants are resistant to *P. infestans* and were taken as control. 48 hours post-treatment half of the plants were inoculated with drops containing about 2500 sporangia of GFP transformant $208m_2$ and the other half was water treated (time 0). 48 hours post-inoculation (hpi) total RNA from uninfected and infected plants was isolated (+48). Ethidium bromide staining of ribosomal RNA shows equal loading.

III.3. DISCUSSION

Various chemicals capable of inducing local or systemic resistance have been discovered (Oostendorp, 2001). Few of them have reached commercialization like the best-studied SA analog Bion® (BTH). Most of the resistance inducing chemicals have an unknown mode of action. Nevertheless, one of the most promising chemical compound proved to induce resistance of various plants against *Phytophthora* species is the D,L-ß-aminobutyric acid commonly named BABA (Jakab et al., 2001). We have recently developped a new pathosystem Arabidopsis-*Phytophthora* and reported that all the known defense pathways in Arabidopsis (salicylic acid, jasmonate and ethylene) are not necessary for resistance. Interestingly, the mutant *pad2-1* is hypersusceptible toward *Phytophthora porri* and it is impaired in salicylic acid and *PR-1* production. However, exogeneous application of BTH or SA does not induce visible resistance (Roetschi et al., 2001). We evaluated the induced resistance effect of BTH by quantifying the *Phytophthora* biomass inside the leaf tissues. In parallel, we tested the capability of BABA to induce resistance in Arabidopsis against *P. porri* in the same manner like it induces resistance in potato (Cohen, 2000). Until now the most widely used protocol to evaluate fungal biomass *in-planta* is to stain the leaf sample with lactophenol trypan blue (Shipton and Brown, 1962; Keogh et al., 1980; Roetschi et al., 2001). However, this staining is destructive, time-consuming and poisonous. An other method based on the quantification of the ß-glucuronidase (GUS) expressed *in planta* by transgenic *Phytophthora infestans* was recently developped (Kamoun et al., 1998) but this method is dependent on the application of a substrate which is inconvenient for certain studies. In contrast, GFP does not require any substrate and more importantly, its expression can be easily followed in living organisms. We constructed a vector containing both GFP and the selectable marker gene nptII under the control of the promoter Ham34 (Judelson et al, 1991). This promoter is constitutively expressed in several *Phytophthora* species like *Phytophthora parasitica* var. *nicotianae* (Bottin et al., 1999), *Phytophthora palmivora* (van West et al.,

1999a) and *Phytophthora infestans* (Judelson et al., 1991; van West et al, 1998). Using this construct we have successfully transformed *Phytophthora porri* and *Phytophthora infestans*. In both species, 85% of the G418 resistant transformants showed a bright-green fluorescence through all the whole life cycle which was easily visible using fluorescence microscope or fluorescence dissecting microscope even after several subcultures during two years. This high stability of the GFP (*S65Tpgfp*) expression observed in 85% of the transformants is not only due to the improved design of the protein by mutating the serine to threonine at amino acid position 65 (Pang et al., 1996) but also because of the design of our construct. The transformation procedure used in our study is different from the co-transformation protocol used until now to introduce reporter genes in *Phytophthora* species (van West et al., 1998; Bottin et al., 1998; Judelson et al, 1995). In this procedure the reporter gene and the marker gene are on separate vectors. Since *Phytophthora* is multinucleated, protoplasts could contain more than one nuclei. The probability that the reporter gene and the marker gene can be integrated in different nuclei is high. By using our vector containing both reporter gene and marker gene this probability is considerably reduced. The rest of the transformants (15%) showed that the drug-resistance marker (nptII) was activated while the GFP was not. Our data is not sufficient to explain this observation and the exact nature of this silencing process. Although it seems that neither deletion nor methylation (Judelson et al., 1995) are the cause of inactivation of integrated genes in *Phytophthora*. Explaining this observation by an internuclear silencing as proposed by van West et al. (1999) is a matter of speculation. Stable *P. porri* and *P. infestans* GFP transformants were analyzed during several asexual life cycles. The fluorescence was strong in all the structures: zoospores, cysts, mycelium and sporangia. During infection both *P. porri* and *P. infestans* GFP transformants showed a bright-green fluorescence easily measurable *in planta* by using an automatic fluorescence reader. Leaf discs were cut from the infected leaf around the infection site without destroying neither the plant nor the oomycete pathogen and allowing a real time quantification of GFP emitted by transgenic *Phytophthora*. The readings are very fast (within 30 secondes) and a large number of plants can be screened in a short period. This is especially important for statistical evaluation of induced resistance by a given chemical. It was recently shown that BTH can not induce resistance in the hypersusceptible mutant *pad2-1* against *Phytophthora porri* (Roetschi et al., 2001). However, using our quantification method we have estimated the resistance induced by BTH in *pad2-1* at about 32 % compared to water treated plants. The microscopic observations did not reveal any difference between BTH and water treated plants. In the BABA treated plants the resistance was 2 time more higher (67%) but not as important as the BABA potato treated plants (97% protection) or BABA treated Landsberg erecta (100% protection). The difference of the efficiency of BABA induced protection between Ler and *pad2-1* could be explained by the fact that the mutant *pad2-1* is too hypersusceptible to be completely protected by BABA treatment. The mutant *pad2-1* is impaired in salicylic acid production thus treating it with BTH is supposed to replace this lack. Despite this fact only 32 % of resistance was induced by this treatment supposing that salicylic acid pathway is not necessary for the resistance against *P. porri*.. This observation can be added as a proof to our previous statement for the existence of a yet unknown pathway in Arabidopsis involved in resistance against *Phytophthora porri*. Referring to the protection quantified in BABA treated plants, BABA probably induces this unknown pathway. Furthermore, BABA induces also defense pathways in potato leading to resistance toward *Phytophthora infestans*. It is tempting to speculate about a common defense pathway present in Arabidopsis and in potato important for resistance toward *Phytophthora* and inducible by BABA. We have used the previously described pathogenesis related protein *StPR-1* (Vleeshouwers et al., 2000) as a marker for BTH treatment in potato. This gene is also transiently induced two days after BABA application. BTH treatment increased *StPR-1* gene expression only four days following application. It is unlikely that this protein plays an important role in resistance against late

blight since it is also induced in the susceptible Bintje. Nevertheless, it is a useful marker for BTH or SA induction in potato. For instance, a more refined time-course could reveal interesting features of *StPR-1*. In conclusion, *Arabidopsis thaliana* can be used as a plant model to study molecular mechanisms of action of BABA and the results can be extrapolated to important crop plants. Other chemicals inducing resistance against *Phytophthora* both in *Arabidopsis thaliana* and *Solanum tuberosum* can be easily screened using our new quantification method. Furthermore, our improved vector can be used to generate thousands of transformants and our new screening method could be used to rapidly identify transformants putatively altered in pathogenicity. Their study will help to better understand the molecular mechanisms governing the pathogenicity of *Phytophthora* in particular and the oomycete in general.

III.4. MATERIEL AND METHODS

III.4.1 Transformation vector

The transformation vector p34GFN (Figure 1) is a pBluescript II SK(+) vector (Stratagene) carrying the selectable marker neomycine phosphotransferase (nptII) and the reporter gene Green Fluorescent Protein (GFP), both fused to the Ham34 promoter and terminator. The GFP gene was excised as a NcoI/EcoRI fragment from pMON30060 (Pang et al., 1996), blunt-ended with Klenow polymerase and inserted in the SmaI site of the plasmid pHAM34H (Judelson and al., 1991) provided by H.S. Judelson. The cassette containing GFP was then subcloned as a HindIII/EcoRI fragment in a pBluescript II SK giving the p34GF plasmid. The cassette containing the nptII gene fused to the Ham34 promoter and terminator was cut from pHAM34N (Judelson and al., 1991) as a HindIII/EcoRI fragment and inserted in a pGEM-7Zf(+) plasmid (Promega) previously digested with the same restriction enzymes. This cassette was further cut out from the pGEM-7Zf(+) vector and inserted as a BamHI/XbaI fragment in the p34GF plasmid giving the transformation vector p34GFN.

III.4.2 Growth of *Phytophthora* and transformation procedure

P. porri isolate HH was grown and maintained as previously described (Roetschi et al, 2001). *P. infestans* isolate CRA 208 or BS88, kindly provided by D. Chavaillaz (Ciba-Geigy Basel, Switzerland), was grown on rye sucrose agar medium (Caten and Jinks, 1967) at 18°C in the dark. These two isolates were used as recipients for transformation according to the protocol of Judelson et al. (1991) with some modifications. All the steps were done under aseptic conditions. For both species 72h old mycelium was used to produce protoplasts. *P. porri* mycelium was produced from a zoospore suspension in water obtained as previously described (Roetschi et al, 2001). The zoospores germinated by adding V8 juice to a final concentration of 10%. For *P. infestans*, the mycelium was produced by adding, to a sporangial suspension in water, the same volume of 2xALBA medium (Bruck et al., 1980). The further steps were the same for both *Phytophthora* spp. and the media used (KC, KC/MT and MT) were exactly the same as described by Judelson et al. (1991). Briefly, mycelium was washed in KC osmoticum and incubated 35 minutes at room temperature with a solution of KC containing 5 mg/ml lysing enzymes from *Trichoderma harzanium* (Fluka, 62815) and 2 mg/ml cellulase (Sigma, C8546). The protoplasts were filtered through a 50-µm nylon mesh, pelleted at 700xg during 4 minutes, washed once with 10 ml KC, once with 10 ml KC/MT and resuspended in MT medium at a concentration of $1.5x10^6$ to $0.5x10^7$ protoplasts/ml for *P. porri* and $2.5x10^6$ to $1x10^7$ protoplasts/ml for *P. infestans*. A mixture of 20 µg vector DNA and 60 µg lipofectin (Life technologies) was prepared, preincubated at room temperature and added to the protoplast supension. 1 ml of (w/v) 50% PEG 3350 (Sigma) was added and after

5 minutes incubation the mixture was diluted in 20 ml of (v/v) V8 10% 1M mannitol medium for *P. porri* and 20 ml rye sucrose 1M mannitol for *P. infestans*. The protoplasts germinated after 24h incubation at 18°C in the dark and propagated on V8 agar containing 20µg/ml of geneticine antibiotic (G418, Life Technologies) for *P. porri* or rye sucrose agar containing 5 µg/ml of the same antibiotic for *P. infestans*. The regeneration rates were determined by microscopic counting of serial dilutions of protoplasts plated on clarified V8 agar or clarified rye sucrose agar.

III.4.3 Plant treatments and infection of Arabidopsis and potato

Arabidopsis ecotype Columbia (Col-0), Lansberg erecta plants and the mutant *pad2-1* (Col-0 background) were grown as previously described (Roetschi et al, 2001). Potato plants (*Solanum tuberosum*) cv Bintje and cv Matilda, commercialy available, were grown in a growth chamber calibrated to 20°C and 14h of light (100 $\mu Em^{-2}s^{-1}$). Four weeks old Arabidopsis plants were soil-drench treated with either 330 µM BTH (Bion®) or 30 mg/l BABA (ß-aminobutyric acid, Sigma) as previously described (Zimmerli et al., 2001). One day after the treatment, the plants were inoculated with plugs of V8 agar containing young growing mycelium according to Roetschi et al. (2001). Potato compound leaves (4^{th} to 6^{th}) were cut out from the plant and the petiole inserted in wet stonewool cubes (Grodan). Adaxial leaf surfaces were sprayed with either 1.5 mM BTH or from 1000 µg/ml to 100 µg/ml BABA with an atomizer. After 2 days, the treated leaves were transferred to a growth chamber maintened at 18°C with 10h of light and infected with 2 to 4 drops/leaf of 10 µl water containing 2500 sporangia. Complete darkness and high RH (80-100%) was kept for the first 24 hours and the plants were then returned to 12h of light and 20°C.

III.4.4 *In planta* GFP emission measurements

Six days after infection discs of 0.5 cm diameter were cut out circularly around the infection site and placed, with the adaxial surface faced up, in a 96-mutliwell black maxisorp plate (Nunc) containing 200 µl sterile distilled water in each well. The fluorescence emitted from GFP *Phytophthora* transformants growing in each Arabidopsis or potato leaf disc was recorded automatically using a fluorescence microplate reader Lambda Fluoro 320 (MWG AG Biotech) using a static mode with 10 reads in each well. The reading parameters were the following: an excitation at 485/20 nm, an emission at 530/25 nm, a sensitivity factor of 110.

III.4.5 Northern-blot analysis

Total RNA from potato leaves was isolated, transferred and hybridized as previously described (Roetschi et al, 2001). The probe used for detecting *Solanum tuberosum* pathogenesis related *StPR-1* (van´t Klooster and al., 1999) transcript accumulation was amplified by PCR from Potato (cv Bintje) genomic DNA using the following primers: 5´CAAATGGGGTTGTTTAACATC3´ (sense primer) and 5´CTTTATGTATTCATGTTTA GTGGAAAC 3´ (antisense primer).

III.4.6 Microscopy

Phytophthora GFP transformants were examined, *in vitro* or *in planta*, using a fluorescence microscope (Leica DMR microscope) with different filter sets: 480/40 nm and 470/40 nm excitation GFP filters. Photographs were taken using Kodak Ektachrome 400 film when the Leica MPS60 camera was used or pictures were acquired using Axiovision 2.05 software when the Axiocam CCD camera (Zeiss) was used.

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CHAPTER IV

Transcriptional profiling of the Arabidopsis-*Phytophthora* pathosystem using oligonucleotide-based arrays

ABSTRACT

A preliminary characterization of the Arabidopsis-*Phytophthora* pathosystem showed that resistance against this oomycete pathogen is independent of the known salicylic acid and jasmonate/ethylene pathways. The resistance toward *Phytophthora* depends on a new pathway which is controlled by PAD2. In order to identify marker genes for this new pathway we profiled 30% of the whole transcriptome of Arabidopsis using oligonucleotide-based arrays also known as Affymetrix GeneChip®. Gene expression levels during infection in the incompatible interaction were compared to the one of the mutant *pad2-1*. Data-mining using GeneCluster 1.0 software highlighted 41 genes which were strongly expressed in the incompatible interaction but not, or barely, induced in the mutant *pad2-1*. Seven genes were chosen for further analysis. The majority of those genes are regulated by SA at different levels. This was confirmed by analyzing their expression in several mutants impaired in salicylic acid accumulation. Since their expression is SA-dependent, they can not be used as marker genes for this new resistance pathway. However, their involvement in resistance toward *Phytophthora* is not excluded and their role in defense mechanisms are discussed.

IV.1. INTRODUCTION

Since the dramatic epidemics that led to Irish potato famine more than 150 years ago, studies aiming to better understand plant defense mechanisms against the oomycete plant pathogen *Phytophthora* have evolved and became more sophisticated. During the last decade new emerging technologies were used by several *Phytophthora* groups in order to identify plant genes encoding key proteins involved in defense signal transduction or encoding proteins with a direct antifungal effect. Different methods were developped to study changes in gene expression during infection with *Phytophthora*. One of the most promising method used was suppression substractive hybridization (SSH). This method allowed a rapid generation of a potato cDNA library enriched for sequences induced during the incompatible interaction (Birch et al., 1999) or during the compatible interaction (Beyer et al., 2001). However, in the international DNA and protein databases the majority of the plant sequences are available from *Arabidopsis thaliana*. Besides, our knowledge of Arabidopsis defense signal transduction pathways evolves very rapidly. Several mutants impaired in salicylic, jasmonate and ethylene defense pathways (reviewed in Glazebrook, 2001) are available and can be used to decipher plant defense strategies. Therefore, using Arabidopsis to study gene expression during pathogen attack is advantageous especially with the emergence of cDNA and high-density oligonucleotide array technology. These two technology platforms allows the parallel interrogation of mRNA transcripts of hundreds to thousands genes. The oligonucleotide probe array, also known as Affymetrix GeneChip®, is more objective because subjective preselection of gene probes to be put on microarray is avoided (Lockhart and Winzeler, 2000). Large-scale profiling of Arabidopsis transcriptome using this technology is now possible. The array contains probes (tethered nucleic acid molecules) for 8734 Arabidopsis genes used to interrogate the experimental samples. This first generation of designed arrays are commercially manufactured using photolithography technology eliminating time-consuming and labor-intensive array fabrication process (Lipshutz et al., 1999). Moreover, possible human errors that occur during the clone tracking process are eliminated. The second generation which will contain the complete Arabidopsis genome (25.498 genes) is currently under development (Zhu and Wang, 2000). The efficiency and the reproducibility of this method in Arabidopsis was already proven by studies of Zhu et al. (2001) and Harmer et al (2000). Microarray technology was used efficiently to monitor mammalian gene expression during pathogenesis on a broad scale offering an unprecedented amount of potential pharmaceutical targets (Coller et al., 2000; Voehringer et al., 2000; Der et al., 1998). In the same manner, one of the most attractive applications of microarrays is characterization of plant-pathogen interactions. Gene expression patterns of resistant plants can be compared to the susceptible plants, to mutants, to transgenic plants or to chemically treated plants allowing a rapid identification of genes involved in resistance. We have recently developped a new *Arabidopsis thaliana -Phytophthora porri* pathosystem which offers an accession-dependent range of phenotypes when infected with the same isolate of *P. porri* (Roetschi et al., 2001). In this study, we aimed to apply oligonucleotide-based array technology to better understand the transcription events during the interaction. In fact, we have previously characterized this pathosystem and showed that the salicylic acid deficient mutant *npr1-1* (Cao et al., 1997), the transgenic plant *nahG* converting salicylic acid to cathecol (Gaffney et al., 1993), the jasmonate mutant *jar-1* (Staswick et al., 1992) and the ethylene mutant *etr1-1* (Hua and Meyerowitz, 1998) are all resistant towards *Phytophthora*. In contrast, the mutant *pad2-1* impaired in both SA and camalexin accumulation is hypersusceptible. However, another biosynthetic mutant deficient in camalexin production *pad3-1* is completely resistant. We concluded that neither SA nor jasmonate/ethylene pathways nor accumulation of camalexin are necessary for resistance towards *Phytophthora* but the function of PAD2 is absolutely

required. Moreover, exogenous application of SA can not rescue the hypersusceptible phenotype of *pad2-1*. All these observations suggest that resistance of Arabidopsis against *P. porri* depends on an yet uncharacterized pathway which is under the control of *PAD2*. In this study, we have hybridized DNA chips with 15 different experimental samples representing interactions with three degrees of phenotype severity: an incompatible interaction (Col-0) characterized by hypersensitive reactions macroscopically visible within 48 hours, a compatible interaction (Ler) where plants are moderatly colonized and the hypersusceptible *pad2-1* mutant (Col-0 background) that was dramatically colonized by *P. porri* within two days. In this study, a preliminar transription profiling of 30% of the Arabidopsis genome will give us some important information about potential markers of the *PAD2* pathway and their role in the resistance as well as their putative role in the defense mechanisms against *Phytophthora porri*.

IV.2. RESULTS

Columbia (Col), Landsberg erecta (Ler) and *pad2-1* Arabidopsis plants were inoculated by using the plug inoculation method and leaf tissue was harvested at different time points (table I).

Table I. Description of the microarray experiment.

	Columbia (Col-0)		**Landsberg erecta (Ler)**		***pad2-1***	
Phenotype	Resistant		Susceptible		Hypersusceptible	
Time-points	Control	Inoculated	Control	Inoculated	Control	Inoculated
0h	Col0	-	Ler0	-	pad20	-
24h	Col24	Col24HH	Ler24	Ler24HH	pad224	pad224HH
48h	Col48	Col48HH	Ler48	Ler48HH	pad248	pad248HH

The experiment was repeated three times. In order to control the reproducibility of the induction of defense genes, total RNA from the three independent experiment were separated on an agarose gel containing formaldehyde, blotted and the membrane was hybridized with radiolabelled *PR-1* probe. *PR-1* showed a similar induction in Ler and Col-0 in the three experiments (figure 1). The earliest induction of *PR-1* is in the compatible interaction (Ler) and *PR-1* transcripts accumulation is completely blocked in *pad2-1*. *PR-1* gene expression was also induced at later time points in the control experimemts performed with plugs without *Phytophthora*. Equal amounts of total RNA from each experiment were then mixed in order to obtain 50 µg of total RNA for each time-point. A cDNA synthesis was performed directly on 5 µg of the mixed total RNA. Biotinylated complementary RNAs (cRNAs) were in vitro transcribed from synthesized cDNAs and used as hybridization probes. The hybridization of the Affymetrix chip was performed at the Torrey Mesa Reseach Institute (TMRI) in San Diego with the support of Syngenta.

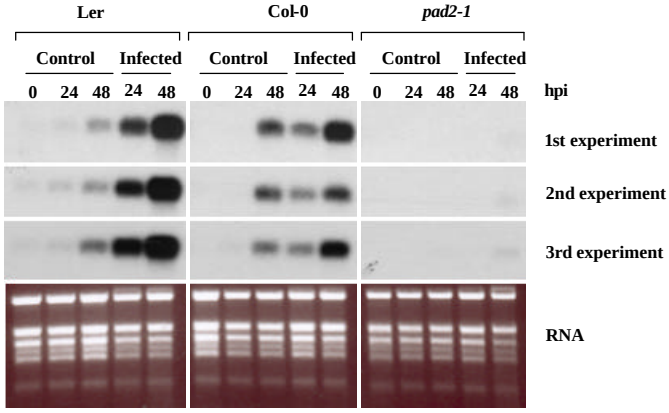


Figure1. PR-1 mRNA accumulation in the three independent experiments.

Landsberg erecta (Ler), Columbia (Col-0) and *pad2-1* representing respectively the compatible interaction, the incompatible interaction and the hypersusceptible mutant were infected and the total RNA was extracted at 24 and 48 hours post-inoculation (hpi). The experiment was repeated three times. One µg of total RNA from each independent experiment was separated on a formaldehyde gel, blotted and the membrane hybridized with *PR-1*. The RNA was stained with ethidium bromide and the bands corresponding to different ribosomal RNA are sharp and well separated proving a high purity ($OD_{260/280}$>2.00)

IV.2.1 Transcriptome pattern

Quality control of the data generated from expression analysis probe arrays was done using the GeneChip® 3.1 software (Affymetrix). In this software, an absolute analysis algorithm uses a metric to compare the sequence-specific perfect match (PM) probe cells with their control mismatch probes cells (MM) for each probe set. These metrics are then used by a decision matrix to determine if a transcript is present (P), marginal (M) or absent (A or undetected). Among the 8300 genes of the dataset about 5000 transcripts are "present" (Figure2).

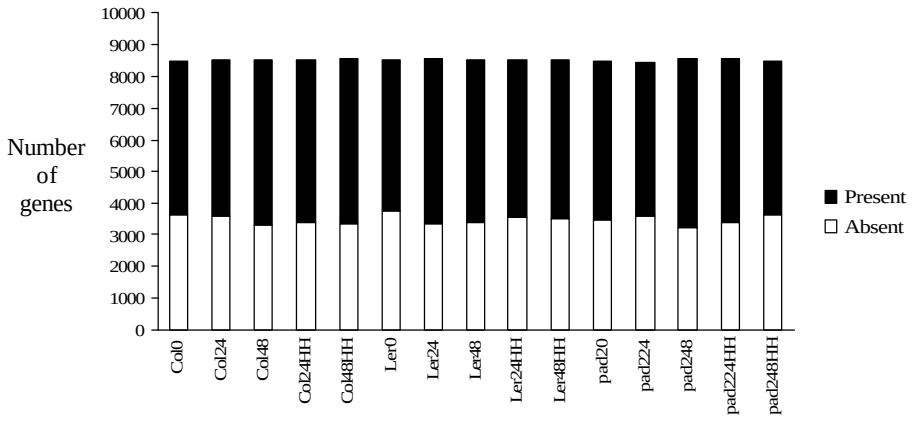


Figure 2. Number of present and absent genes in the 15 samples.

Transcripts in the 15 experiments were designated present or absent using the GeneChip® 3.1 software. The abbreviations are mentioned in Table I.

Gene expression levels (average signal difference between PM and MM) of the detectable genes were extracted, $\log_{10}$ transformed and plotted against the frequency at which they occur in each interaction (Figure 3). Normal distributions of the transcripts were then performed for all types of interactions. Each distribution was separated in two groups: control plants at 24h

and 48h and infected plants with *P. porri* at the same time-points. This abundance distribution of transcripts reflects the complexity of the transcriptome. The pattern of expression is quite similar in all the interactions. In the 6 distributions, the median was centered at approximately 100, quite similar to the profiles of human HIV-infected T lymphocytes, mouse olfactory epithelium, rat brain, yeast (Lockhart et al., 2000) and developping Arabidopsis (Zhu et al., 2001). Uninfected plants present a similar transcription profile in all the interactions. However, transcriptome of the infected plants is more complex. Interestingly, the incompatible interaction (Col-0 infected) presents the most elevated overall gene expression. This could be due to a significant increase in the expression levels of genes involved in the resistance response towards *Phytophthora*.

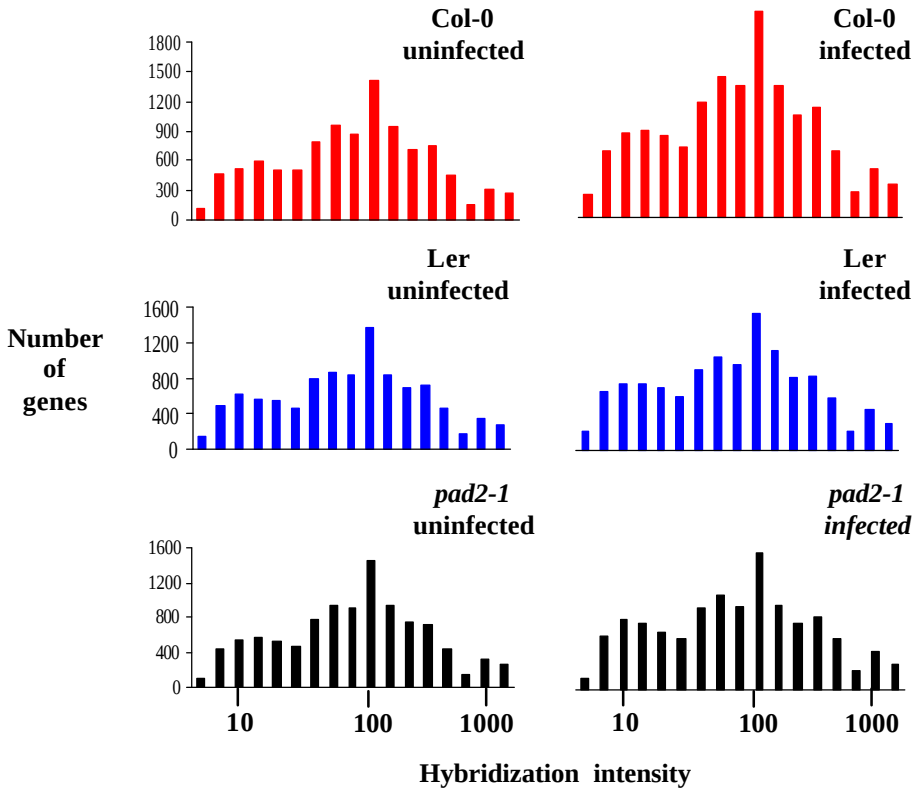


Figure 3. Complexity of the transcriptome in the 3 interactions.

Normal distributions of the 3 interactions: resistant (Col-0), suceptible (Ler) and hypersusceptible (*pad2-1*) showed that the resistant interaction upon infection has the most elevated gene expression levels.

IV.2.2 Gene expression levels

The median expression level for all the "present" genes in the 15 arrays is 250, exactly the same value was reported for organs at different developmental stages in Arabidopsis plants (Zhu et al., 2001). Before data mining we performed northern blot analysis to correlate the chip gene signal with a visual signal on a northern blot. For this analysis we used ^{32}P radiolabelled probes to detect pathogenesis related *PR-1* and the heat shock protein *HSP83*

transcripts. These probes were chosen because of their different patterns of expression and different levels of hybridization. *PR-1* is highly expressed in the compatible and in the incompatible interaction but completely absent in the highly susceptible mutant *pad2-1* (Figure 4, A). In contrast, *HSP83* transcript accumulation is higher in *pad2*-1 but absent in Col-0 and Ler (figure 4, B). The intensity of the northern signals correlates well with the expression levels obtained in the microarray experiments. The strongest expression of *PR-1*, corresponding to an intensity of 2764 is found after 48 hours post-infection with *P. porri* in Landsberg. The weakest visible signal is 271 corresponding to the expression level of *HSP83* in Landsberg plants after 48 hours post inoculation (figure 4, B). According to the figure 4, we estimate that the weakest hybridization intensity detectable by northern analysis is approximately 300.

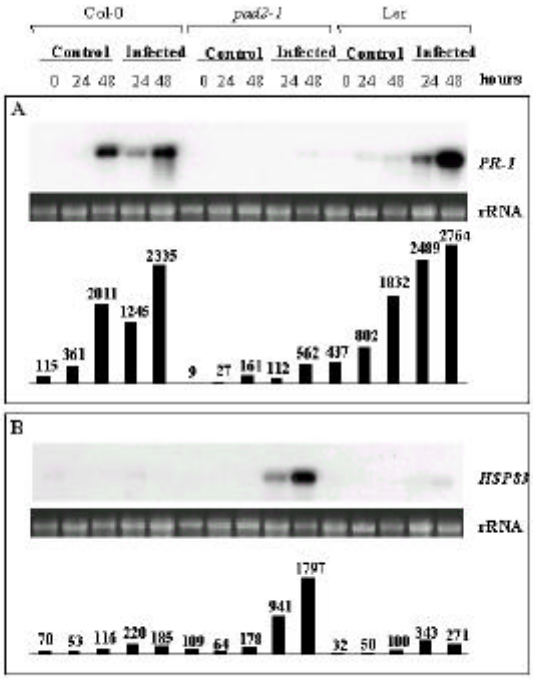


Figure 4. Correlation between northern blot analysis signals and microarray hybridization intensities.

Total RNA from 3 independent experiments were extracted at 0, 24 and 48 hours from the incompatible interaction (Col-0), compatible interaction (Ler) and the highly susceptible mutant *pad2-1* and mixed.
RNA gel blots were performed with 8 µg of total RNA and probed with *PR-1* (**A**) and *HSP83* (**B**). Ethidium bromide staining of ribosomal RNA shows equal loading. Hybridization levels (average signal difference between perfect match and mismatch) for each sample are indicated in the histogram.

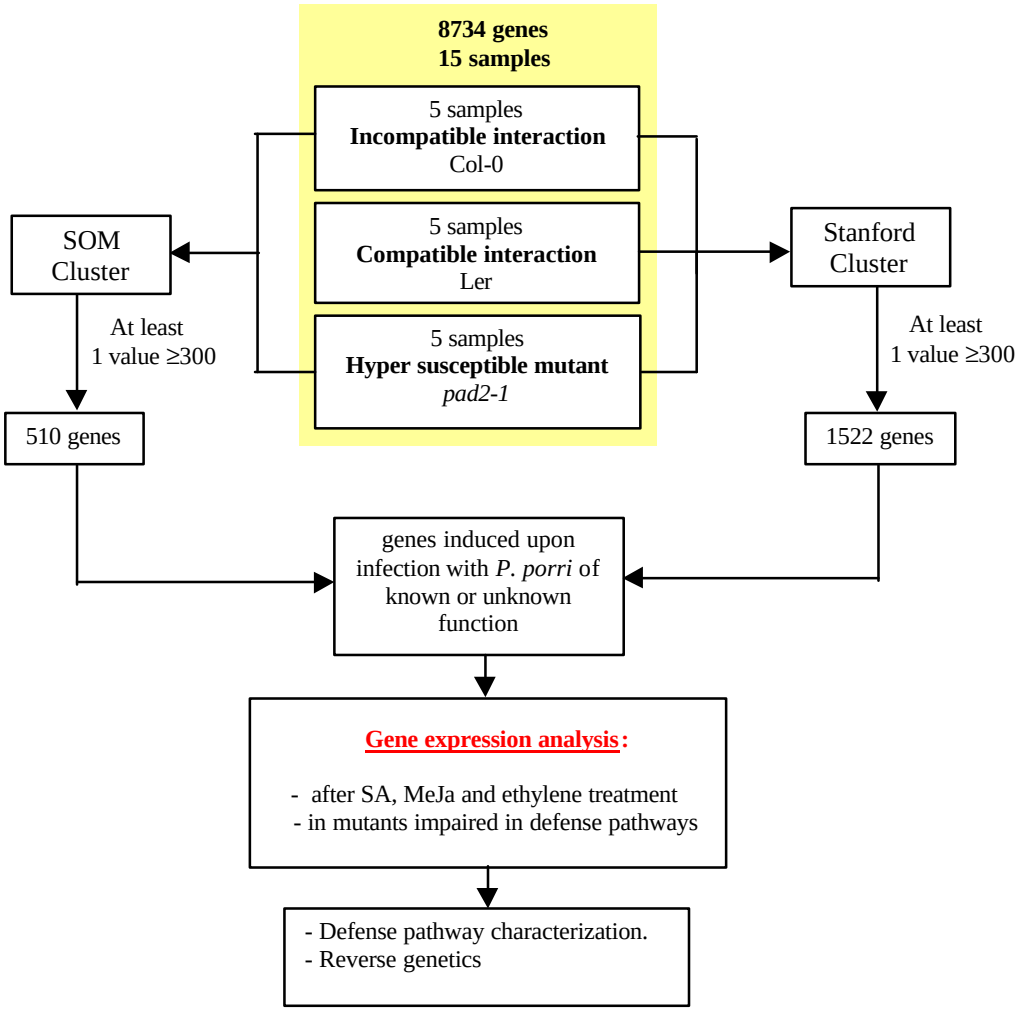


Figure 5. Diagrammatic scheme representing data mining approach used in our study.

Dataset from the 3 interactions were subjected to clustering using either Self-organized map (SOM) or Stanford Cluster and Treeview software. Expression levels of genes involved in defense mechanisms of known or unknown function could be further studied in detail. Gene expression analysis of the selected genes was performed under different conditions and in several mutants impaired in defense mechanisms. Possible roles of those genes in a known or unknown defense pathway could be proposed according to the previous gene expression analysis. Finally, a reverse genetic approach using T-DNA tagged lines could be used to evaluate the effect of the mutated selected gene on the resistant phenotype.

IV.2.3 Dynamics of gene expression after *Phytophthora* infection

A tab-delimited text file containing dataset corresponding to gene expression levels in the infected plants at 24h and 48h after infection and their corresponding controls was created using Microsoft Excel. The data mining was done according to the diagram scheme shown in Figure 5. In order to visualize the whole transcriptome after *P. porri* infection we used the Cluster and Treeview software (Eisen et al., 1998). 1522 genes showed at least 1 observation with an absolute value over 300 in any of the twelve time-points. Using the Stanford Cluster software, these genes were $\log_2$ transformed, centered and normalized and both genes and arrays were hierarchically clustered. The resulting tree was then visualised using the Treeview software. Two main array clusters containing 5 distinct gene sub-clusters were identified

(Figure 6). The arrays corresponding to the infected plants are more related and within this cluster the susceptible interactions are much closer in term of gene expression patterns. The majority of the 1522 genes are more highly expressed in the infected plants of all the interactions but not in the control plants (Group IV) or vice-versa (Group II). The group III (figure 7) contains genes strongly expressed in the mutant *pad2-1* with low expression in the resistant plants Columbia. 55% of these genes are involved in metabolism and 29% are of unknown function (figure 10, B). Transcription factors (3%) present in this group are mainly ethylene responsive element binding factors (Fujimoto et al., 2000). The group V (figure 8) shows genes which are expressed in Columbia plants and in the *pad2-1* mutant but not in Landsberg erecta. The major part (figure 10, C) are metabolic enzymes (44%). The only defense gene in this group (1% of total genes) is similar to the RPS2 resistance disease protein. Group I clusters genes that show a high expression level in the incompatible interaction and moderately elevated in the compatible interaction. However, they are below the mean in the highly susceptible mutant *pad2-1* in control and inoculated plants (figure 9). An important part of those genes are unknown (16%) but the majority (44%) is involved in metabolism (figure 10, A and table II). 13% of those genes are related to defense mechanisms. Interestingly, within this group different resistance proteins are expressed (table II). Some of them are putative but the disease resistance protein *RPP1-WsC*, a gene involved in the specific resistance of Arabidopsis (ecotype Ws) against the oomycete pathogen *P. parasitica* isolate Noco2, was well described and encodes a NBS-LRR-type R protein (Botella et al., 1998). Other genes are known to be expressed upon *Phytophthora* infection, like the beta-1,3-glucanases (Beerhues and Kombrink, 1994; Dong et al., 1991). *NPR1* controls systemic acquired resistance (Cao and al., 1997). *AIG1* is induced by the *Pseudomonas syringae* pv *maculicola* strain ES4326 carrying the avirulence gene *avrRpt2* (Reuber et Ausubel, 1996). *DAD-1* suppresses apoptosis in Arabidopsis and it is homologue to the mammalian gene "defender against apoptotic death 1". EIN3 encodes a nuclear localized protein and is required for ethylene mediated effects including gene expression, triple response, cell growth inhibition and accelerated senescence (Chao et al., 1997). The kinases represent 5% of the group I and could be an important component of the defense reaction.

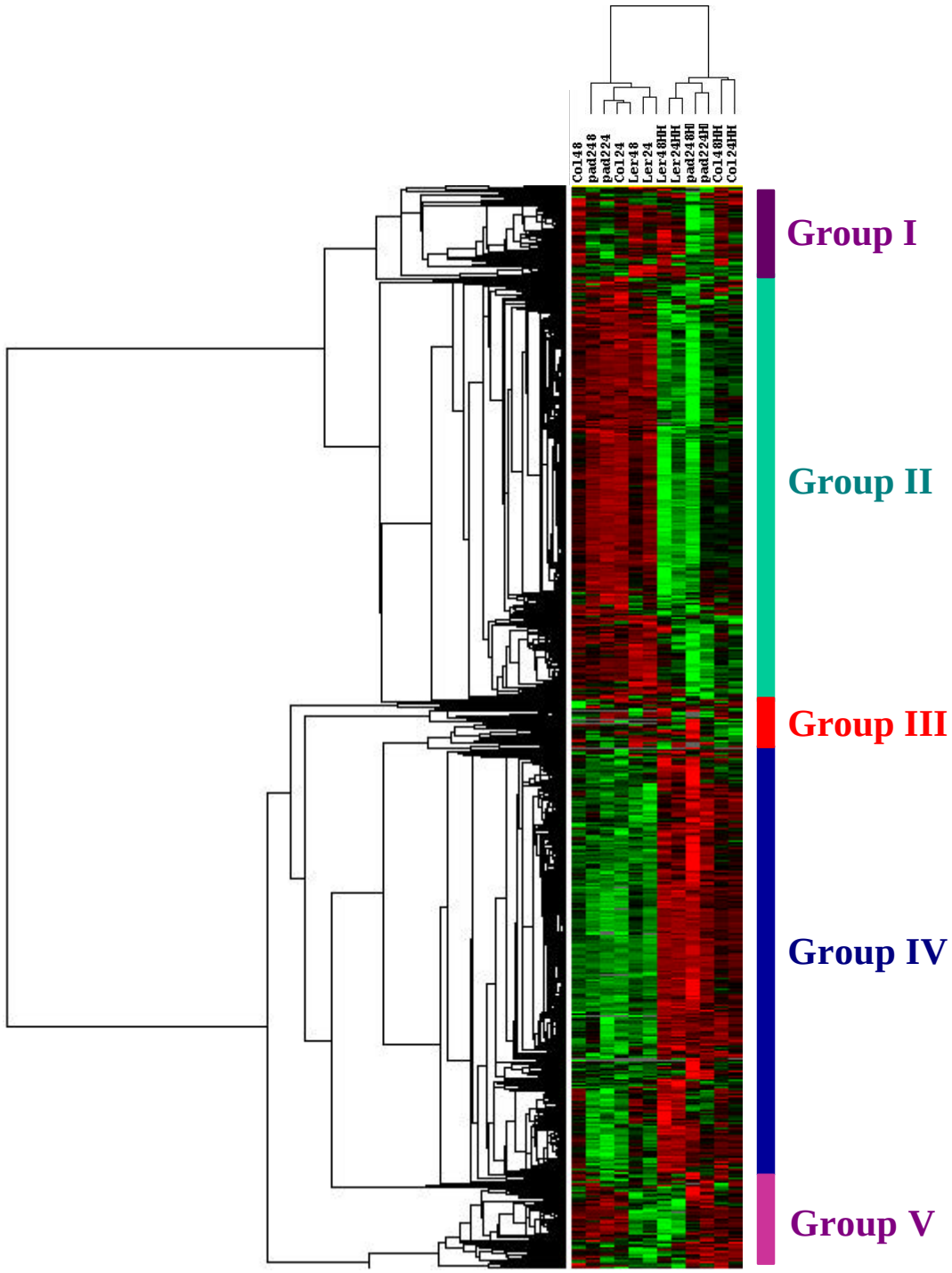


Figure 6. Cluster analysis of the Arabidopsis-*Phytophtthora* pathosystem.

All datasets from the 3 interactions, incompatible, compatible and highly compatible representing 1522 genes and 12 samples were clustered using Stanford cluster and Treeview software (Eisen et al., 1998). Colour codes are as follows: ■ expressed above the mean, ■ expressed below the mean and ■ mean. We have divided this cluster in 5 groups. Group I: high expression level in the incompatible interaction and in the compatible interaction and below the mean in the highly susceptible mutant *pad2-1*; GroupII: genes highly expressed in the control plants of all the interactions but with low expression in the infected plants; GroupIII: genes strongly expressed in the mutant *pad2-1* and lowly expressed in the resistant plants Columbia; Group IV: genes highly expressed in the infected plants of all the interactions but not in the control plants and group V: genes expressed in Columbia plants and in the *pad2-1* mutant but not in Landsberg erecta.

Table II . Genes contained in group I showing high expression level in the incompatible and in the compatible interaction and low expression in *pad2-1*.

Probe set ID	Description
	Defense (13%)
19665_s_at	emb|CAA64837.1| (X95585) DAD-1 homologue
14110_i_at	emb|CAB36854.1| (AL035528) putative disease resistance protein
13625_s_at	emb|CAB42924.1| (AL049862) putative disease resistance protein
13212_s_at	emb|CAB68132.1| (AL137080) beta-1, 3-glucanase 2 (BG2)
16578_s_at	emb|CAB68132.1| (AL137080) beta-1, 3-glucanase 2 (BG2)
12364_at	gb|AAA32756.1| (M58464) beta-1,3-glucanase
16365_at	gb|AAC04495.1| (AC003974) putative disease resistance protein
12879_s_at	gb|AAC49282.1| (U40856) AIG1
17544_s_at	gb|AAC49282.1| (U40856) AIG1
14239_s_at	gb|AAC49611.1| (U76707) regulatory protein NPR1
16602_s_at	gb|AAC49611.1| (U76707) regulatory protein NPR1
15205_s_at	gb|AAC49749.1| (AF004216) ethylene-insensitive 3
19426_i_at	gb|AAC72979.1| (AF098964) disease resistance protein RPP1-WsC
	Kinase (5%)
15616_s_at	emb|CAA08794.1| (AJ009696) wall-associated kinase 1
16140_s_at	emb|CAB42872.1| (AJ012423) wall-associated kinase 2
12353_at	gb|AAC23641.1| (AC004684) putative receptor-like protein kinase
12354_at	gb|AAC23641.1| (AC004684) putative receptor-like protein kinase
16393_s_at	gb|AAD28318.1|AC006436_9 (AC006436) putative receptor-like protein kinase
12497_at	gb|AAD32284.1|AC006533_8 (AC006533) putative receptor-like protein kinase
	Metabolism (44%)
17063_s_at	dbj|BAA00831.1| (D01026) small GTP-binding protein
15684_s_at	dbj|BAA03090.1| (D13983) chloroplast envelope Ca2+-ATPase precursor
18672_s_at	dbj|BAA03090.1| (D13983) chloroplast envelope Ca2+-ATPase precursor
14652_s_at	dbj|BAA06103.1| (D29017) squalene synthase
15113_s_at	dbj|BAA11944.1| (D83531) GDP dissociation inhibitor
15630_s_at	dbj|BAA22504.1| (AB005560) AtGDI2
18011_s_at	dbj|BAA22504.1| (AB005560) AtGDI2
13559_at	emb|CAA17567.1| (AL021961) caffeoyl-CoA O-methyltransferase - like protein
12893_a	emb|CAA57973.1| (X82647) class III ADH, glutathione-dependent formaldehyde dehydrogenase.
12893_at	emb|CAA57973.1| (X82647) class III ADH, glutathione-dependent formaldehyde dehydrogenase.
17450_s_at	emb|CAA59061.1| (X84318) NADH dehydrogenase
15982_s_at	emb|CAA66863.1| (X98190) peroxidase ATP2a
17413_s_at	emb|CAA67551.1| (X99097) peroxidase
16036_i_at	emb|CAA70691.1| (Y09482) HMG1
19974_s_at	emb|CAA72363.1| (Y11650) cyclic phosphodiesterase
13264_s_at	emb|CAA72973.1| (Y12295) glutathione transferase
18730_at	emb|CAA78057.1| (Z12022) calmodulin
18731_at	emb|CAA78059.1| (Z12024) calmodulin
13185_at	emb|CAA84612.1| (Z35475) thioredoxin
13133_s_at	emb|CAB36530.1| (AL035440) ubiquitin-like protein
17489_s_at	emb|CAB36849.1| (AL035528) glycine-rich RNA-binding protein AtGRP2-like

Table II. Continued.

17990_at	emb|CAB41312.1| (AL049711) putative calmodulin
20555_s_at	emb|CAB45975.1| (AL080318) copper amine oxidase like protein (fragment2)
15585_s_at	gb|AAB63528.1| (L36939) dynamin-like GTP binding protein
20499_at	gb|AAB80665.1| (AC002332) putative NAM (no apical meristem)-like protein
15690_f_at	gb|AAB96832.1| (U40399) cytosolic cyclophilin
15181_s_at	gb|AAB96879.1| (U70424) O-methyltransferase 1
12750_s_at	gb|AAC17620.1| (AC002131) Identical to aspartic proteinase cDNA gb|U51036 from A. thaliana.
15967_at	gb|AAC72872.1| (AF104919) contains similarity to cysteine proteases
18322_i_at	gb|AAC73040.1| (AC005824) putative AAA-type ATPase
20446_s_at	gb|AAC80600.1| (AC005106) T25N20.21
18581_at	gb|AAC95204.1| (AC004561) putative tropinone reductase
13692_s_at	gb|AAD10151.1| (AC005917) putative WD-40 repeat protein, MSI4
17917_s_at	gb|AAD12002.1| (AC004261) calcium binding protein (CaBP-22)
17962_at	gb|AAD21729.1| (AC006931) putative citrate synthase
14720_s_at	gb|AAD34236.1|AF083913_1 (AF083913) annexin
15620_s_at	gb|AAD47191.1|AF106084_1 (AF106084) 4-coumarate:CoA ligase 1
	Ribosomal (4%)
16772_at	emb|CAB10447.1| (Z97341) ribosomal protein
16920_at	emb|CAB43407.1| (AL050300) putative ribosomal protein S14
16544_s_at	gb|AAB87692.1| (AF034694) ribosomal protein L23a
16527_at	gb|AAC73028.1| (AC005824) 60S acidic ribosomal protein P2
17910_at	gb|AAC73029.1| (AC005824) 60S acidic ribosomal protein P2
	Stress (3%)
15190_s_at	dbj|BAA04049.1| (D16628) ATsEH
13165_at	emb|CAA16552.1| (AL021635) HSP associated protein like
16447_at	gb|AAB63606.1| (AC002343) HSP90 isolog
16448_g_at	gb|AAB63606.1| (AC002343) HSP90 isolog
	Structural (5%)
15200_s_at	dbj|BAA24804.1| (AB010946) AtRer1B
17562_at	dbj|BAA84651.1| (AB005903) AtPH1
19919_i_at	emb|CAA71879.1| (Y10986) hypothetical protein 194
15534_f_at	gb|AAA33476.1| (M13377) histone H4 [Zea mays]
15180_s_at	gb|AAB57799.1| (AF001535) AGAA.4
19940_at	gb|AAD20090.1| (AC006532) putative endosomal protein
	Transcription/Translation (4%)
16463_at	emb|CAA52751.1| (X74733) elongation factor-1 beta A1
16464_g_at	emb|CAA52751.1| (X74733) elongation factor-1 beta A1
17011_at	emb|CAB36546.1| (AL035440) putative DNA binding protein
17489_s_at	emb|CAB36849.1| (AL035528) glycine-rich RNA-binding protein AtGRP2-like
17030_s_at	gb|AAB68038.1| (U78866) gene1000

Table II. Continued

	Transport (6%)
18328_at	emb|CAA47325.1| (X66857) sugar transport protein
20369_s_at	emb|CAB41109.1| (AL049656) ammonium transport protein (AMT1)
16060_s_at	emb|CAB64732.1| (AJ249967) putative sugar transporter
16056_s_at	gb|AAB72112.1| (U79960) vacuolar sorting receptor homolog
13670_s_at	gb|AAC15999.1| (AF061570) potassium channel beta subunit homolog.
17499_s_at	gb|AAD19610.1| (AF107726) cyclic nucleotide gated channel
	Unknown (16%)
12112_at	emb|CAA17560.1| (AL021961) putative protein
13641_at	emb|CAB38788.1| (AL035678) putative protein
14249_i_at	emb|CAB43438.1| (AL050300) putative protein
14250_r_at	emb|CAB43438.1| (AL050300) putative protein
19563_s_at	emb|CAB45907.1| (AL080283) putative protein
14964_at	gb|AAB60905.1| (AC001229) F5I14.4 gene product
19369_at	gb|AAB81674.1| (AC002354) unknown protein
20017_at	gb|AAC16079.1| (AC004521) unknown protein
17901_at	gb|AAC27469.1| (AC003672) unknown protein
14699_at	gb|AAD15461.1| (AC006067) unknown protein
14704_s_at	gb|AAD15461.1| (AC006067) unknown protein
15846_at	gb|AAD15461.1| (AC006067) unknown protein
20184_at	gb|AAD36957.1|AC000107_3 (AC000107) F17F8.3
12232_at	gb|AAD41433.1|AC007727_22 (AC007727) EST gb|T41993 comes from this gene.
14703_at	gb|AAD46040.1|AC007519_25 (AC007519) ESTs gb|H36253 and gb|AA04251 come from this gene.
14736_s_at	gb|AAF26761.1|AC007396_10 (AC007396) T4O12.15 protein in budding yeast

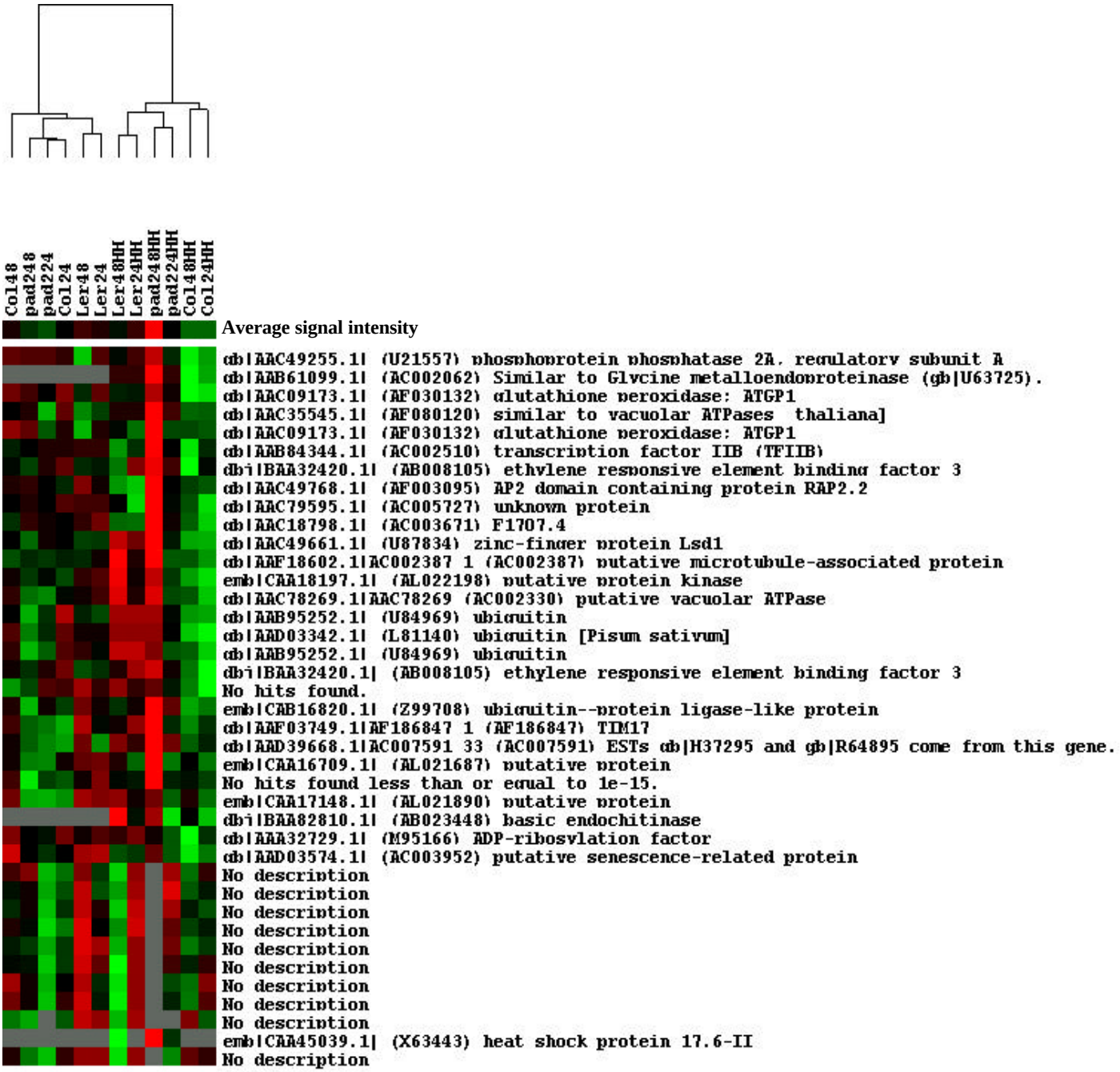


Figure 7. Cluster of genes strongly expressed in the hypersusceptible mutant *pad2-1* during infection.

The area containing genes of group III (see Figure 6) was zoomed and genes strongly expressed in the mutant *pad2-1* with low expression in the resistant plants are described.

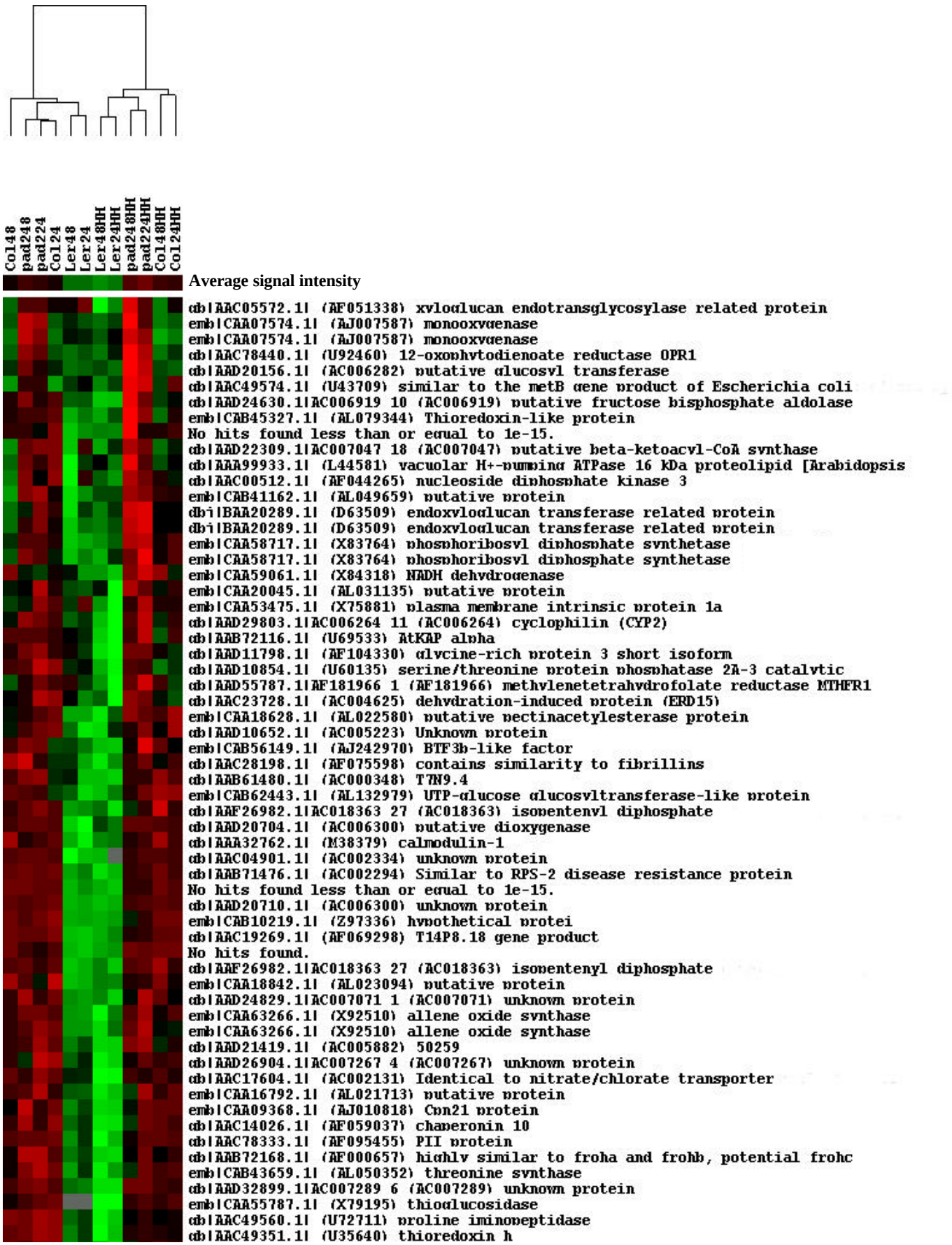


Figure 8. Cluster of genes which are expressed in Columbia plants and in the *pad2-1* mutant but not in Landsberg erecta.

The area containing genes of group V (see Figure 6) was zoomed and genes strongly expressed in the Col-0 and *pad2-1* plants but low expression in Ler are described.

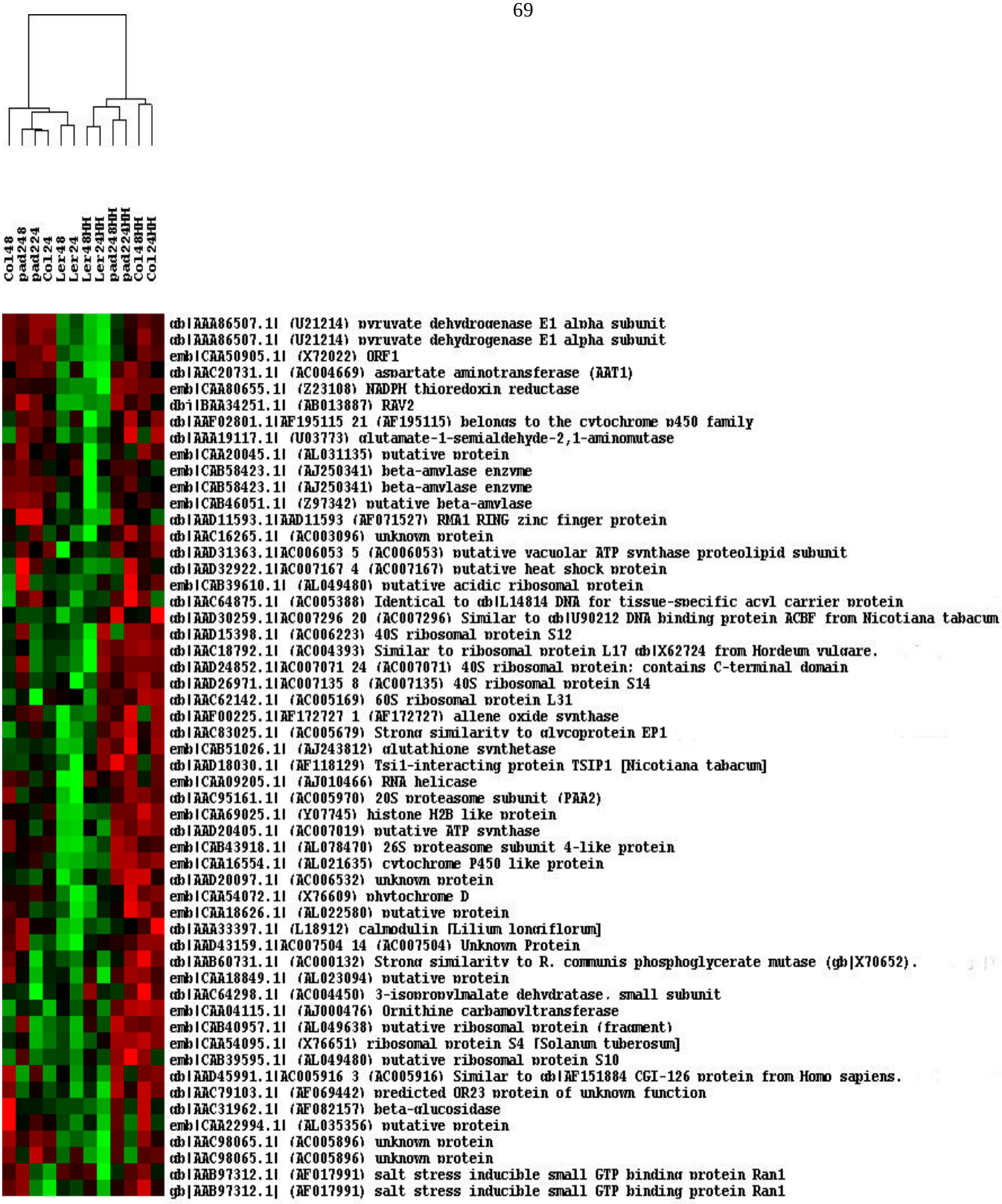


Figure 8. Continued.

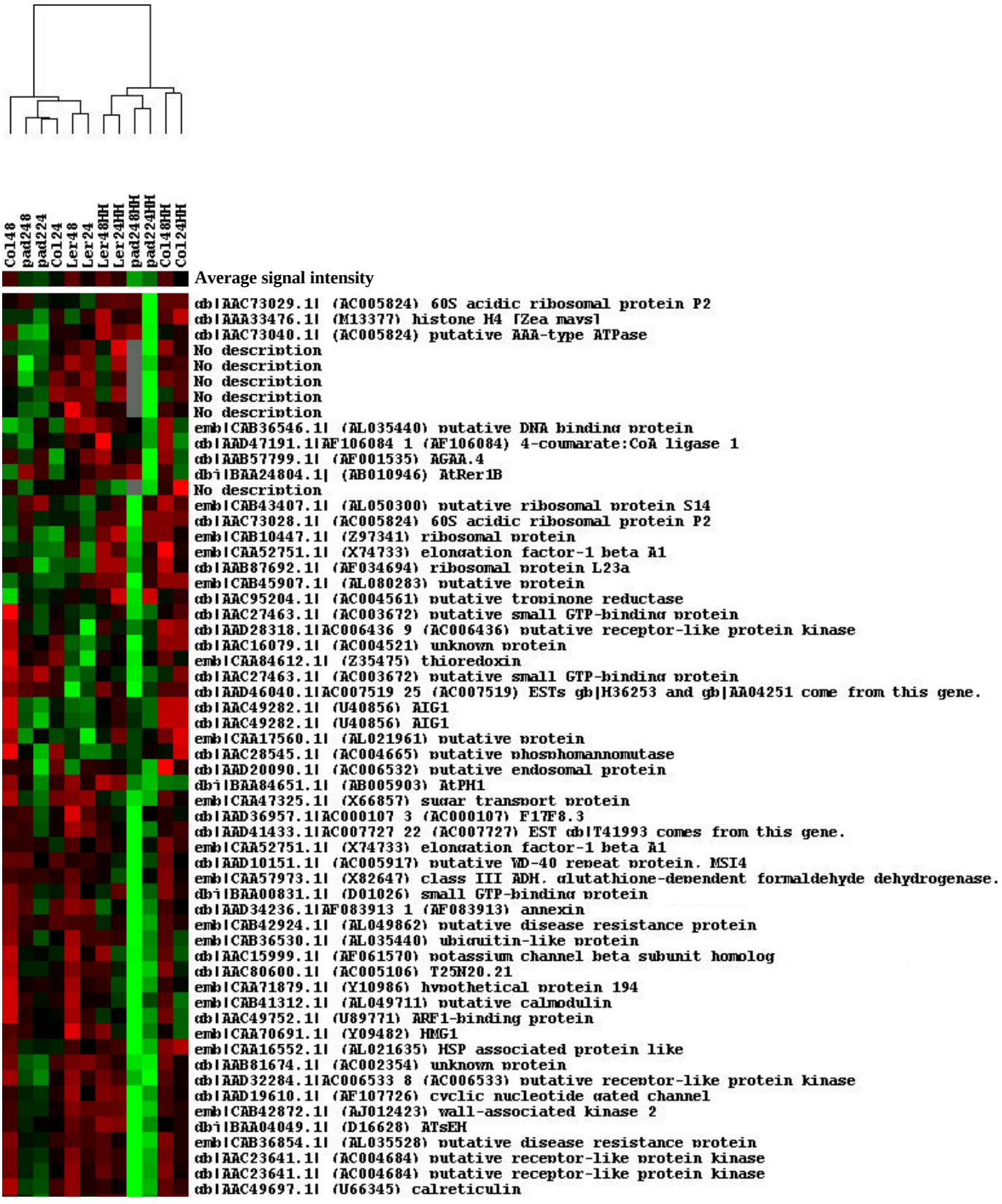


Figure 9. Genes lowly expressed in *pad2-1*and strongly expressed in the incompatible interaction (Col-0).

Genes of group I (see Figure 6) were clustered and described. Those genes show a high expression level in the incompatible interaction, moderatly elevated in the compatible interaction and below the mean expression value in the highly susceptible mutant *pad2-1*.

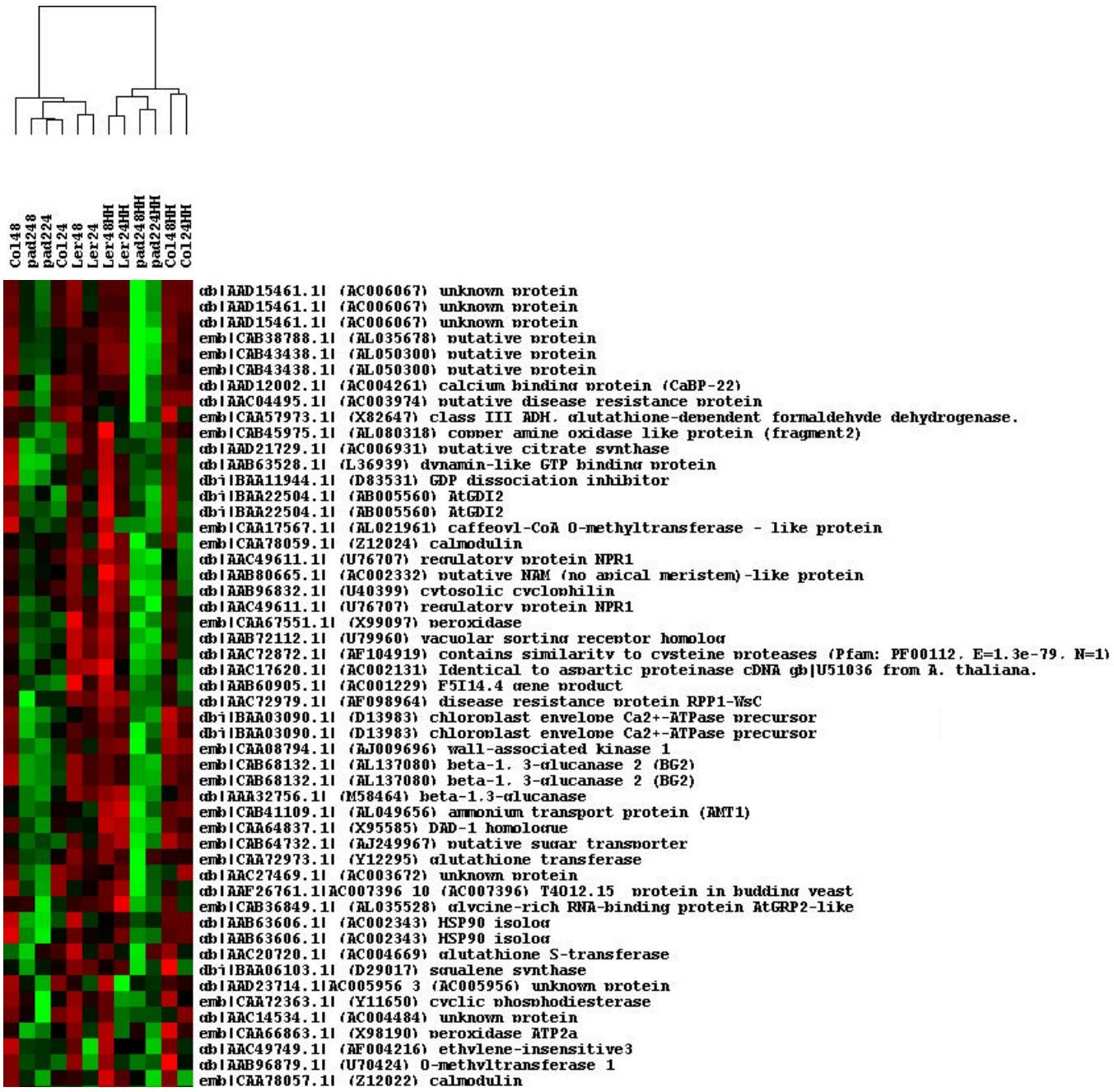
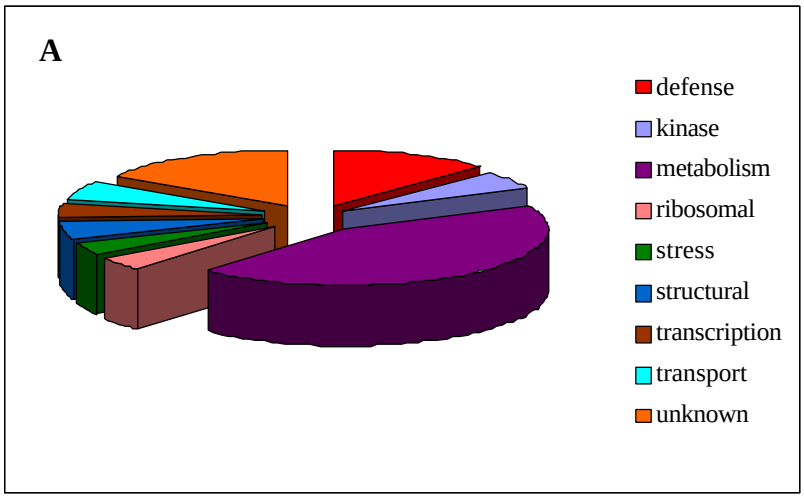
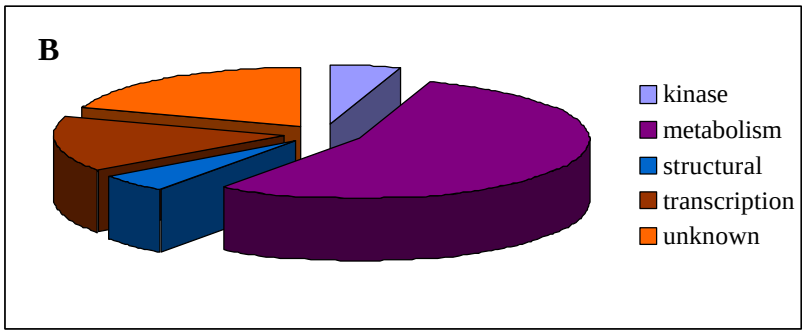


Figure 9. Continued.





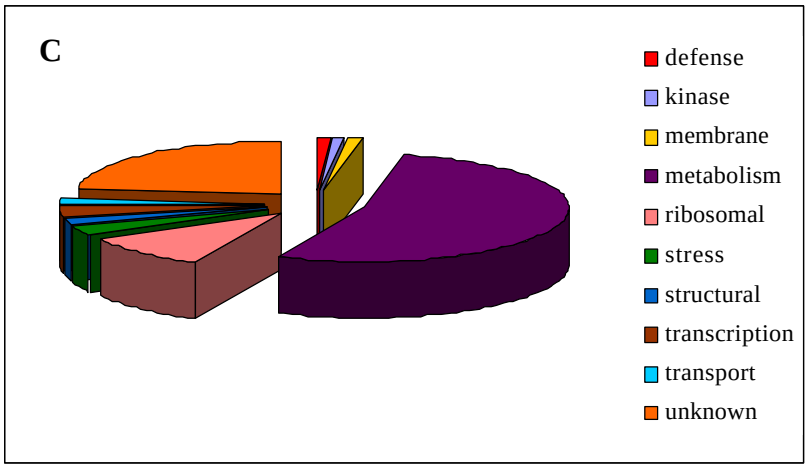


Figure 10. Function of the genes clustered in the groups I, III and V.

Genes contained in group I (A), group III (B) and group V (C) were classed according to their function (see figure 7, figure 8 and figure 9) .

IV.2.4 Identification of genes upregulated in the incompatible interaction

In order to identify genes highly expressed in the incompatible interaction, a SOM (Self Organized Map) clustering (Tamayo et al., 1999) was performed using datasets corresponding to both the incompatible interaction Col-0 and the highly susceptible mutant *pad2-1* and the corresponding controls. Because of the different genetic background from Col-0 and pad2-1 (Col-0 background), the dataset of the Landsberg plants was not subjected to SOM analysis. Using parameters described in material and methods 4 (2x2) clusters, representing 510 genes which showed at least 1 observation with an absolute value over 300 in any of the twelve time-points, were obtained. The majority of the genes (252) are either upregulated in *pad2-1* (c0) or downregulated in both interactions (c3).The cluster 1 "c1" shows 41 genes upregulated in the resistant plants but expressed at low levels in *pad2-1* (figure 11). In this cluster, the majority of the genes are of unknown function or have no homology to any sequence in the databases or are not described by the manufacturer of the chip (31%) followed by genes involved in defense (27%), genes involved in metabolism (24%), kinases (9%), stress related genes (6%) and the last 3% are ribosomal genes (figure 12 and table III). This cluster was analyzed in more detail. Expression levels in Col-0 were divided by the expression level in *pad2-1*. A group of 14 genes clearly showed high ratios and seven of them were selected for further studies (figure 13). *PR-1* (14635_s_at and 17128_s_at) , *PR-2* (16578_s_at and 13212_s_at) and the gene with probe set ID *14145_at* are also contained in this group but were either previously characterized (Roetschi et al., 2001) or not described and therefore not selected. Among the 7 selected genes 3 are defense related genes, the previously characterized genes *AIG1* (for *avrpt2*-induced gene; Reuber and Ausubel,1996), *PAD4* (Jirage et al., 1999) and a putative disease resistance protein. Three other genes are of unknown function and the seventh selected gene is involved in the metabolism (Table III). Sequence homology search showed that the gene *20017_at* contains a lipid-transfer protein (LTP) conserved motif and the gene *18224_s_at* contains a putative peptide sulfoxide reductase enzymatic domain. *AIG1* showed the highest ratio (27 time fold difference) at 24h post-infection and *20017_at* the highest ratio at 48 hours (105 time fold difference).

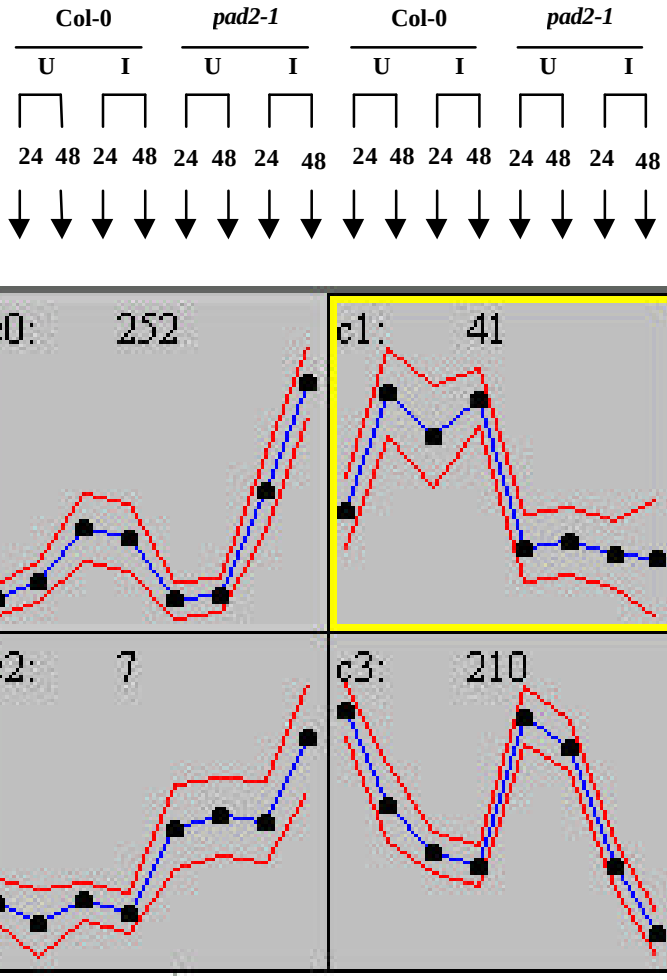


Figure 11. Self-oragnized map (SOM) analysis of gene expression in the resistant Col-0 plants and in the hypersusceptible *pad2-1*.

Datasets of genes expressed in both the incompatible interaction (Col-0) and the highly susceptible mutant *pad2-1* 24 and 48 hours post-inoculation were subjected to SOM analysis. Each cluster of genes showed a distinct pattern of gene expression. The cluster highlighted with a yellow line, contains genes upregulated in the incompatible interaction but not in *pad2-1*and was studied in more detail. The blue lines represent the average expression level of the genes within a cluster and the red lines represent the standard deviation. U means uninfected and designate the control plants and I stands for infected.

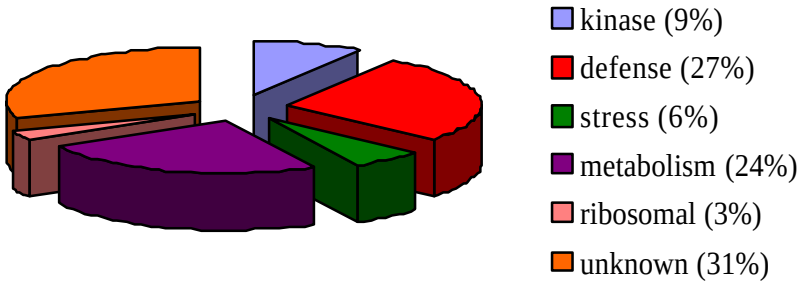


Figure 12. Function of the genes upregulated in the incompatible interaction with low expression in the hypersusceptible mutant *pad2-1*.

Genes within cluster 1 (see figure 11) were grouped according to their function. The majority of these are of unknown function (31%) followed by genes involved in defense mechanisms (27%).

Table III. Genes upregulated in the incompatible interaction. The asterisk * indicates the seven genes selected for further analysis

Probe set ID	Description
	Kinase (9%)
12353_at/12354_g_at	gb|AAC23641.1| (AC004684) putative receptor-like protein kinase
16140_s_at	emb|CAB42872.1| (AJ012423) wall-associated kinase 2
13659_at*****	emb|CAA18462.1| (AL022347) serine/threonine kinase-like protein
	Defense (27%)
14636_s_at/16153_s_at	gb|AAF21072.1|AC013258_10 (AC013258) thaumatin-like protein
19426_s_at	gb|AAC72979.1| (AF098964) disease resistance protein RPP1-WsC
14635_s_at/17128_s_at	gb|AAC69381.1| (AC005398) pathogenesis-related PR-1
12879_s_at/17544_s_at*****	gb|AAC49282.1| (U40856) AIG1
16365_at*****	gb|AAC04495.1| (AC003974) putative disease resistance protein
12364_at	gb|AAA32756.1| (M58464) beta-1,3-glucanase
13212_s_at/16578_s_at	emb|CAB68132.1| (AL137080) beta-1, 3-glucanase 2 (BG2)
14249_i_at/14250_r_at*****	emb|CAB43438.1| (AL050300) PAD4
14111_s_at	emb|CAB36854.1| (AL035528) putative disease resistance protein
	Stress (6%)
13165_at	emb|CAA16552.1| (AL021635) HSP associated protein like
15190_s_at	dbj|BAA04049.1| (D16628) epoxide hydrolase
	Metabolism (24%)
17008_at*****	gb|AAD23027.1|AC006585_22 (AC006585) putative tyrosine aminotransferase
20446_s_at	gb|AAC80600.1| (AC005106) anthocyanin 5-0-glucosyltransferase homologue
17097_s_at	gb|AAC49697.1| (U66345) calreticulin
13187_i_at/13188_r_at	gb|AAC49356.1| (U35829) thioredoxin h
17109_s_at	gb|AAC31962.1| (AF082157) beta-glucosidase
13177_at	emb|CAB40989.1| (AL049640) growth factor like protein
19974_s_at	emb|CAA72363.1| (Y11650) cyclic phosphodiesterase
16463_at	emb|CAA52751.1| (X74733) elongation factor-1 beta A1
	Ribosomal (3%)
17910_at	gb|AAC73029.1| (AC005824) 60S acidic ribosomal protein P2
	Unknown (31%)
14096_at	No hits found
13718_at	No description
14145_at	No description
14146_at	No description
14201_at	No description
14217_at	No description
14704_s_at/15847_g_at	gb|AAD15461.1| (AC006067) unknown protein
20017_at*****	gb|AAC16079.1| (AC004521) unknown protein
12521_at	gb|AAC14413.1| (AF049236) unknown
18224_s_at*****	emb|CAA17150.1| (AL021890) putative protein

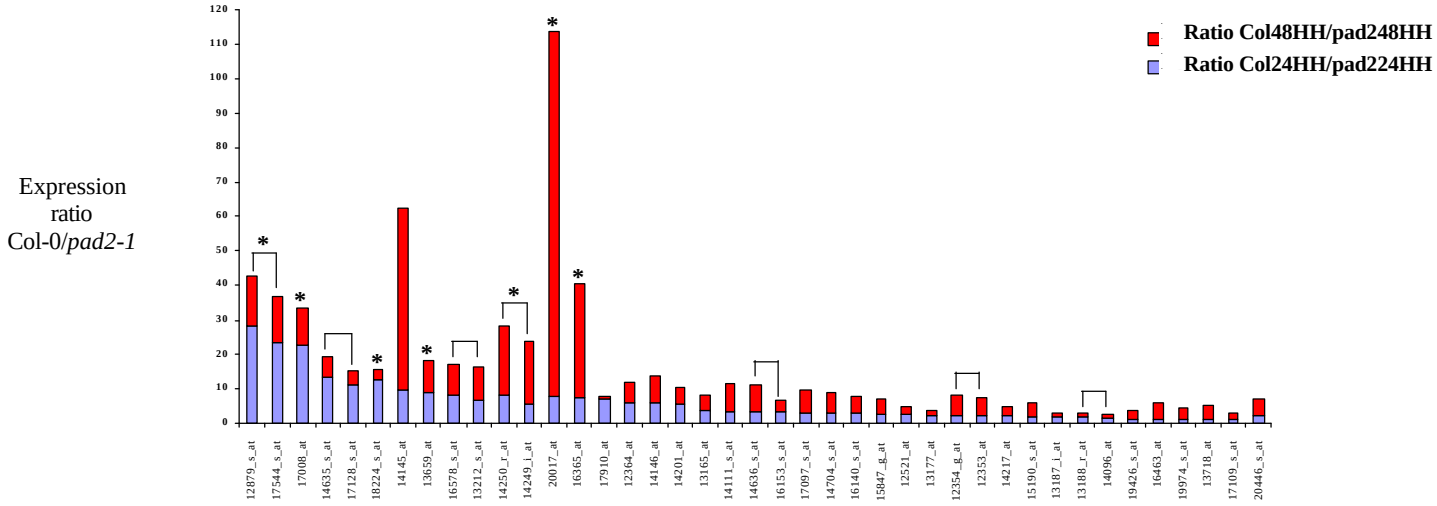


Figure 13. Expression ratios Col-0/pad2-1 of the genes upregulated in the incompatible interaction.

Expression ratios at 24h (Col24HH/pad224HH) and at 48h (Col48HH/pad248HH) were calculated. The diagram represents ratios of the 41 genes contained in cluster c1 (see figure 11). Some genes are represented as duplicates on the Affymetrix chip and were joined by a line. Only genes showing highest fold inductions were selected for further northern blot analysis (represented with an asterisk). The gene with the probe set ID 14145_at was not yet described at the time of our study.

IV.2.5 Gene expression analysis of the selected genes using mutant lines and chemically treated plants

From our previous study, we concluded that resistance against *P. porri* is SA- and jasmonate/ethylene-independent and it is dependent of an unknown defense pathway controlled by PAD2 (Roetschi et al., 2001). By microarray analysis we aimed to find marker genes for this new pathway which are not induced by SA, methyl jasmonate and ethylene accumulation. We therefore analyzed the expression of the seven selected genes (Figure 13 and table III), upregulated in Columbia but not in *pad2-1*, in plants treated with SA, methyl jasmonate and ethylene. For convenience, the genes with a putative or unknown function will be, hereafter, named with their probe set ID. The majority of these genes are induced by SA treatments at different levels except *18224_s_at* but none of them are induced by methyl jasmonate or ethylene (figure 14). As control we have used the marker genes *PR-1* for salicylic acid treatment and *PDF1.2* for both methyl jamonate and ethylene treatments. Indeed, exogenous application of SA strongly induced *PR-1* and both jasmonate and ethylene treatment induced PDF1.2 (figure 14). In order to characterize more precisely the role of salicylic acid in the expression of the selected genes, several mutants impaired in SA accumulation were inoculated with *P. porri*. Two days after inoculation the total RNA was extracted, separated on an agarose gel containing formaldehyde, blotted and the membrane hybridized with radiolabelled cDNAs of the selected genes. The SA-deficient lines used are the non-expresser of PR genes 1 *npr1-1* (Cao et al., 1997), the salicylic acid-induction deficient *sid2* (Nawrath and Métraux, 1999), the phytoalexin deficients *pad2-1* and *pad4-1* (Glazebrook et al., 1997), the non-race-specific disease resistance *ndr1-1* (Century et al., 1995) and the transgenic *nahG* (Gaffney et al., 1993). All of them are in Col-0 background.

The enhanced disease susceptibilty *eds1-1* SA mutant (Parker et al., 1996) is in Wassilewskija (WS) background thus WS plants were also used as wild type control. All these mutants are impaired in *PR-1* mRNA accumulation except *ndr1-1* mutant which expresses *PR-1* at a low level compared to wild-type controls (figure 14). Despite the fact that *ndr1-1* is impaired in SA accumulation, it was recently shown that this mutant is able to express *PR-1* (Shapiro and Zhang, 2001). All the mutants as well as the wild-type plants express strongly *PDF1.2* indicating that *P. porri* effectively induced defense mechanisms in all the inoculated plants. However, the strong expression of *PDF1.2* in *pad2-1* and *eds1-1* is not correlated with the observed susceptible phenotypes confirming our previous conclusion that neither the jasmonic acid nor the ethylene resistance pathway are involved in resistance toward *Phytophthora* (Roetschi et al., 2001). All the selected genes are strongly expressed in inoculated Col-0 plants. In contrast, these genes are not or barely induced in inoculated *pad2-1* plants. In *nahG*, only *18224_s_at* and *AIG1* are expressed. *18224_s_at* is not induced by SA treatment. Together this indicate that the induction of *18224_s_at* by *P. porri* is not SA-dependent. However, the expression of 18224_s_at is strongly down regulated in *pad2-1* at 24 hours post-inoculation but not at 48 hours post-inoculation. The effect of *pad2* on the expression of *18224_s_at* appears to be time dependent (Figure 13). The expression of *AIG1* appears to be more complex since it is induced weakly in *nahG* and it is induced by SA treatment. One explanation for this is that *AIG1* is induced in separate pathways by both SA and an unknown factor. This gene is strongly expressed in *npr1-1* indicating a NPR1-independent induction of *AIG1*. Interestingly, Rate et al. (1999) reported a similar expression pattern of *AIG1* in the "activated cell death 6" mutant (*acd6*) upon infection with *Pseudomonas syringae* carrying the avirulence gene *avrpt2*. Moreover, all the selected genes are induced in *npr1-1* to some extent. *20017_at* and *16365_at* are the most weakly induced in *npr1-1*. Despite the fact that *sid2* does not accumulate SA (Nawrath and Métraux, 1999), all the selected genes are induced in this mutant upon infection with *P. porri* except the putative tyrosine aminotransferase *17008_at*. All the selected genes induced by SA treatment are expressed in *ndr1-1* but not in *eds1-1*. It was previously shown that a mutation in *NDR1* and *EDS1* block gene-for-gene resistance that is mediated by some resistance genes specific *to Peronospora parasitica* or *Pseudomonas syringae* (Aarts et al., 1998). The mutant *eds1-1* is susceptible to *P. porri* and it is tempting to speculate that EDS1 is required for resistance mediated by resistance genes to *Phytophthora porri.*

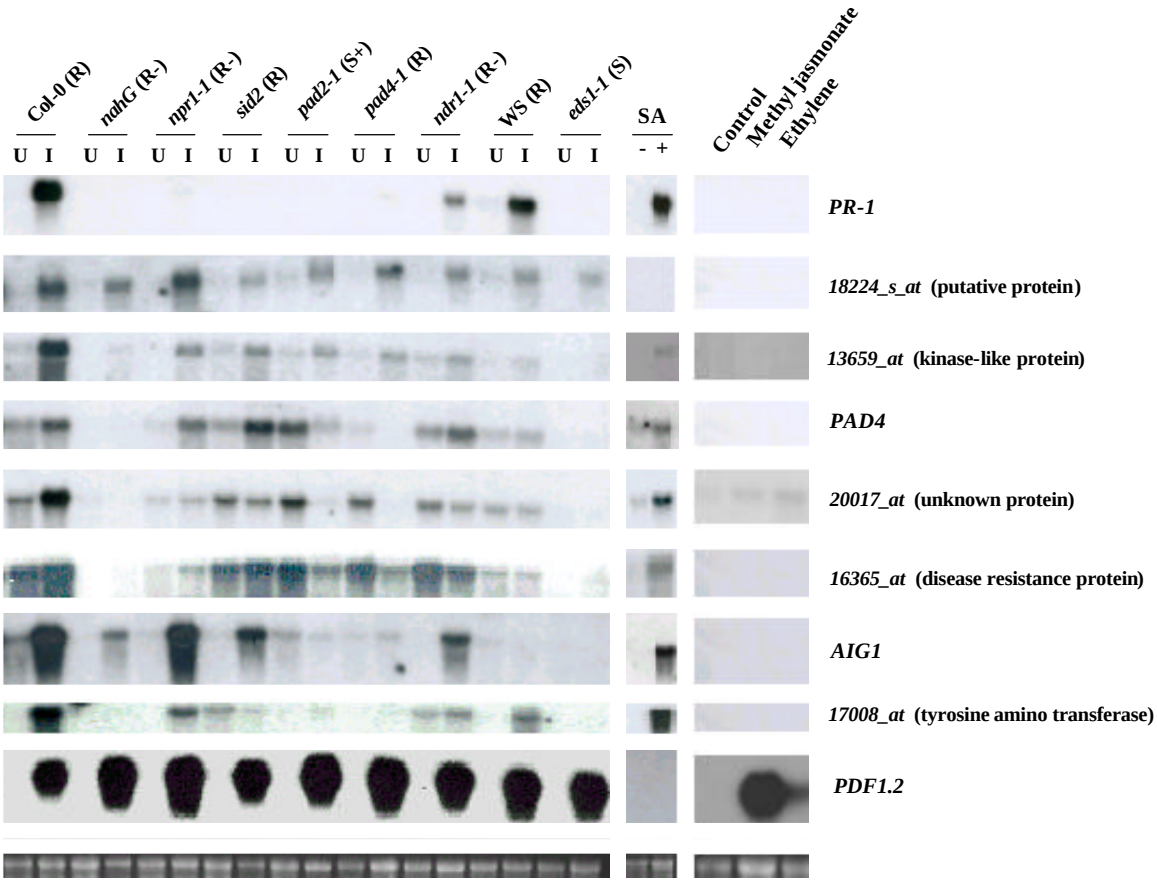


Figure 14. Northern blot analysis of the selected genes.

Northern blots were performed using 8 µg of total RNA extracted from plants 48 hours after infection. Selected genes were used as probes. Ribosomal RNA stained with ethidium bromide show an example of the equal loading. Northern analysis were performed twice with the same results. The phenotype of each mutant is indicated between gaps in the top of the figure with the following legend R: resistant, R-: resistant shifted toward susceptibility, S: susceptible and S+: highly susceptible.

IV.3. DISCUSSION

One advantage of the Arabidopsis-*Phytophthora* pathosystem is the diversity of symptoms that different hosts can show towards the same isolate (HH in our study). The wild-type accession Columbia is completely resistant, the accession Landsberg erecta is susceptible and the mutant *pad2-1* (Col-0 background) is hyper susceptible. These three interactions were subjected to microarray analysis. About 2/3 of the 8734 genes on the array were "present" and most of them are expressed at a hybridization intensity of about 100. This value was also reported for different organisms (Lockhart et al., 2000; Zhu et al, 2001). The resistant interaction during the infection process showed the overall highest hybridization intensities and therefore the most active transcriptome. Using the Stanford Cluster and Treeview software (Eisen et al., 1998) all the genes which did not show at least one value ≥ 300 in any of the time point were eliminated. 1522 genes changed significantly through the experiment if the 3 different interactions were compared. However, this analysis comparing Col-0, Ler and *pad2-1* was complicated by the fact that the transcription profiles are compared in different ecotypes. Because the Genechip® uses gene specific oligos designed for the Col-0 genome

the expression profile of Ler is potentially influenced by not perfectly matching gene sequences. We have previously shown that resistance toward *P. porri* is independent from the salicylic, jasmonate and ethylene signalling but is dependent on a new pathway controlled by PAD2 (Roetschi et al., 2001). By comparing transcription profiles of the resistant plants Col-0 and the mutant *pad2-1* we aimed to identify genes which could serve as markers for this new pathway. Those genes should be upregulated upon infection with *P. porri* in Col-0 but not *pad2-1*. In order to identify these marker genes, we used the GeneCluster 1.0 software which gives a better overview of the different clusters than the Stanford Cluster software does. 510 genes showed at least one hybridization intensity ≥ 300 in the time points. After clustering, 41 genes upregulated in the incompatible interaction and weakly or not in the hypersusceptible mutant *pad2-1* were selected. Among them seven genes were selected for further analysis. Most of these seven genes have a putative function.

The putative protein 18224_s_at contains a domain associated with the peptide methionine sulfoxide reductase (PMSR) enzymatic domain pfam01625. Recently, Sadanandom et al. (2000) showed that Arabidopsis PMSRs are induced by pathogens and are probably involved in the protection of key proteins from oxidative damage caused by ROS.

The gene corresponding to the probe set ID 13659_at encodes a serine/threonine kinase. This gene is a member of a large cluster on chromosome IV consisting of 21 receptor-like kinases arranged in a tandem array. All the SA-responsive RLK genes as well as the 13659_at have a TTGAC sequence in the upstream region of the coding region (Ohtake et al., 2000). This sequence has been suggested to be important for induced expression of many plant defense genes (Eulgem et al., 1999; Eulgem et al., 2000). It was found, using microarray-based profiling of Arabidopsis transcriptome during systemic acquired resistance SAR, that this sequence is the common promoter element in genes of the "*PR-1* regulon" which binds plant-specific transcription factor WRKY (Maleck et al., 2000). Nevertheless, WRKY DNA-binding sites are not unique to RLKs and disease resistance genes (Du and Chen, 2000). The function of RLKs is not clear yet. They could be involved in race-specific disease resistance like the rice disease resistance gene *Xa21* (Song et al., 1995) or in the SA dependent hypersensitive cell death (Richberg, 1998).

The gene with the probe set number 20017_at encodes a lipid transfer-like protein. The exact role of LTP proteins in defense mechanisms is not yet clear (Kader, 1996). However, antimicrobial and antifungitoxic effects of LTPs have been demonstrated (Salzman et al., 1998; Segura et al., 1993) and a direct antifungitoxic effect of 20017_at on *Phytophthora* can not be excluded.

17008_at corresponds to a putative tyrosine amino transferase (TAT). TAT converts tyrosine to 4-hydroxyphenylpyruvate (OHPP) which is then converted to homogentisate by the OHPP-dioxygenase. Homogentisic acid is the precursor of both α-tocopherol and the reduced form of plastoquinones which are known as scavengers for reactive oxygen species (ROS).
Chlorophyll breakdown and thus ROS are formed after pathogen attack and have to be neutralized by prenylquinones (Lopukhina et al., 2001). Another proposed role of TAT could be the production of phenolic compounds which might have a role in building physical barriers in order to stop pathogens. Therefore a high demand of TAT, the first enzyme in the pathway, may explain its increased expression.

AIG1 is a putative GTP-binding protein expressed upon AvrRpt2-RPS2 gene products interaction probably participating in the control of programmed cell death process (Poirier et al., 1999). Indeed, expression of *AIG1* is correlated with cell death occuring in Arabidopsis upon infection with *P. syringae* carrying *Avrpt2* and controlled by *ACD6* (Rate et al., 1999).
Finally, the seventh selected gene is *PAD4* which encodes a lipase-like protein that is required for SA accumulation (Jirage et al., 1999).

Specific probes of these genes were designed and a northern blot analysis was performed to analyze their expression after treatment with SA, methyl jasmonate and ethylene. None of

these genes are induced by methyl jasmonate or ethylene. All the genes are induced by SA, except the putative protein with a probe set ID *18224_at*. This gene is also expressed in all the SA mutants studied in figure 14. The experiment with SA mutants and the transgenic *nahG* revealed that all the selected genes, except *18224_s_at*, are not expressed in the *eds1-1*mutant. Since the induction of all these genes is SA-dependent, their expression is completely blocked in the *eds1-1* mutant. This is also confirmed by the fact that all the genes are not expressed in the SA degradating transgenic *nahG*. *nahG* and presumably e*ds1-1* have a strong SA minus phenotype than the other mutants and a minimal SA level is required to induce the selected genes. All the selected genes, except *17088_at*, are induced in *sid2*. It was recently shown that *SID2* encodes an isochorismate synthase and that *sid2* contains constitutive low level of SA (Wildermuth et al., 2001). This low level of SA present in the *sid2* mutant is probably sufficient for the expression of the selected genes, except *17088_at*. All the genes are expressed in the *npr1-1* indicating that their expression is SA-dependent but NPR1-independent. Our goal in this study was to find marker genes for the new pathway controlled by PAD2 which are not regulated by SA. However, except *18224_s_at*, all the genes we have selected are inducible by SA and the northern analysis using mutant lines impaired in SA accumulation confirmed this. The only gene which is not inducible by SA is the putative protein with a probe set ID 18224_at but it is induced in all the mutants and its pattern of expression does not correlate with the observed phenotypes. It is, therefore, not a useful marker gene for this new resistance pathway. It is not surprising that the microarray analysis highlighted genes which are SA-dependent. Indeed, since *pad2-1* is defective in SA accumulation it is logical that by clustering transcriptomes of Col-0 and *pad2-1*, all the SA-dependent genes will be highlighted in Col-0. The expression of these genes could be probably brought to the expression level of the wild-type Col-0 by spraying SA on *pad2-1* mutant plants before microarray analysis. In this way only the SA-independent genes regulated by PAD2 will be identified. Nevertheless, all these genes are upregulated during the resistant interaction and are not or barely induced in the *pad2-1* mutant and in the *eds1-1* mutant which are both susceptible. Therefore, their role in resistance toward *Phytophthora* can not be excluded. A knock-out of those genes could help to better understand their functions and their involvement in resistance toward *Phytophthora*. A reverse genetic approache using insertion lines whose genomic regions flanking the T-DNA tag have been rescued and sequenced could be coupled to the microarray analysis. In fact, precise location of the insertion within the gene of interest can be determined using the Notthingham stock center (http://nasc.nott.ac.uk/blast.html) or the Torrey Mesa Research Institute (http://www.tmri.org/pages/collaborations/garlic_files/GarlicAnalysis.html) searchable databases for sequenced tagged lines so that the phenotype can be determined very rapidly. At the time of our study we could not find a flanking sequence matching an ORF or a 3´UTR of any of the seven selected genes. We have in this study focused our efforts only on genes highly upregulated in the resistant interaction. It is possible that the marker genes for the new PAD2 pathway are not so highly expressed and a less stringent analysis than we have performed could reveal them. It is also possible that these marker genes have very low expression values and therefore have been excluded from the analysis after filtering the data. Finally, the Affymetrix GeneChip® used in this study covers only ~ 30% of the Arabidopsis genome and the marker genes that we aimed to find in this study could be located in the other 70% of the genome.

IV.4. MATERIAL AND METHODS

IV.4.1 Plant material

Arabidopsis plants were grown as previously described (Roetschi et al, 2001). Wild-type ecotype seeds (Columbia, Col-0 and Landsberg erecta, Ler) were purchased from Lehle Seeds (Round Rock, TX). The *nahG* seeds were obtained from J. Ryals (Novartis, Research Triangle Park, NC). The mutant *pad2-1* was supplied by J. Glazebrook (Torrey Mesa Research Institute, CA) and *pad4-1* obtained from Nottingham Arabidopsis Stock Center (NASC, UK). *npr1-1* seeds were provided by X. Dong (Duke University, Durham, NY) and *sid2* mutant by C. Nawrath (University of Fribourg, Switzerland). *eds1-1* and the corresponding wild-type control Wassilewskija (WS-0) were kindly provided by J. Parker (Sainsbury laboratory, Norwich, UK) and *ndr1-1* seeds were supplied by B.J Staskawicz (University of California, Berkeley, CA).

IV.4.2 Plant treatments and infections

For both microarray experiment and northern blot analysis, 5 weeks old Arabidopsis plants were infected using plugs of V8 agar (0.3 cm diameter) containing young growing mycelium (5 days) of *P. porri* (isolate HH, CBS782.97). The infection conditions were as previously described (Roetschi et al., 2001). Chemical treatments were also done on 4 weeks old plants. For salicylic acid (SA) treatment, a solution of 1mM SA containing 0.02 % (v/v) Silwet L-77 was sprayed on leaves. The corresponding control was sprayed with water containing 0.02 % Silwet L-77. For gazeous treatments with methyl jasmonate (MeJa) and ethylene, the plants were kept in a tightly closed atmosphere containing 100 ppm ethylene or 2 ppm MeJa. The corresponding control contained pure atmosphere.

IV.4.3 RNA isolation for microarray hybridization and data processing

Leaf discs of 1.4 cm diameter around the infection site, were cut at the indicated time points and pulverised in liquid nitrogen. Total RNA was isolated using RNAwiz isolation reagent (Ambion) and further cleaned using RNeasy columns (Qiagen) until a purity $OD_{260/280} > 2.00$. For each time point equal amount of RNA from three independent experiments was mixed. cDNA synthesis, cRNA synthesis, array hybridization and data normalization were processed as described by Zhu et al. (2001). GeneChip Suite 3.1 algorithm (Affymetrix) was used for data normalization.

IV.4.4 Microarray data analysis

Average signal differences between perfect match (PM) and mismatch probes (MM) were taken as relative indicators of the level of expression of the transcripts (Lipshutz et al., 1999). Tab-delimited text files were created using Microsoft Excel. Table rows represented genes and columns represented time-points. Different comparisons of time-points were performed in this study using different algorithms for cluster analysis. All rows that do not have at least 1 observation with absolute value greater than 300 were removed. The filtered data were adjusted by $\log_2$ transformation, mean centered and normalized. Average hierarchical linkage clustering was performed using Stanford Cluster software (Eisen et al., 1998). The resulting tree was visualised using the Stanford Treeview software (Eisen et al., 1998). The data were also subjected to the self-organizing map (SOM) algorithm GeneCluster 1.0 (Tamayo et al., 1999). Genes that did not change significantly across the experiments were excluded using the filter option of Genecluster 1.0. Genes which did not show a relative change of 2 and an

absolute change of 300 were eliminated. Expression levels were normalized within the time points for each experiment (mean=0 and variance=1). Candidate genes were then selected by visualising the clusters.

IV.4.5 Northern blot analysis

Total RNA from control plants, *P. porri* infected leaves and from treated leaves was isolated, separated on an agarose gel containing formaldehyde, transferred and hybridized as previously described (Roetschi et al., 2001). Different probes were used in this study: *PR-1* (Genbank accession N° M90508, Uknes et al., 1992), *PDF1.2* (Genbank accession N° T04323, Pennincks et al., 1999), *HSP83* (accession N° M62984, Conner et al., 1990) and the probes hybridizing specifically to the selected genes listed hereafter:

Probe set ID	Description	Size of the PCR product	Primer name[a]	Primer sequence 5´-3´
12879_s_at and 17544_s_at	*AIG1*	704bp	F-12879 R-12879	TATTGGAAGATGACGGTATGACATT CAAAAGCTGACTCATAACAGGC
14249_i_at and 14250_r_at	*PAD4*	551 bp	F-PAD4 R-PAD4	ACTCGACATTGCGAATTTCTACA AGAATATATAGTAACATTCATCAGAAAGTCTTGC
16365_at	putative disease resistance protein	729 bp	F-16365 R-16365	GTGAACTGGAAAGCATCATCACG CTAACGCTTTCTGCGTTTATTCA
18224_s_at	putative protein	426 bp	F-18224 R-18224	ATGACCGCGGCAGCA TTATTGGGAGGAACCAGCAGA
13659_at	serine/threonine kinase-like protein	536 bp	F-13659 R-13659	AGCAGAACACAAGCAGAATAGTTGG TCAACGAGGATCTAAATCAGACATT
17008_at	putative tyrosine aminotransferase	674 bp	F-17008 R-17008	GCTCCGGTGATCACGCT GCTATTTTTCTTGATTGTGTTCCTG
20017_at	unknown protein	615 bp	F-20017 R-20017	GAGTCACGGAAGATTAAAGTAATGGC CTAAACGAGGATGAAAATATAAAAAATTACG

a: F= forward, sense primer and R= reverse, antisense primer.

Specific primers were designed on the 3´ region of the genes using their available ORF sequences on http://www.tmri.org/gene_exp_web/index.html . For some genes the antisense primer was designed on the 3´ UTR according to Arabidopsis published genomic sequences (http://www.ncbi.nlm.nih.gov). Probes synthesis was done by RT-PCR. A cDNA synthesis was performed on the total RNA isolated from Col-0 after 48h of infection with *P. porri*. This RNA was isolated using RNeasy columns (Qiagen). The reverse transcription was performed using Omniscript reverse transcription kit (Qiagen) according to the manufacturer protocol. cDNAs were further amplified using a Taq Polymerase (Sigma) and the PCR products cloned directly into TOPO PCR 2.1 vector (Invitrogen). Cloned fragments were confirmed by sequencing (Microsynth, Basel).

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CHAPTER V

Determination of the genome size of *Phytophthora porri* by flow cytometry using *Arabidopsis thaliana* as internal standard.

ABSTRACT

Phytophthora porri was recently shown to be a true pathogen of Arabidopsis rending this oomycete attractive for molecular genetic and gene-knockout studies. It is homothallic thus the transformed strain is self-fertile and the recovery of the progeny that are homozygous at the mutated locus is possible. The knowledge of the genome size of *Phytophthora porri* and its ploidy is therefore important. We have estimated the genome size of *P. porri* by flow cytometry and genomic reconstruction to be 110± 10 Mbp. Using flow cytometry analysis, *P. porri* was found to be diploid. To test the reliability of the method, we have estimated by flow cytometry the genome sizes of *P. sojae* and *P. infestans* to be respectively 86±4 Mbp and 250±19 Mbp which is in agreement with previously published genome sizes. Flow cytometry is a fast method for genome size estimations and ploidy determination using Arabidopsis as internal standard and propidium iodide as a fluorescent DNA stain. The use of isolated nuclei provides a high resolution and accurate estimations.

V.1 INTRODUCTION

Despite the economic importance of many *Phytophthora* species it is only in the last decade that molecular genetic studies of *Phytophthora* were improved. This is partially due to the difficulty in applying the sophisticated experimental procedures which were developed to the ascomycetes and basidomycetes. Nowadays, after considerable efforts, modern procedures such as routine DNA transformation (Judelson et al., 1991 and 1996; Kamoun et al., 1998; van West et al., 1998), genetic mapping (van der Lee, 1997 and Whisson et al., 1995) and gene silencing (van West et al., 1998) are available to *Phytophthora* researchers. All these technical developments promise to bring *Phytophthora* research to an exciting research level. Furthermore, cDNA libraries constructed from RNA extracted from mycelium, cysts, germinating cysts or zoospores of *P. infestans*, *P. sojae* and *P. porri* are being sequenced (http://www.ncgr.org/pgi and Kamoun et al., 1999). The available gene transfer technology coupled with a large amount of DNA sequence data is a prelude for a large scale gene knockout project. Silencing (van West et al., 1999), homologous recombination and transposon mutagenesis have been proposed as methods for targeted gene knock-out in *Phytophthora* spp (Kamoun, 2000). *Phytophthora* spp. are diploid at the vegetative stage. Using homothallic species such as *P. sojae* or *P. porri* instead of the heterothallic *P. infestans* facilitate the selfing of the transformed strain and the recovery of the progeny that are homozygous at the mutated locus. Indeed, we have recently developped a new pathosystem *Arabidopsis thaliana - Phytophthora porri* (Roetschi et al., 2001) in which both partners are amenable to genetic analyses and are both transformable (chapter IV). In order to develop such knockout facilities it is crucial to know not only the genome size of *P. porri* but also its ploidy. Different methods were used to estimate genome size of *Phytophthora* species. Reassociation kinetic associated to genomic reconstruction (Francis et al, 1990) were succesfully applied to estimate the genome size of *Phytophthora sojae* (syn. *Phytophthora megasperma* f. sp. *glycinea* Kuan and Erwin) and revealed a genome size of 62 Mbp (1C = 0,063 pg; Mao and Tyler, 1991). Contour-clamped homogeneous electric field (CHEF) electrophoresis was also used to estimate the genome size of *P. sojae* (Tooley and Carras, 1992) but the authors reported a lower genome size of 46,5 Mbp. This was explained by the fact that several chromosomes are concentrated in a single band or were larger than the marker chromosomes, rending the interpretation of CHEF gels erroneous. In addition, *P. infestans* chromosomes failed to separate despite a more extreme ramping. Using Feulgen cytophotometry several isolates of *P. infestans* (Mont.) de Bary genome sizes were estimated (Therrien et al., 1989, 1993). In average its genome size is 250 Mbp (2C = 0,52 pg, Tooley and Therrien, 1987). In fact, Feulgen staining coupled with absorbance photometry or image analysis was the most applied method for genome size determination of plant pathogenic fungi such as *Verticillium* spp. (Typas and Heale, 1980), *Tilletia indica* (Therrien et al., 1988) or several Peronosporales (Volgmayr and Greilhuber, 1998). All these methods have their advantages but have in common to be time-consuming and labor intensive. A fast method allowing high resolution of genome size is flow cytometry. Despite its intensive use in plant breeding, systematic and taxonomy (Brown and Bergounioux, 1989; Benett and Leitch, 1995) and despite the attempts of Volgmayr and Greilhuber (1998) to use this technique for genome size determination in Peronosporales, it was rarely applied to estimation of genome size of plant pathogenic fungi (Eilam et al., 1994). Flow cytometry has two main disadvantages: the substantial cost of flow cytometers and because it is a sophisticated instrument, a skilled operator is required to obtain an optimum performance. However, because of the continuing demand of these instruments in clinical research and diagnostic, the price is slowly decreasing and most modern instruments are designed to maximize their user friendliness. The choice of the stain is important for high resolutions and Propidium iodide (3,8-diamino-5-

diethylmethylamino-propyl-6-phenylphenanthridium diiodide) is the most recommended stain for absolute genome size estimation. Another important parameter for accurate estimates is the internal standard to be used. Several reference standards can be chosen like *Glycine max* which has a DNA content of 1C = 1,134 pg (Greilhuber and Obermayer, 1997) , *Hordeum vulgare* cv. Sultan (2C = 11,12 pg), *Vicia faba* (2C = 26,66 pg) or *Allium cepa* cv. Ailsa Craig (2C = 33,55 pg) recommended by Johnston et al. (1999) or chicken erythrocyte nuclei (2,41 pg) used as a standard by Tooley and Therrien (1987).

Here, we report, the use of flow cytometry for the determination of the genome size of *Phytophthora porri* using *Arabidopsis thaliana* as an internal standard and propidium iodide stained nuclei. As a reference control we have also estimated the genome size of *Phytophthora infestans* and *Phytophthora sojae* using the same experimental conditions. Genomic reconstruction method (Carr and Shearer, 1998) was used as an independent method to verify the genome size obtained by flow cytometry.

V.2. RESULTS

Flow cytometry is an excellent technique for the estimation of genome size. This, however, depends to a great extent on the quality of the sample and the selection of a proper internal standard (Price and Johnston, 1996). The internal standard should not overlap but should also not be too different from *Phytophthora* to avoid linearity problems. Moreover, it should be readily available and its genome size should be known, as precise as possible. *Arabidopsis thaliana* was therefore chosen as internal standard because of its known genome size of 125 Mbp confirmed recently by sequencing (The Arabidopsis initiative, 2000).

V.2.1 Arabidopsis standard curve determination

The DNA content of Arabidopsis can be determined by flow cytometry using protoplasts or isolated nuclei (Galbraith et al., 1997). Protoplasts can be prepared from plant tissue by enzymatic digestion of cell wall. In our preliminary assays, the resolution obtained using Arabidopsis protoplasts revealed important variations in the flow cytometry histograms. High resolution flow cytometry histograms have been obtained in most plant species using suspension of nuclei (Ulrich and Ulrich, 1991; Doležel and Göhde, 1995). Galbraith et al. (1983) reported that suspensions of intact nuclei may be prepared by chopping a small amount of plant tissue with a razor blade, and that these nuclei suspensions are suitable for DNA content analysis. This fast and easy protocol was combined to buffers commercially available (Partec CyStain PI absolute P). Those buffers were optimized to preserve the integrity of nuclei, to protect DNA from degradation and to provide optimal conditions for propidium iodide DNA staining. Propidium iodide quantitatively intercalates into double stranded nucleic acids thus, digestion of the double stranded RNA by DNase free-RNase A is required. The time of staining is also an important parameter and we obtained important variations if the samples were not stained for, at least, eight hours. Microscopic observations of PI stained nuclei, prior to analysis, revealed the uniformity of the staining and a very low background. Samples were then analyzed by flow-cytometry. The channel number represents the intensity of fluorescense which is directly proportional to the amount of DNA. Convenient representation of these various peaks was obtained by plotting the data on a logarithmic scale (figure 1, A). The peaks of fluorescence emission are narrow and well defined and show a regular pattern. When displayed on a linear scale the channel numbers corresponding to those peaks are respectively 15, 30, 56 and 112 corresponding to 2C, 4C, 8C and 16C nuclei. These values correspond to an arithmetic progression (base two) similar to the one reported by Galbraith et al. (1991) showing endopolyploidy in Arabidopsis vegetative tissues (Samoylova

et al., 1996). The Arabidopsis nuclei population sorted in this analysis was extracted from three weeks old plants and the distribution of the multiploidy obtained is in accordance with the distribution obtained by Galbraith et al. (1991). Indeed, these authors reported that the 2C peak is smaller than the 4C peak in older leaves which is exactly the case in our study. The fluorescence was plotted against the DNA content in pg calculated for each ploidy. Assuming that 1 pg = 0,965 10^9 bp and assuming that *Arabidopsis thaliana* has a genome size of 125 Mbp then Arabidopsis 2C DNA content = 0,26 pg, the 4C DNA content the double and so forth. The curve showed a linear progression (figure 1, B).

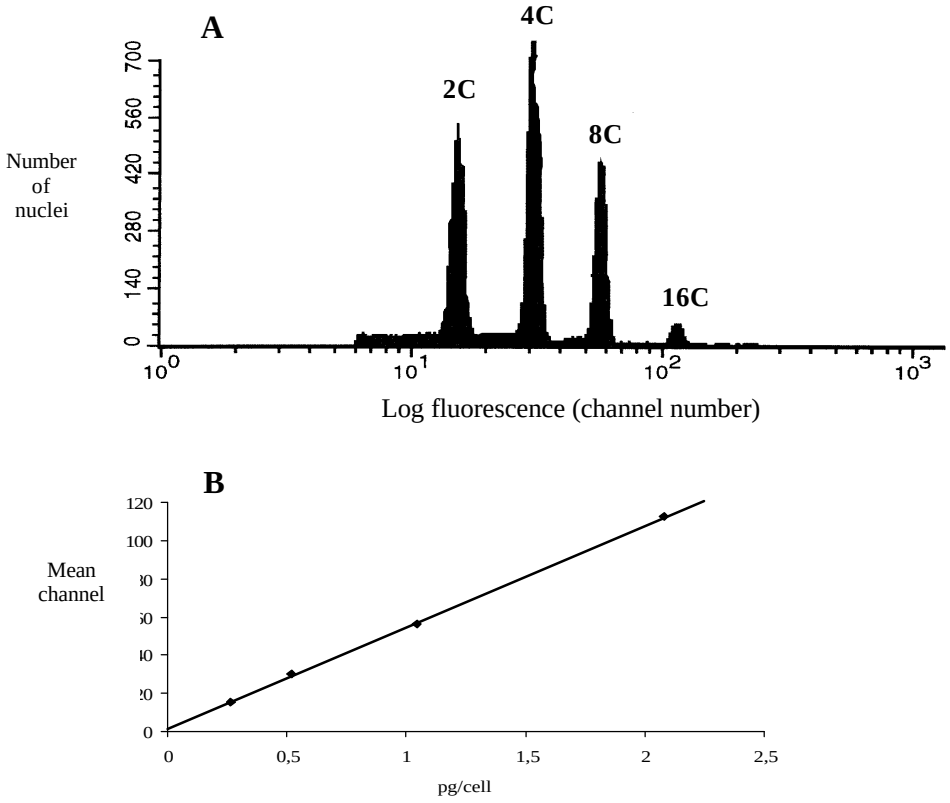

Figure 1. Flow cytometry histogram of nuclei from Arabidopsis.

In panel A, four, narrow, well defined peaks were obtained by sorting Arabidopsis nuclei suspension. The mean channel fluorescences of the 2C, 4C, 8C and 16C peaks were plotted against the DNA content in pg (panel B). These DNA contents were calculated on the basis of a haploid genome of 125 Mbp.

V.2.2 Determination of the genome size of *Phytophthora* spp. using Arabidopsis as internal standard

To estimate the genome size of *Phytophthora* species we used internal and external standardization approaches (Doležel , 1997). We compared the G_0-G_1 peak position of each *Phytophthora* spp. with the G_0-G_1 peak of the internal standard Arabidopsis in a single histogram or in individual histograms obtained under identical conditions. In order to eliminate variability in the preparation of samples, the same nuclei isolation procedure was applied to both Arabidopsis and *Phytopththora* species. *P. porri* HH, *P. sojae* P6497 or *P. infestans* 88069. Mycelia and Arabidopsis leaves were chopped in the same petri dish (internal standardization) or separately (external standardization). Three independent nuclei suspensions of each *Phytophthora* species were then sorted three times and the peak corresponding to the 2C DNA content was identified as the major peak. Three independent nuclei suspensions of each mix combination (*Phytophthora* spp. + Arabidopsis) were also sorted three times. All the analysis were performed within one hour without changing any parameter of the flow cytometer. Peaks of each *Phytophthora* species were first located in the external standardization histogram and then located in the internal standardization histogram and compared to the Arabidopsis peaks 2C, 4C, 8C and 16C. The G_0-G_1 peak with the lowest value corresponds to *P. sojae* with a mean fluorescence of 10,4±0,78 followed by *P. porri* G_0-G_1 peak with a channel mean of 15±1,8. *P. infestans* G_0-G_1 peaks appeared just before the 4C peak of Arabidopsis and has a mean fluorescence of 30±3,29 (Figure2). This indicate clearly that DNA contents of *P. sojae* and *P. porri* are smaller than the one of *P. infestans* and that *P. infestans* has a bigger DNA content than Arabidopsis. Absolute genome sizes were calculated according to the equation: sample 2C DNA content (pg DNA) = sample G_1 peak mean/standard G_1 peak mean x standard 2C DNA content (Galbraith et al., 1997). As expected *P. sojae* and *P. porri* have the smallest 2C DNA content of 0,18±0,01 pg and 0,25±0,03 pg respectively (Table I). The 2C DNA content of *P. infestans* is 0,51±0,05 pg and is in agreement with the average value of 0,52 pg reported by Tooley and Therrien (1987). To convert the DNA content in base pairs we multiplied the 1C DNA content values of each *Phytophthora* spp. by 0,965 x 10^9 bp (Galbraith et al., 1997). The calculated genome size of *P. sojae* is 86,8 ± 4,8 Mbp followed by *P. porri* with 120,6 ± 14,5 Mbp and then *P. infestans* with 250,9 ± 19,3 Mbp (Table I and figure 3).

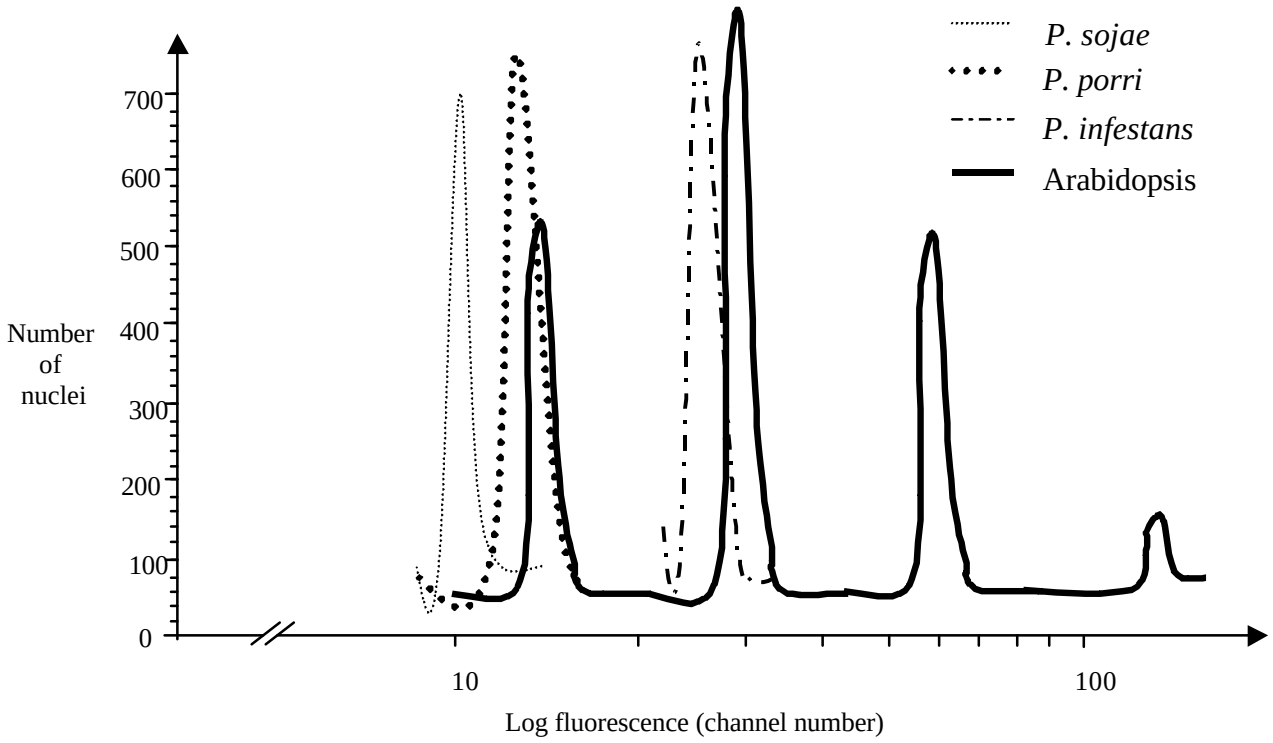

Figure 2. Flow cytometry analyses of *P. sojae, P. porri* and *P. infestans* using Arabidopsis as internal standard.

A mix of nuclei of *P. sojae, P. porri* or *P. infestans* and Arabidopsis were stained with propidium iodide and analyzed by flow cytometry. Each *Phytophthora* spp. G_0-G_1 peak corresponds to 2C DNA content (diploid). They were compared to the G_0-G_1 diploid peak of Arabidopsis (2C). Arabidopsis has a particular pattern of endopolyploidy as shown by the 2C, 4C, 8C and 16C well defined peaks. Convenient representation of these various peaks was obtained by plotting the data on a logarithmic scale.

Table I. DNA contents and genome sizes of *P. sojae, P.porri* and *P. infestans* using Arabidopsis as internal standard.

Samples	**Channel mean of the G_0-G_1 peak**	**DNA content[a] (2C) in pg**	**DNA content (1C) in pg**	**Haploid genome size[b] (Mbp)**
Arabidopsis 2C	15.2 ± 1.01	0.26	0.13	125
P. sojae P6497	10.4 ± 0.78	0.18 ± 0.01	0.09 ± 0.05	86,8 ± 4,8
P. porri HH	15 ± 1.80	0.25 ± 0.03	0.125 ± 0.015	120,6 ± 14,5
P. infestans 88069	30 ± 3.29	0.51 ± 0.05	0.255 ± 0.025	250,9 ± 19,3

a: Channel mean of the G_0-G_1 of each *Phytophthora* species was used to calculate the 2C DNA content according to the equation: sample 2C DNA content (pg DNA) = sample G_1 peak mean/standard G_1 peak mean x standard 2C DNA content
b: DNA content values (1C) are expressed in Million of base pairs (Mbp) using the following formula: 1pg DNA=0,965 x 10^9 bp.

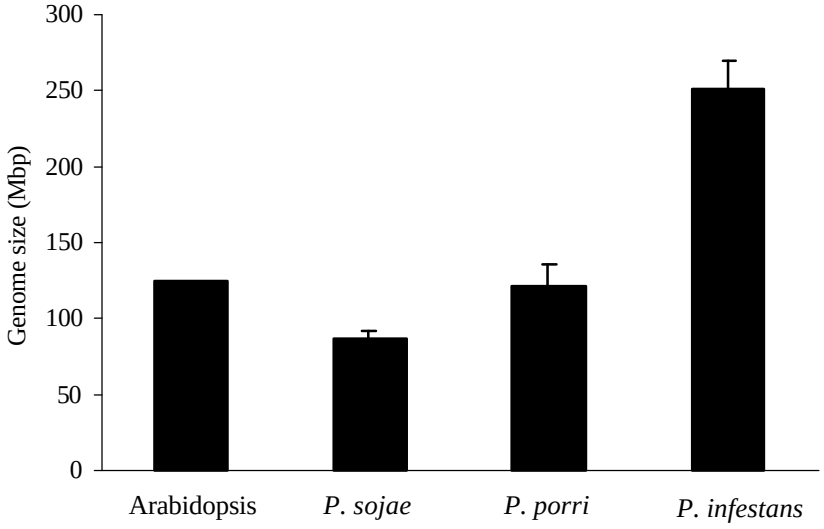

Figure 3. Genome sizes of *P. sojae, P.porri* and *P. infestans.*

Using flow cytometry the genome sizes of *Phytophthora* spp. have been estimated to be: *P. sojae* P6497, 86,8 ± 4,8 Mbp; *P. porri* HH, 120,6 ± 14,5 Mbp and *P. infestans* 88069, 250,9 ± 19,3 Mbp. The internal standard Arabidopsis has a genome size of 125 Mbp.

V.2.3 Genomic reconstruction

As an independent method for genome size estimation, genomic reconstruction experiment with a single-copy gene was performed. Genomic DNA from *P .porri* and cloned DNA from *P. porri* single-copy gene mucin (intronless) were carefully quantified and then applied to a nylon membrane. The membrane was hybridized with the radiolabeled mucin cDNA and the band intensities were revealed using a phosphorimager (Biorad). The intensities of the cloned DNA were plotted against the amount (in pg) of mucin (Figure 4, B). The resulting standard curve was then used to quantify the amount of mucin in a known concentration of genomic DNA. The slope was calculated and is equal to 719,5. The intensity of the band corresponding to 750 ng genomic DNA has an intensity of 2770 a.u. and is identical to the band corresponding to 3,75 pg of cloned mucin which has an intensity of 2657 (Figure 4, A). Thus, the actual genome size of *P. porri* was then estimated from this point at which the genomic to cloned DNA hybridization ratio was 1.0 (2770/2657= 1,04). Indeed, if the intensity of the 750ng band (2770 a.u.) is divided by the slope (719,5) the result is 3,85 pg. With these data the amount of genomic DNA required to yield one copy of mucin (per haploid genome) can be easily calculated. Thus 750ng of genomic DNA was found to contain 3,75 pg of the 500 bp mucin probe. The calculated haploid genome size is then 100 Mbp (0,00375ng/750ng = 500bp/haploid genome size). Since the haploid genome estimated by genomic reconstruction is lower than the one estimated by flow cytometry, we performed additionnal DNA measurements. The result of those additional replications confirmed that genome size of *P. porri* estimated by genomic reconstruction is 100±2.5 Mbp.

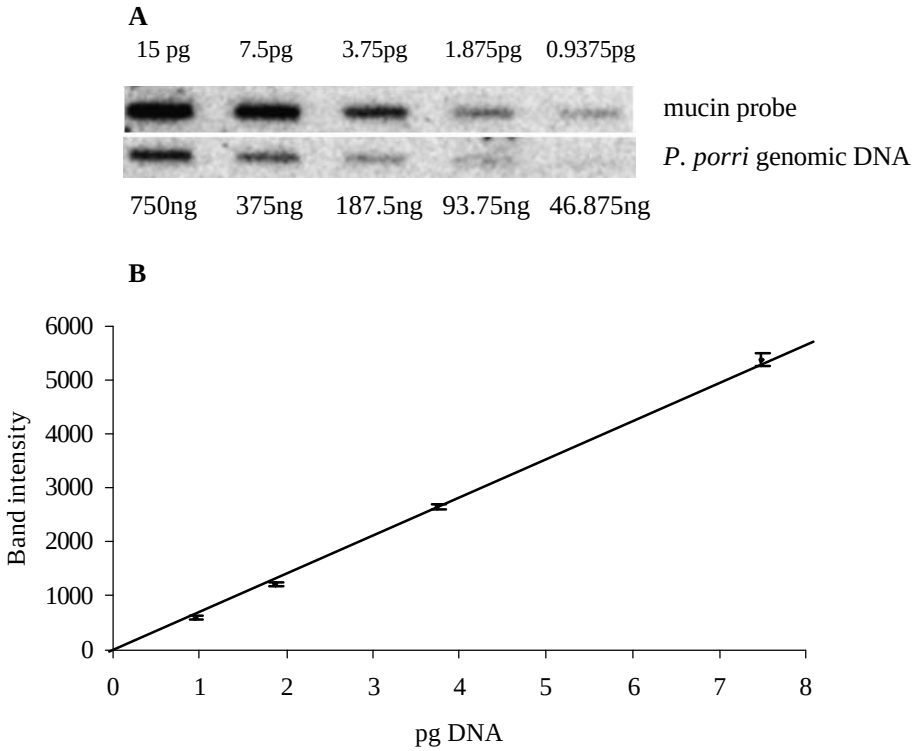

Figure 4. Genomic reconstruction using a *P. porri* mucin probe from *P. porri*.

Panel A shows a representative autoradioph of *P. porri* DNA and mucin DNA. The lane on the top shows twofold serial dilutions of *P. porri* mucin probe starting with 15 pg and the lane below shows twofold serial dilutions of *P. porri* genomic DNA starting with 750 ng (panel A). The nylon blot was probed with radiolabeled *P. porri* mucin probe. The hybridization signal was quantified with the help of a phosphorimager. Background intensity was substracted from the band intensities and the intensities were plotted against the amount of mucin (panel B). The standard curve is linear and the slope used to quantitate the amount of mucin in known concentration of genomic DNA. Each bar shows the standard deviation of triplicate measurements.

V.2.4 Ploidy

It is generally recognized that *Phytophthora* spp. have gametangial meiosis and a diploid somatic phase (Brasier and Sansome, 1975). However, Tooley and Therrien (1987) described diploid, triploid, tertraploid and aneuploid isolates of *P. infestans.* For further genetic analysis using *P. porri* it is crucial to know not only the genome size but also the ploidy. To determine the ploidy of *P. porri* flow cytometry is the method of choice. Generally, DNA triploidy, tetraploidy or aneuploidy is defined as a single G_0-G_1 peak corresponding to a mean channel 1.5 , 2 or *n* times (DNA index) bigger than the value of the G_0-G_1 peak corresponding to the diploid stage. In the histograms corresponding to the external standardization experiments no distinct peak appeared after the 2C peak for all the *Phytophthora* spp. In the *P. porri* histogram a discrete peak with a Gaussian distribution representing about 8% of the total

population appeared (Figure 5). According to the criteria reported by Steck and El-Naggar (1998) this peak should be more considered as G_2/M rather than a tetraploid G_0-G_1 peak. Thus, *P. sojae* isolate P6497, *P. infestans* isolate 88069 and *P. porri* isolate HH are diploid.

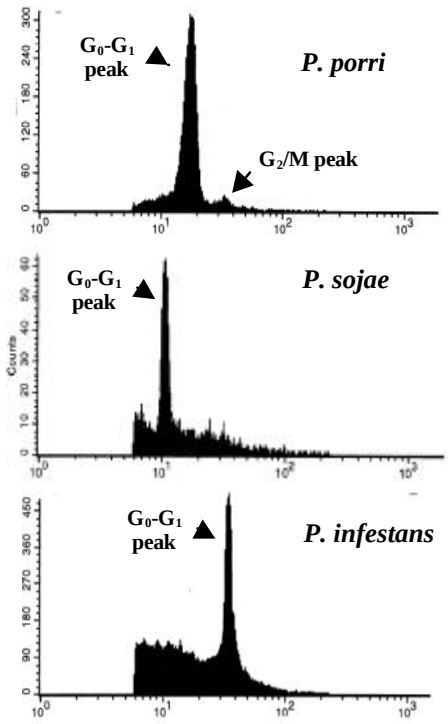

Figure 5. Flow cytometry histograms of *P. porri*, *P. sojae* and *P. infestans.*

Nuclei were isolated from mycelium, stained with propidium iodide and analyzed by flow cytometry. The only apparent and clear peak is the G_0-G_1 peak corresponding to the 2C DNA content. A peak with Gaussian distribution in *P. porri* histogram is rather a G2/M peak than a tetraploid peak. All the isolates tested in this study are diploid.

V.3. DISCUSSION

Different methods are available to estimate the *Phytophthora* genome size in absolute units (pg or bp): reassociation kinetics, CHEF gel electrophoresis, genomic reconstruction and flow cytometry. So far, only *P. infestans* and *P. sojae* genome sizes were determined. Using Feulgen staining, an absolute average genome size of 2C = 0,52 pg (or an haploid genome of 250 Mbp) was reported for *P. infestans* by Tooley and Therrien (1987). By using reassociation kinetic and genomic reconstruction an haploid genome size of 62 Mbp (2C = 0,064 pg) was reported for *P. sojae* (Mao and Tyler, 1991). However, flow cytometry is faster, easy and allows high resolution analysis. We report on a genome size determination of *Phytophthora* by flow cytometry. To determine a genome size in absolute units an internal standard should be added and processed simultaneously. In our preliminary assays we have determined that *P. porri* is between *P. sojae* or *P. infestans* in term of genome size. We

have therefore chosen *Arabidopsis thaliana* as an internal standard because it has a genome size (125 Mbp) bigger than *P. sojae* but smaller than *P. infestans*. Moreover, it is readily available and taking advantage of its endopolyploidy (Galbraith et al., 1991) a standard curve can be easily drawn. Indeed, the 2C, 4C, 8C and 16C peaks showed an arithmetic progression and the progression is linear (Figure 1, B). Using Arabidopsis as an internal standard we have determined the genome size of *P. porri* by flow cytometry to be 120,6 ± 14,5 Mbp. To confirm the reliability of the data obtained with *P. porri* we have also determined the genome size of *P. sojae* and *P. infestans* using the same experimental conditions. For *P. sojae* we have determined a genome size of 86,8 ± 4,8 Mbp which is 1,4 times higher than the genome size of 62 Mbp reported by Mao and Tyler (1991). Interestingly, Volgmayr and Greilhuber (1998) using Feulgen image analysis also reported genome sizes for *P. sojae* between 86,03 ± 5,9 Mbp and 97,57 ± 3,56 Mbp. Our result is in good agreement with their estimation particularly if we consider that the same *P. sojae* isolate P6497 was used. Mao and Tyler (1991) used the reassociation kinetics method to estimate a genome size of *P. sojae* and in their calculation they considered a GC content of 48 %. However, recently the GC content of *P. sojae* P6497 was reported to be 58% (Qutob et al., 2000). Since the complexity of the genome increase with higher GC content we suppose that the genome size calculated by Mao and Tyler (1991) using reassociation kinetics was probably underestimated. Our estimation of *P. infestans* genome size by flow cytometry is 250,9 ± 19,3 Mbp which is exactly the average genome size reported by Tooley and Therrien (1987) confirming that there is a good correlation between flow cytometry and Feulgen cytophotometry as reported by Michaelson et al. (1991). More important, since our estimation of the genome sizes of *P. sojae* and *P. infestans* by flow cytometry, using Arabidopsis as internal standard, is in agreement with published values using other independent methods, we assume that the genome size of *P. porri* that we estimated using the same conditions is correct. Our flow cytometry measurement data seem to be of good quality since the maximal coefficient of variation in our study is 10,99. However, critical parameters for accurate estimation are a long PI staining (at least eight hours) and the use of commercially certified buffers like the Partec Cystain PI absolute P used in the present study. Indeed, when other buffers were used (Galbraith et al., 1997) we obtained important variations. By microscopy analysis we concluded that those buffers did not preserve the integrity of the nuclei and showed a strong background fluorescence.

As an independent method we used genomic reconstruction and we estimated the genome size of *P. porri* to be 100 ± 2.5 Mbp. This is 1,2 time less than the estimation by flow cytometry but considering the standard deviations, this value is, more or less, in agreement with the flow cytometry estimation. Our data and data reported by Carr and Shearer (1998), indicate that genomic reconstruction can give genome size estimations close to the flow cytometry. Using genomic reconstruction as an independent method for genome size estimations in parallel with flow cytometry is a good choice. Moreover, because they are fast and reproducible these two methods, combined together, give accurate estimations of *Phytophthora* genome sizes. We estimate the *P. porri* genome size, based on average by flow cytometry (120,6 Mbp) and genomic reconstruction (100 Mbp), to be 110,3 ± 10,3 Mbp. With such relatively small genome size, *P. porri* could be a good representative of *Phytopththora* spp. for genetic and molecular genetic studies such as mutant analysis, the molecular cloning of avirulence or pathogenicity genes and the construction of a detailed physical map of its genome.

Our study shows that estimation of *Phytophthora* spp. genome sizes and ploidy by flow cytometry using Arabidopsis as an internal standard is fast and accurate. This method could be used in the future for rapid characterization of genomes and ploidy of other *Phytophthora* species like the well studied *P. palmivora* and *P. parasitica.*

V.4. MATERIAL AND METHODS

V.4.1 Growth of Arabidopsis and *Phytophthora*

Arabidopsis seeds accession Columbia (Col-0) were purchased from Lehle Seeds (Round Rock, TX). Plants were grown on sterile soil mix of humus/perlite (3:1) under 12 hours light and 12 hours dark. *Phytophthora porri* isolate HH (CBS782.97), *Phytophthora sojae* isolate P6497 and *Phytophthora infestans* isolate 88069 were provided by F. Govers (University of Wageningen, Netherlands). *P. porri* was grown routinely on (v/v) V8 20% agar plates at 18°C in the dark. *P. sojae* was maintained on clarified (v/v) V8 20% agar plates (Erwin and Ribeiro, 1996) at room temperature. *P. infestans* was routinely grown on rye sucrose agar (Caten and Jinks, 1967) at 18°C in the dark. For liquide cultures, young growing mycelium was scratched from the agar cultures and resuspended in sterile Erlen Meyers containing 20 ml of (v/v) V8 10% for *P. porri* and *P. sojae* and 20 ml of ALBA medium (Bruck et al., 1980) for *P. infestans*.

V.4.2 Nuclear extraction and flow cytometry

The commercially available kit "Cystain PI absolute P" (Partec, Münster, Germany) was used to extract nuclei from both *Phytophthora* and Arabidopsis. Nuclei were extracted from leaf tissues of 3 weeks old Arabidopsis plants and from 10 days old *Phytophthora* mycelium growing in liquid medium. For external standardization, approximately 100 mg of Arabidopsis leaves or 100 mg of *Phytophthora* mycelium were put in a 55 mm plastic dish and 400 µl of extraction buffer was added. The material was chopped using a sharp razor blade for 30 to 60 seconds at, approximately, 5 chops/sec to homogenize the tissue and to release the nuclei. For internal standardization Arabidopsis leaves and *Phytophthora* mycelium were chopped simultaneously in the same petri dish. The samples were then filtered through a sterile 50 µm mesh and the staining solution containing 50 µg/ml propidium iodide and 200 µg/ml DNAse free-RNase A was added. The samples were kept at least eight hours at 4°C before processing flow cytometry. The process was monitored through epifluorescence microscopy and PI stained nuclei were observed via excitation at 510 nm to 560 nm. The nuclei suspension was vortexed briefly to reduce clumping and analyzed using a Becton-Dickinson LASER FACScan Cell Analyzer according to the manufacturer´s recommandation. Acquisition and analysis were performed using the Cell Quest software.

V.4.3 Genomic DNA extraction

To ensure a correct quantification in the genomic reconstruction experiment, genomic DNA has to be of a high purity. *P. porri* genomic DNA was extracted using Qiagen genomic columns with improved buffers for filamentous fungi genomic extraction (Qiagen Basel, Switzerland). Zoospores of *P. porri* were released as previously described (Roetschi et al., 2001) and added to a solution of (v/v) V8 10% to allow germination. Approximately 100 mg of mycelium was pulverised in liquid nitrogen and resuspended in lysis buffer containing: 20 mM EDTA, 10 mM Tris (pH7.9), 1 mg/ml lysing enzymes (Fluka), 1% Triton X-100, 500 mM Guanidine-HCl and 200 mM NaCl and incubated 1 hour at 37°C. After digestion with DNAse-free RNase A (20 µg/ml) and proteinase K (0.8 mg/ml) the debris were pelleted 20 minutes at 12.000 x g. The clarified lysate was then transferred to the Qiagen genomic tip and the following steps were performed according to the manufacturer´s recommandations.

V.4.4 Genomic reconstruction

Genomic reconstruction analysis was done according to the protocols of Carr and Shearer (1998) and Francis et al. (1990). DNA concentration was determined by quantifying the fluorescence intensity of the ethidium bromide stained DNA using image analysis and a plasmid of certified known concentration (Invitrogen) as a standard. Serial twofold dilutions of *P. porri* genomic DNA were prepared to yield 750 to 46,875 ng in 200 µl of TE buffer. The same procedure was performed with the single-copy gene mucin. The mucin was found by screening randomly an EST bank of *P. porri* for single copy genes and by PCR cloning the full length cDNA from genomic DNA into a TOPO 2.1 vector (Invitrogen). The mucin gene is intronless and has a size of 500 bp. The mucin was cutted from the plasmid with EcoRI, purified using Qiagen columns and was prepared to yield 15 to 0,47 pg in 200 µl of TE buffer. In both cases, salmon sperm DNA was added as a carrier to constantly obtain 800 ng of total DNA per 200 µl aliquot. The final samples were prepared as previously described (Carr and Shearer, 1998) and vacuum filtered on a nylon membrane (Hybond-N, Amersham Pharmacia biotech, Little Chalfont, UK) using a slot blot apparatus (Biorad). The membrane was probed with ^{32}P-labeled mucin and the bands revealed using a phosphorimager (Biorad). The hybridization intensities were quantified using the "Multinanalyst" software (Biorad).

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CHAPTER VI

General conclusion

Economic damage to crops caused by *Phytophthora* species are estimated to billion of dollars annually (Duncan, 1999). At present, there are only few measures available to control *Phytophthora*. These include host resistance breeding, chemical control and both biological and cultural control measures (Erwin and Ribeiro, 1996). In many instances these control measures are no longer effective. It was for a long time thought that vertical resistance or race-specific resistance would successfully control *Phytophthora* species. However, new races of *Phytophthora*, especially within *P. infestans*, rapidly overcome the single gene resistance. Furthermore, these new races of *P. infestans* have become resistant to metalaxyl, a phenylamide widely sprayed to control various downy mildews, *Phytophthora* and *Pythium* species (Gisi and Cohen, 1996; Judelson, 1997). Antagonistic fungi, sanitation, and crop rotation can be effective in preventing spread of diseases caused by *Phytophthora* but are not efficient if the soil already contains sexual oospores which are viable for several years and serve as an inoculum source (Drenth et al., 1995). Different new transgenic potato cultivars showed high resistance toward *P. infestans* and it was believed that their wide application could replace the chemical control (Tadege et al.,1998; Abad et al., 1997). However, because of the resistance of the public to genetically modified plants, especially in Europe, those projects were not followed up by commercial applications. Nowadays, the chemical control is the only widely used method to control *Phytophthora* species but this is not the best solution for the future. What are the alternatives ? We believe that exploring more deeply the *Phytophthora*-plant interaction at the molecular level will lead to the development of new control strategies. In this sense, we have developed a new pathosystem *Arabidopsis thaliana*-*Phytophthora porri*. The genome of Arabidopsis thaliana is completely sequenced (The Arabidopsis initiative, 2000) and by using this plant as a model, the defense pathways leading to resistance toward *Phytophthora* can be deciphered. Basic knowledge of the molecular mechanisms of this resistance is essential to come up with new control strategies.

The Arabidopsis-*Phytophthora* pathosystem: a new resistance pathway controlled by PAD2 is required for Arabidopsis defense against *Phytophthora*

Phytophthora porri was originally described as a pathogen for leek. Using seven isolates of *P. porri* which were known to be infective on *Brassicaceae*, a screening was performed on wild-type Arabidopsis accessions in order to identify susceptible and resistant hosts. Columbia was found to be resistant and Landsberg erecta was susceptible. Two defense pathways are studied intensively in Arabidopsis: the salicylic acid (SA) pathway and the jasmonate/ethylene pathway. Several mutants impaired in these pathways are available. By a combined microscopic and northern analysis we have concluded that neither the salicylic acid pathway nor the jasmonate/ethylene pathway are involved in the defense against *Phytophthora*. Moreover, we have found that the *pad2-1* mutant defective in both camalexin and SA, is hypersusceptible. Another mutant deficient in camalexin, *pad3-1*, is completely resistant indicating that this phytoalexin is not important for resistance (Chapter II). A new pathway independent of SA, jasmonate, ethylene, and camalexin and controlled by PAD2 is involved in the resistance toward *Phytophthora*. These observations open future prospects. In fact, several strategies can be adopted to characterize more precisely this new pathway and genes interacting with it. It is clear that by cloning *PAD2*, this new pathway can be unraveled. However, until this gene is cloned other methods can be used to find marker genes for this new pathway. Genomic tools have been developed for Arabidopsis research and methods for profiling the transcriptome during certain conditions are available. We used the oligonucleotide-based array to find marker genes for the SA-independent resistance pathway mentionned above.

The microarray analysis did not reveal marker genes for the PAD2 pathway

The first generation of oligonucleotide-based array covers about 30% of the Arabidopsis genome, for instance 8300 unique genes. Some genes are in replicate and the array contains in total 8734 genes. Using this technology we aimed to find putative markers of the PAD2 pathway. The induction of those genes should be SA-independent. By profiling the transcriptome of both resistant Columbia and the hypersusceptible *pad2-1* we aimed to find those markers. We restricted the analysis to genes which are highly upregulated in the incompatible interaction. Using the GeneCluster 1.0 software, seven genes highly expressed in Columbia but not in *pad2-1*, were highlighted (Chapter IV). Their expression analysis after SA treatment revealed that they are all SA-inducible except a putative protein. However, this putative protein is expressed in all the mutants used in northern analysis and its expression is not correlated with the phenotypes of the studied mutants. We could not find a marker gene which is not induced by SA. What are the reasons ? The mutant *pad2-1* is defective in SA accumulation. We suppose that all the genes which are normally induced by SA in the wild-type Columbia are not expressed in *pad2-1*. Therefore, by comparing Col-0 and *pad2-1* using microarray, they were highlighted in the resistant plant. Nevertheless, they could be involved in the resistance. It would be interesting to characterize more precisely their function by overexpressing them in *pad2-1* or by silencing them in Col-0. The subsequent phenotype will indicate if their role is important during resistance to *Phytophthora*. A second microarray analysis could be performed using *pad2-1* plants pretreated with SA. In this way, all the SA-inducible genes will be expressed in both wild-type and *pad2-1* plants, thus the analysis could be restricted only to genes which are affected by the *pad2* mutation. Another alternative solution is to find a chemical compound which could induce resistance in *pad2-1*. It is likely that such chemical could also induce resistance genes involved in the PAD2 pathway. We have successfully induced resistance in Arabidopsis using the ß-aminobutyric acid (BABA). The inducing activity of this compound was reported to be not dependent on salicylic acid, jasmonate, ethylene and camalexin (Zimmerli et al., 2000). BABA could be used as chemical inducer of PAD2 pathway for future microarray analysis.

BABA induces resistance toward *Phytophthora* in both Arabidopsis and potato

It was previously shown that BABA induces resistance toward *P. infestans* in both potato and tomato (Cohen, 2000; Cohen et al., 1994). We were interested to see if BABA could also induces resistance in Arabidopsis toward *P. porri*. In order, to quantify the BABA induced resistance in Arabidopsis and potato plants, we have transformed both *P. infestans* and *P. porri* with the green fluorescent protein (GFP). Both *Phytophthora* species constitutively expressed the GFP. In this way, we could quantify the GFP emitted *in planta* by the transgenic *Phytophthora*. The intensity of the GFP is proportionnal to the fungal biomass. This method allowed an easy scoring of the infection. We also used the SA analog benzothiadiazole (BTH) to confirm that SA is not required for resistance against *Phytophthora* (Chapter III). We obtained 100% protection in BABA treated Arabidopsis plants and 97% protection in BABA treated potato. No significant protection was obtained with BTH confirming that resistance against *Phytophthora* is clearly SA-independent. As mentionned above BABA can be used as an inducer for this new pathway controlling resistance to late blight. Our data is not sufficient to say if this pathway is similar in Arabidopsis and in potato. In the future, the Arabidopsis putative genes found to be BABA-inducible, SA-independent and not expressed in *pad2-1* could be putative markers for this new resistance pathway. Homologies to potato genes can be searched and the involvement of the putative paralog genes in resistance toward late blight can be studied in potato.

Is *P. porri* a model for studying *Phytophthora* species ?

One advantage of the Arabidopsis-*Phytophthora* pathosystem is that both partners can be studied separately in parallel. It is important to develop molecular techniques to study *Phytophthora porri*. Different strategies have been employed to clone genes that are important during pathogenesis. Differential hybridization procedures have been used to identify *in planta* expressed genes of *P. infestans* (Pieterse et al., 1994). Positional cloning is currently being used to identify avirulence genes in *P. infestans* (van der Lee et al., 1997). Insertional mutagenesis is another method which can be used to isolate genes involved in pathogenesis or in avirulence. It was applied successfully to knock-out genes in plant pathogenic ascomycetes and basidiomycetes (Kronstad et al., 1989; Stahl and Schafer, 1992). It was only at this time that the first transformation of *Phytophthora* has been reported (Judelson et al., 1991). However, traditional mutagenesis approaches are not feasible in *P. infestans*. The majort part of *Phytophthora* spp. is diploid at the vegetative stage. It is necessary to use homothallic species such as *P. porri* instead of the heterothallic *P. infestans*. This facilitate the selfing of the transformed strain and the recovery of the progeny that are homozygous at the mutated locus. This is a solid argument for using *P. porri* as a model for other *Phytophthora* spp. especially that its genome size is relatively small. We have estimated it to be 110 ± 10 Mbp which is about 2 times smaller than the genome size of *P. infestans* (Chapter V). Furthermore, *P. porri* is transformable and sexual oospores can be produced.

Future perspectives

It is only now, 150 years after the Irish late blight epidemic, that we start to understand processes occuring during infection and colonization of plants by *Phytophthora*. Progress has been extremely slow compared to other organisms. The Arabidopsis-*Phytophthora* pathosystem offers new strategies to control *Phytophthora*. However, big efforts have to be made to reach the goal. We hope and anticipate, that future employment of the techniques developed during this thesis will help to identify both Arabidopsis resistance genes and *Phytophthora porri* pathogenicity or avirulence genes. Finally, we hope that results obtained using microarrays will help to improve the use of this technique.

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