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

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Summary

***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.

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

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

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*.

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.

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 1a,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).

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*.

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.

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 signalling 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 signalling (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-

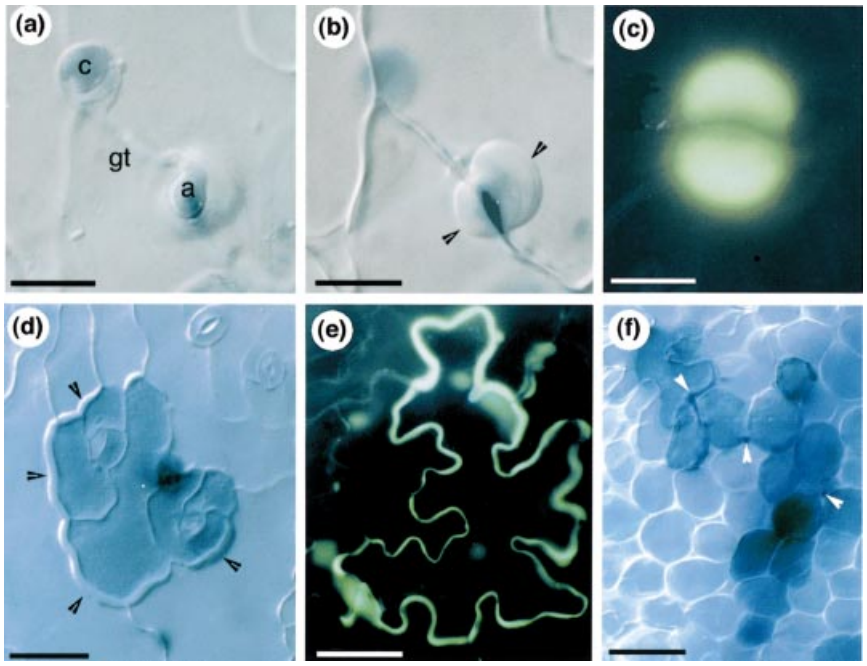

Figure 1.

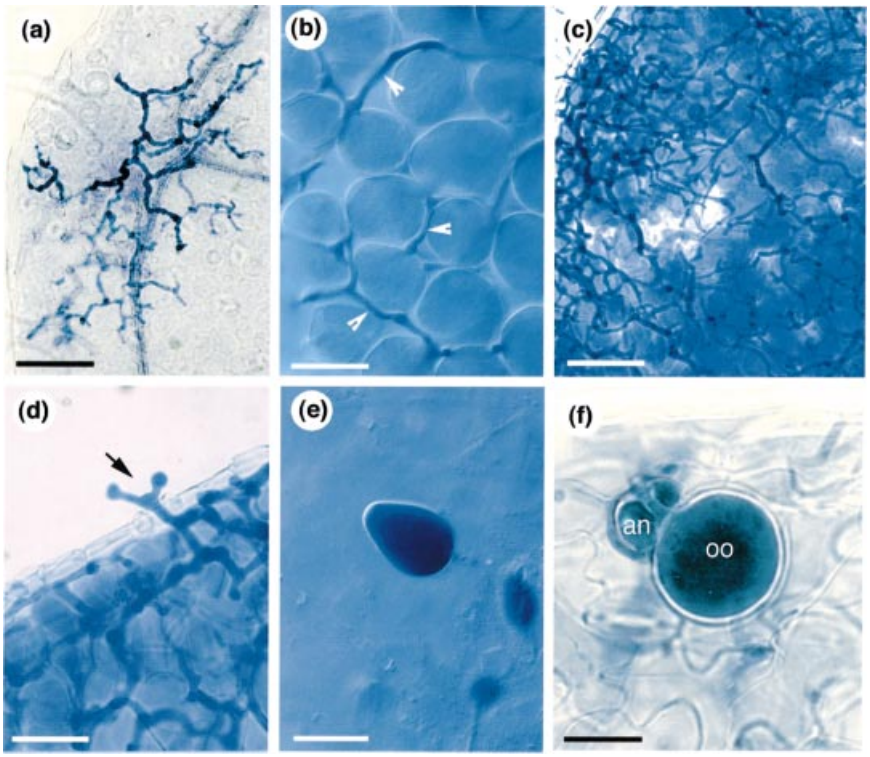

Figure 2.

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 signalling 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 signalling 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).

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

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.

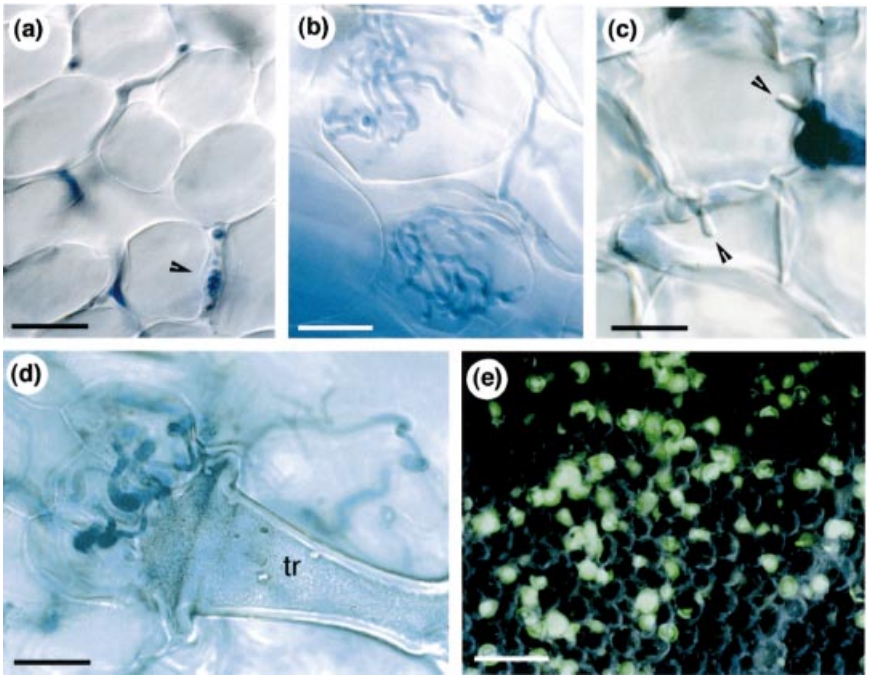

Figure 3. Cytological characterization of the interaction of *P. porri* with the hyper-susceptible *pad2-1* mutant and the jasmonate-insensitive mutant *jar1-1*. (a,b,c) Differential interference contrast (DIC), (d) bright-field, and (e) fluorescence micrographs; (a–d) show lactophenol–trypan blue-stained 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.

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 signalling 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*.

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

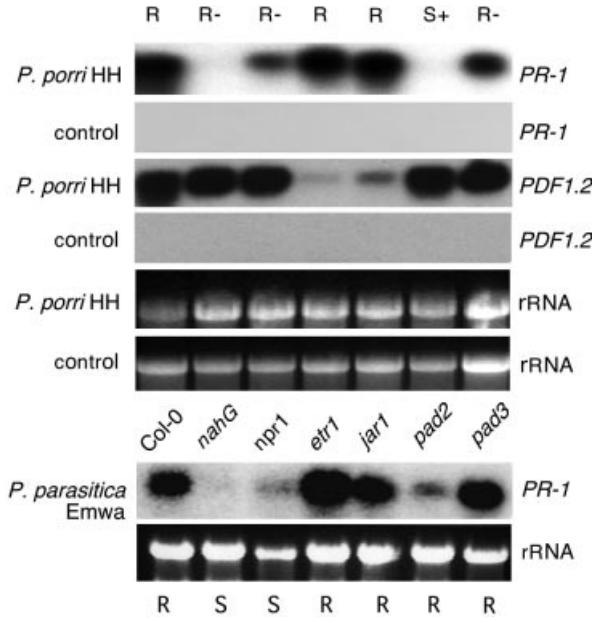

Figure 4. *PR-1* and *PDF1.2* marker gene expression in different *Arabidopsis* genotypes in response to inoculation with *P. porri*.
PR-1 and *PDF1.2* gene-specific probes were used for RNA gel blot analysis of the indicated genotypes (Col-0, *nahG*, *npr1*, *etr1*, *jar1*, *pad2*, *pad3*). Ethidium 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.

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

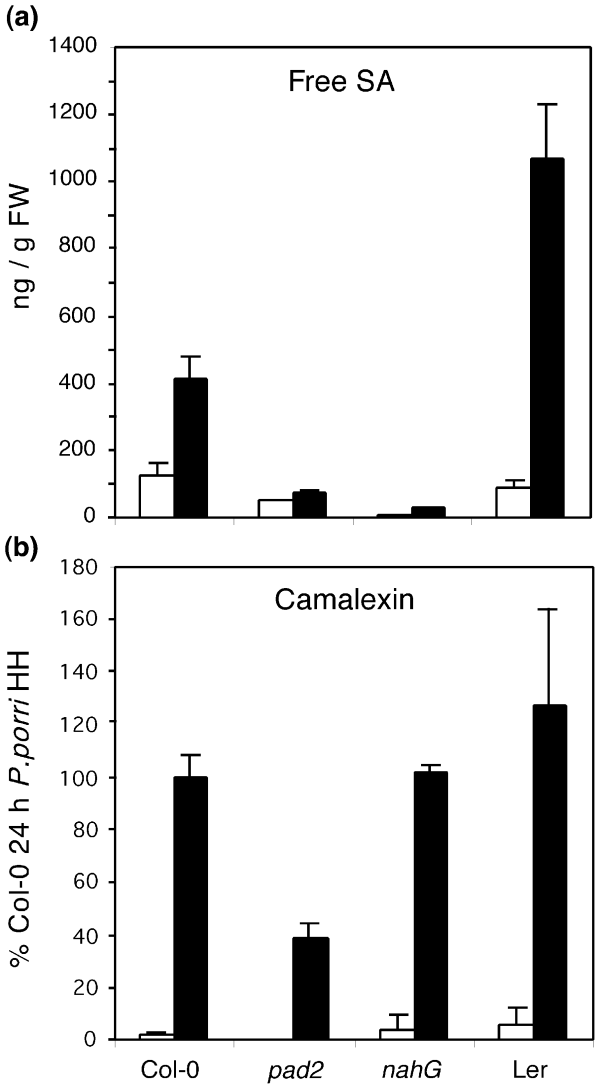

Figure 5. Accumulation of free SA and camalexin in Col-0, *pad2*, *nahG* and Ler after inoculation with *P. porri* isolate HH.
Five-week-old plants were inoculated with *P. porri* isolate HH and leaves were harvested 24 h later. The values represent the average of two independent samples ± SE. (a) Levels 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.

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.

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.

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 F_2 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 F_1 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 signalling 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 signalling 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 signalling 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 signalling 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 signalling (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 signalling 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 signalling 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 signalling 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 signalling 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 signalling (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.

Experimental procedures

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).

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 Aridopsis 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.

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.

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 10^4 conidia ml^{-1} as described previously (Mauch-Mani and Slusarenko, 1994).

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 μM in the soil. The resistance-inducing treatment was applied 1 day prior to challenge with *P. porri*.

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.

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 μg) 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).

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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