

ups1, an *Arabidopsis thaliana* camalexin accumulation mutant defective in multiple defence signalling pathways

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Summary

We report the characterization of an *Arabidopsis thaliana* mutant, *ups1*, isolated on the basis of reduced expression of phosphoribosylanthranilate transferase, a tryptophan biosynthetic enzyme. *ups1* also exhibits defects in a wide range of defence responses. After infection with *Pseudomonas syringae* or *Botrytis cinerea*, the expression of genes regulated by both the salicylic acid and jasmonic acid/ethylene pathways is reduced in *ups1* compared with wild type. Camalexin accumulation in *ups1* is greatly reduced after infection with these two pathogens, as well as after amino acid starvation or oxidative stress. Reactive oxygen species (ROS)-mediated gene expression is also compromised in *ups1* indicating that this mutant is defective in signalling pathways activated in response to both biotic and abiotic stress. The fact that all three major defence signalling pathways are disrupted in *ups1*, together with the oxidative stress phenotype, leads us to suggest that UPS1 is involved in ROS signal transduction.

Keywords: camalexin, tryptophan, salicylate, jasmonate, defence, reactive oxygen species.

Introduction

Plant defence against invading pathogens is governed by a complex web of signalling pathways, many components of which have been identified through mutational analysis (see review by Feys 2000). *Arabidopsis thaliana* is an excellent model system for such forward genetics approaches and a variety of *Arabidopsis* pathosystems have been set up (Thomma *et al.*, 2001). Three major signalling pathways have been identified, mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene. These signalling pathways result in induction of a range of defence responses aimed at restricting pathogen growth and symptoms, including accumulation of antimicrobial compounds/proteins and expression of defence-related genes [such as pathogenesis-related (*PR*) genes]. Ultimately, broad long-lasting systemic acquired resistance is induced (Hammond-Kosack and Jones, 1996). Defence responses appear to be activated rapidly during gene-for-gene resistance, mediated by recognition of pathogen avirulence proteins by specific resistance proteins in the host (Dangl and Jones, 2001). Triggering of defence responses after infection with virulent

pathogens, where such gene-for-gene recognition does not take place, appears to occur more slowly.

A common defence response is the synthesis and accumulation of phytoalexins, compounds with broad antimicrobial activity *in vitro*. The major phytoalexin in *Arabidopsis* is camalexin (3-thiazol-2'-yl-indole) (Glazebrook and Ausubel, 1994; Tsuji *et al.*, 1991), which accumulates after both biotic and abiotic stress (Zhao and Last, 1996; Zhao *et al.*, 1998). Diverse pathogens induce camalexin accumulation, including *Pseudomonas syringae* (Tsuji *et al.*, 1991; Zhao and Last, 1996), *Alternaria brassicicola* (Thomma *et al.*, 1999) and *Cochliobolus carbonum* (Glazebrook *et al.*, 1997). However, other pathogens do not appear to elicit camalexin accumulation (Brader *et al.*, 2001) and pathogens vary in their sensitivity to this toxic compound (Thomma *et al.*, 1999). The importance and role of camalexin in defence is likely to be pathogen-specific as *pad3* mutants, defective in camalexin synthesis, show increased susceptibility to infection by the fungal pathogen *A. brassicicola*, but not *Botrytis cinerea* (Thomma *et al.*, 1999). Similarly, the

growth of avirulent and virulent *P. syringae* is similar to wild type in *pad3* mutants (Glazebrook and Ausubel, 1994).

Several studies using transgenic and mutant plants have added to our understanding of the synthesis and regulation of camalexin accumulation. Camalexin accumulation is not triggered by SA, JA or ethylene application alone (Thomma *et al.*, 1999), however SA is required for accumulation of camalexin in response to pathogen infection (Zhao and Last, 1996). *pad4* mutants do not accumulate camalexin in response to *P. syringae* (Glazebrook *et al.*, 1996) and treatment with SA can partially rescue this defect (Zhou *et al.*, 1998). Camalexin accumulation appears to be regulated via both *PAD4* (and SA)-dependent and *PAD4*-independent pathways as the *pad4* mutation does not prevent high camalexin levels after infection with *C. carbonum* or avirulent *P. syringae* (Zhou *et al.*, 1998). In addition, the *eds5* and *sid2* mutations exhibit low levels of SA, yet accumulate wild-type levels of camalexin after infection with avirulent *P. syringae* (Nawrath and Metraux, 1999).

The exact pathway of camalexin biosynthesis is not known. However, this indolic phytoalexin is synthesized from tryptophan via indole-3-acetaldoxime with the thiazol ring originating from cysteine (Glawischnig *et al.*, 2004; Zook and Hammerschmidt, 1997). The cytochrome P450 encoded by the *PAD3* gene is highly likely to be an enzyme required for synthesis of this phytoalexin (Zhou *et al.*, 1999). The expression of *PAD3* appears to mirror the accumulation of camalexin. *PAD3* is upregulated by *P. syringae* infection (both virulent and avirulent strains) and is not induced in *pad1* and *pad4* mutants after infection with virulent *P. syringae*. Interestingly, induction of *PAD3* by avirulent *P. syringae* in the *pad4* mutant is not defective correlating with wild-type levels of camalexin under these conditions (Zhou *et al.*, 1999). The involvement of reactive oxygen species (ROS) in camalexin regulation has been suggested from studies showing camalexin accumulation in response to compounds causing an increase in ROS (Tierens *et al.*, 2002; Zhao *et al.*, 1998).

Trp biosynthetic enzymes, including anthranilate synthase (ASA) and phosphoribosylanthranilate transferase (PAT), are co-ordinately upregulated at both the mRNA and protein level during biotic and abiotic stress (Zhao and Last, 1996). We screened for Arabidopsis mutants that were defective in induction of the trp biosynthetic enzymes following infection with a virulent *P. syringae* strain or following amino acid starvation. By targeting defence-related responses not used in previous mutant screens, we hoped to identify novel loci involved in defence signalling. Here we report the isolation and characterization of a recessive mutation (*ups1*) from the amino acid starvation screen. This mutant is defective in a range of defence responses, including camalexin accumulation, and appears to be unresponsive to ROS.

Results

ups1 is defective in the regulation of trp biosynthetic enzymes

The *ups1* (*underinducer after pathogen and stress*) mutant was identified in a screen for Arabidopsis mutants defective in trp biosynthetic pathway regulation. PAT is an early enzyme in this pathway that catalyses the formation of 5-phosphoribosyl anthranilate from anthranilate and 5-phosphoribosyl-1-pyrophosphate. The Arabidopsis AR208 line contains a chimeric *PAT::GUS* transgene where the promoter of the single copy Arabidopsis *PAT* gene is fused to the coding region of the *GUS* reporter gene (Rose and Last, 1997). Individual M₂ plants grown from ethyl methanesulphonate-mutagenized Arabidopsis AR208 seed (Col-0 ecotype) were screened for reduced GUS staining following 3 days of treatment with lysine and threonine (K + T) at a final concentration of 2 mM each. This treatment leads to methionine starvation by inhibiting aspartate kinase (Galili, 1995) and upregulates the mRNA and protein levels of PAT and other trp biosynthetic enzymes (Zhao *et al.*, 1998). A number of plants were selected and rescreened in the next generation. *ups1* showed attenuated GUS staining in the M₃ generation indicating reduced expression of the *PAT::GUS* construct (Figure 1). Reduced levels of endogenous PAT protein after K + T treatment in the *ups1* mutant indicated that the mutation was a *trans*-acting mutant and not a *cis* mutation in the transgene (Figure 1). M₆ progeny of *ups1* were used to generate a backcross to wild-type AR208 and this backcrossed line (BC1) was used in all experiments reported here unless otherwise stated. Furthermore, a second backcross (BC2) was used to verify key phenotypes (Supplementary Figures S1–S3 and Figure 5b). The effect of K + T treatment on ASA1 protein

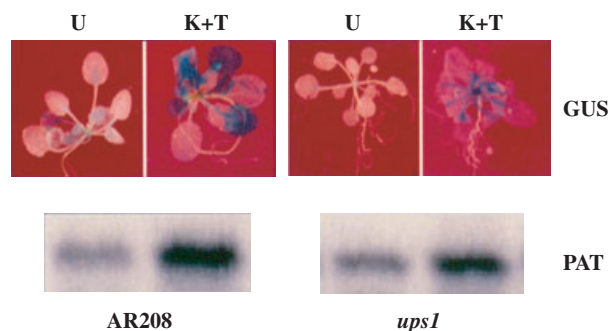


Figure 1. Reduced PAT1 expression in *ups1* mutants. Wild type (AR208) and *ups1* seedlings were grown on plates for 2 weeks and treated with 2 mM K + T or water (untreated) for 2 days. (a) Blue staining indicates expression of GUS from the *PAT1* promoter. (b) Immunoblot analysis of endogenous PAT1 protein levels. Total protein was extracted and 10 µg loaded per sample. Affinity-purified antisera raised against PAT1 protein was used at 1:1000 dilution. The experiment was performed twice with similar results.

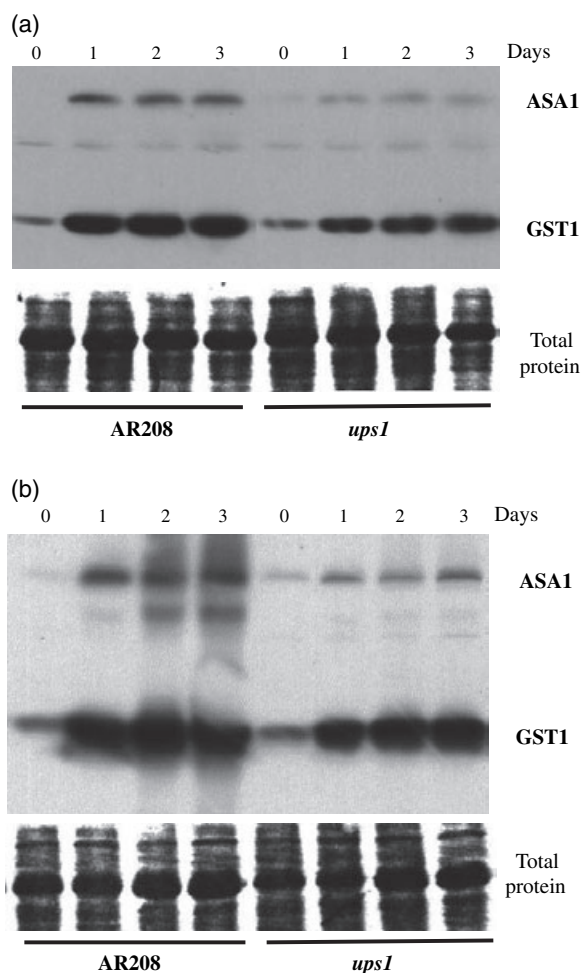


Figure 2. ASA1 protein induction in *ups1* and wild-type plants after abiotic stress.

ASA1 and GST1 protein levels in wild type (AR208) and *ups1* seedlings following 0, 1, 2 or 3 days of treatment with: (a) 2 mM K + T and (b) 0.03 mM acifluorfen. Ten micrograms of a total protein extract was loaded per lane and the total protein panel shows the Ponceau S staining of membranes prior to immunoblotting. Antisera raised against ASA1 and GST1 were used at 1:1000 dilution. This experiment was performed two (acifluorfen) or four (K + T) times with similar results.

levels, the first enzyme specific to trp biosynthesis, is shown in Figure 2(a) for the *ups1* BC1 mutant.

The trp biosynthetic enzymes are upregulated in response to a variety of abiotic stresses (Nyogi *et al.*, 1993; Zhao and Last, 1996; Zhao *et al.*, 1998) and we investigated whether the effect of *ups1* mutation on the regulation of ASA1 is specific for methionine starvation induced by K + T treatment. Oxidative stress was induced by treating plate-grown seedlings with acifluorfen, which inhibits haem and chlorophyll biosynthesis leading to the production of free radicals (Matringe *et al.*, 1989). Treatment with acifluorfen induced the accumulation of ASA1 protein in wild-type plants as previously reported (Zhao and Last, 1996; Zhao *et al.*, 1998).

In contrast, induction of ASA1 protein by acifluorfen was lower in the *ups1* mutant (Figure 2b) indicating a role for *UPS1* in regulation of this trp biosynthetic enzyme by oxidative stress.

Expression of *GST1* (*glutathione S-transferase 1*) is upregulated in response to methionine starvation and oxidative stress at both the mRNA and protein levels (Zhao *et al.*, 1998). GST1 protein levels were found to increase in wild-type plants after K + T and acifluorfen treatment as expected. However, the *ups1* mutation appeared to reduce GST1 accumulation as GST1 protein levels were lower in the mutant plants after both treatments (Figure 2a,b). The *ups1* mutation therefore hinders both trp biosynthetic enzyme (PAT/ASA1) and a known stress-inducible enzyme (GST1) accumulation in response to methionine starvation and an ROS-inducing herbicide.

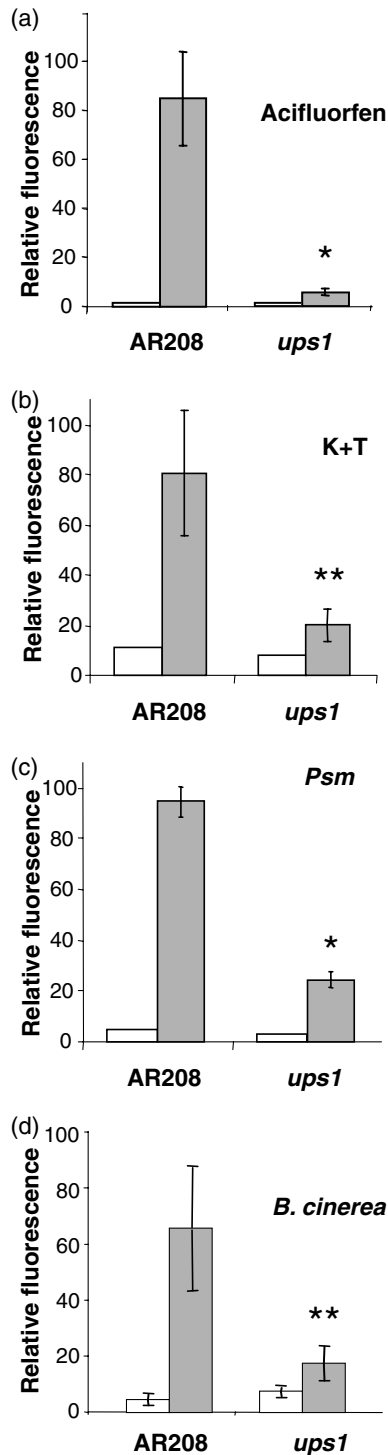
ups1 is defective in camalexin accumulation

Camalexin accumulation is closely coupled to the induction of the trp pathway enzymes in response to a variety of stimuli including oxidative stress and amino acid starvation (Zhao and Last, 1996; Zhao *et al.*, 1998). We therefore investigated whether *ups1* showed a defect in accumulation of camalexin after both abiotic and biotic stress. Acifluorfen and K + T treatments led to the accumulation of camalexin in wild-type plants as expected, and this induction was dramatically reduced in *ups1* mutants (Figure 3a,b). Infection of wild-type plants with virulent *P. syringae* pv *maculicola* (*Psm*) also led to the production of camalexin; again the accumulation of camalexin was significantly lower in *ups1* plants compared with wild type (Figure 3c).

To identify whether the reduced accumulation of camalexin in *ups1* occurred in response to a dramatically different pathogenic microbe, we tested plants following infection with the necrotrophic fungal pathogen *B. cinerea*. Infection with *B. cinerea* resulted in accumulation of camalexin in wild-type AR208 plants by 3 days post-infection and again levels were much lower in *ups1* mutants (Figure 3d). Camalexin accumulation in *ups1* was therefore significantly reduced after both abiotic and biotic stress, typically reaching levels of only 10–20% of those attained in wild-type AR208 plants. Interestingly, the lack of camalexin accumulation in *ups1* after both *Psm* and *B. cinerea* infection is in contrast to the *pad4* mutant, which is defective in camalexin accumulation in response to *Psm* but has normal accumulation after infection with *C. carbonum* or *A. brassicicola* (Glazebrook *et al.*, 1997; van Wees *et al.*, 2003). PAD4 appears to regulate camalexin accumulation in specific situations as camalexin does accumulate in *pad4* mutants after infection with *Psm* carrying the avirulence gene *avrRpt2* (Glazebrook *et al.*, 1997).

Expression of defence genes is compromised in *ups1*

Camalexin accumulation is only one of many defence responses that *Arabidopsis* plants launch following pathogen infection. To determine whether other responses are altered in *ups1*, the expression of several defence-related genes was examined after infection. The *ups1* mutant showed delayed



induction of PR1 and PR5 proteins in response to *Psm* infection compared with wild type (Figure 4a). The difference in expression is most obvious 1 day after infection, when PR1 and PR5 proteins are undetectable in the mutant. *PR2* mRNA levels were also lower in *ups1* compared with the wild-type AR208 plants (Figure 4b). Normalization of rRNA intensity to signal indicated that *PR2* mRNA levels were halved in *ups1* compared with wild type. We also investigated the expression of the putative camalexin biosynthetic gene *PAD3* (Zhou *et al.*, 1999). As expected, *PAD3* expression was induced in wild-type AR208 plants following *Psm* infection (Figure 4c), however, levels of *PAD3* mRNA were reproducibly lower in *ups1* plants.

The effects of *ups1* on induction of the *PDF1.2* defensin gene were tested during *B. cinerea* infection. As shown in Figure 5(a,b), *PDF1.2* mRNA accumulates 3 days after infection with *B. cinerea* in wild-type plants and maximal induction of this defensin gene was reduced in the *ups1* mutant. In addition, we found reduced expression of *PR1* and *PAD3* mRNA in mutant plants after *B. cinerea* infection (Figure 5a,c). Expression of the defensin gene, *PDF1.2*, was not reproducibly induced after infection with *Psm*. However, in experiments when *PDF1.2* was induced in wild-type plants, expression levels were always lower in *ups1* (data not shown).

The expression of PR1 and PR5 proteins and *PR2* and *PAD3* mRNAs is regulated by SA. Reduction of SA in NahG plants reduces the expression of these three *PR* genes during pathogenesis (Delaney *et al.*, 1994; Nawrath and Metraux, 1999; Uknes *et al.*, 1992) and *PAD3* is constitutively expressed in *Arabidopsis* mutants with high endogenous SA levels (Zhou *et al.*, 1999). The expression of *PDF1.2* is mediated by JA and ethylene (Manners *et al.*, 1998; Penninckx *et al.*, 1998). The *ups1* mutant therefore shows defects in induction of both SA-regulated and JA/ethylene-regulated gene expression suggesting that the *ups1* mutation is acting early in defence signalling (to activate all three pathways) or affecting positive interactions between these signalling pathways preventing full activation (Schenk *et al.*, 2000). The *ups1* mutant is not generally defective in responding to

Figure 3. Camalexin accumulation in response to abiotic stress and pathogen infection in wild type and *ups1* plants.

Graphs showing the levels of camalexin in wild type (AR208) and *ups1* plants after indicated treatments. Camalexin was extracted from 100 mg fresh weight samples and is expressed as a percentage of the highest fluorescence value in each experiment. Data represent the mean and standard deviation of three to five replicate samples for each graph. Data sets marked with an asterisk are significantly different from wild type as assessed by the Student's *t*-test: **P* < 0.001, ***P* < 0.04.

(a) Seedlings treated with 0.03 mM acifluorfen (black bars) or water (open bars) for 4 days.

(b) Seedlings treated with 2 mM K + T (black bars) or water (open bars) for 2 days.

(c) Leaves of whole plants inoculated with *Psm* (OD₆₀₀ = 0.02) (black bars) or mock inoculated with MgSO₄ alone (open bars) for 2 days.

(d) Detached leaves inoculated with *Botrytis cinerea* spores (1 × 10³ spores ml⁻¹) (black bars) or grape juice only (open bars) for 3 days.

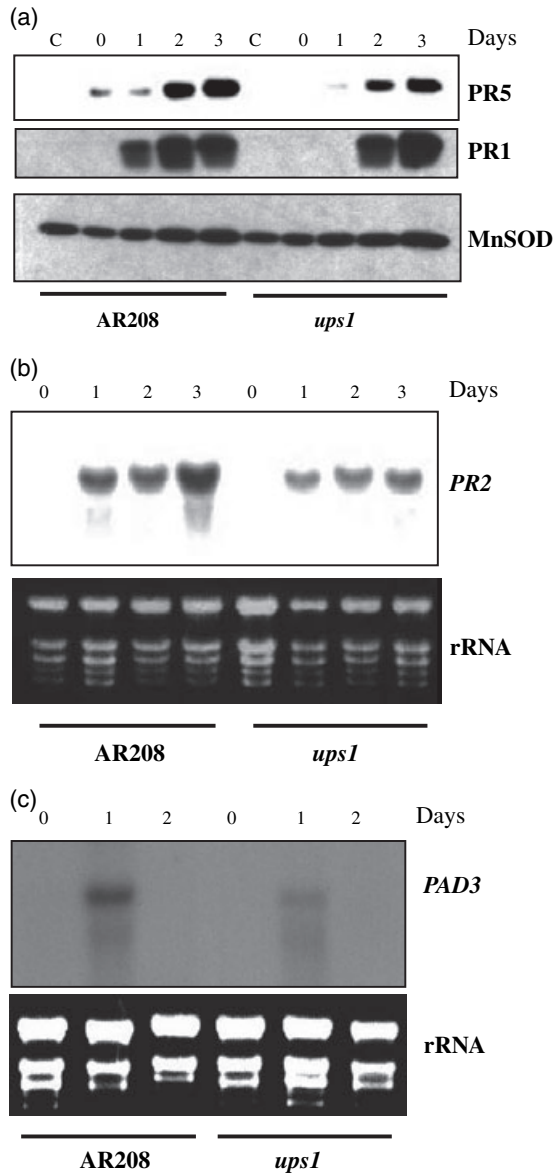


Figure 4. Defence gene expression after *Psm* infection is defective in *ups1* mutant plants.

(a) Immunoblot analysis showing the increase in PR1 and PR5 protein in wild type (AR208) and *ups1* plants following infection with *Psm* ($OD_{600} = 0.02$). Samples were harvested at time 0 and up to 3 days after inoculation. C indicates samples from leaves that were mock inoculated with 10 mM $MgSO_4$ only, and harvested after 3 days. Ten micrograms of total protein was loaded onto the gels and MnSOD protein levels are shown as a control for loading. Antisera raised against PR1 and PR5 proteins were used at a dilution of 1:250 and 1:1000 respectively. This experiment was performed three times with similar results.

(b) RNA blot hybridization analysis of *PR2* mRNA in wild type (AR208) and *ups1* plants following infection with *Psm* ($OD_{600} = 0.02$). Ten micrograms of total RNA was loaded in each lane and the rRNA panel indicates ethidium bromide staining of RNA. This experiment was repeated with similar results.

(c) Accumulation of *PAD3* mRNA in wild type (AR208) and *ups1* plants following infection with *Psm* ($OD_{600} = 0.02$). Ten micrograms of total RNA was loaded in each lane and the rRNA panel indicates ethidium bromide staining of RNA. This experiment was repeated with similar results. A smeary doublet was always observed corresponding to the *PAD3* gene probe.

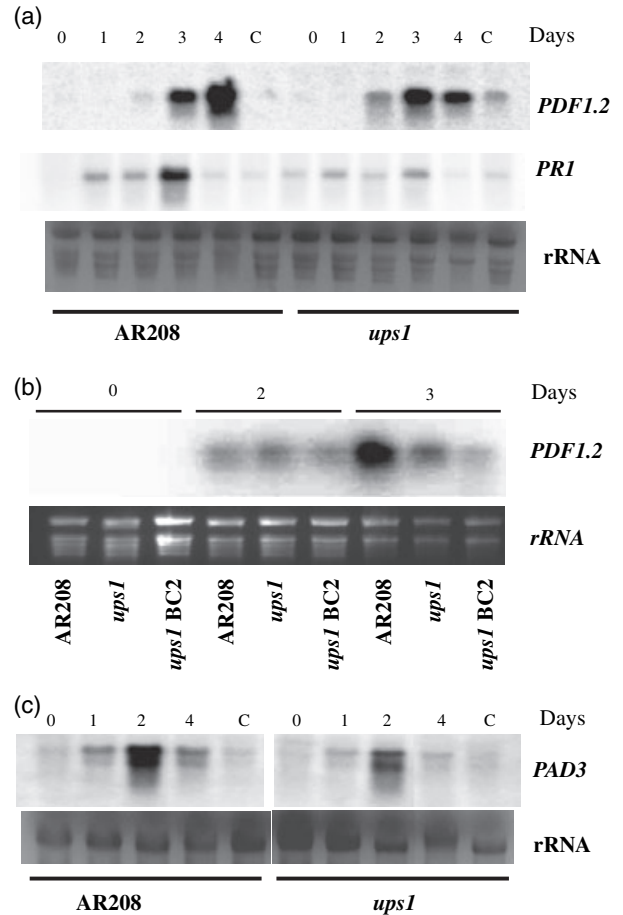


Figure 5. Induction of *PR1*, *PDF1.2* and *PAD3* in *ups1* plants following *Botrytis cinerea* infection.

Detached leaves from wild type (AR208) and *ups1* mutant plants were inoculated with a drop of *B. cinerea* [1000 (a, c) or 5000 (b) spores ml^{-1} in grape juice]. Panel (b) includes the *ups1* mutant BC2 line. Leaves were harvested at time 0 and up to 4 days after inoculation. Control samples (C) were leaves inoculated with a drop of grape juice only and harvested after 4 days. RNA blot hybridization analyses were performed with 10 μg of total RNA extracted from each sample and ethidium bromide staining of RNA is indicated by the rRNA panel. This experiment was repeated with similar results obtained. Membranes were hybridized with probes detecting (a) *PR1* and *PDF1.2* mRNA, (b) *PDF1.2* and (c) *PAD3* mRNA.

environmental signals as responses to addition of JA and ET are normal (shown below).

Responses to SA are compromised in *ups1*

The hypothesis that *UPS1* is necessary for SA-mediated gene induction was directly tested. The expression of the SA-inducible proteins, PR1 and PR5, was monitored in wild type and *ups1* plants following treatment of leaves with SA (Figure 6). The accumulation of PR1 protein was reduced in *ups1* plants compared with wild-type AR208. As expected, PR1 was not detected in *npr1* mutant leaves. Plants mutant for *ups1* and *npr1-1* exhibited lower expression of PR5

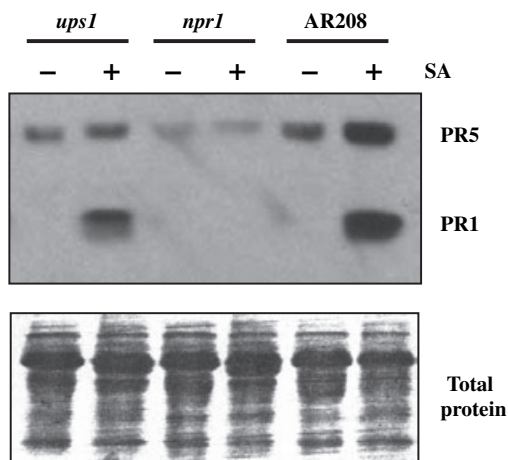


Figure 6. Reduced accumulation of salicylic acid (SA)-inducible PR proteins in *ups1* plants. PR5 and PR1 protein accumulation in leaves from soil-grown wild type (AR208), *ups1* and *npr1* mutant plants. Leaves were hand-infiltrated with 1 mM SA (+) or water (-) and samples harvested 2 days later. Fifteen micrograms of total protein was loaded per lane. The total protein panel indicates Ponceau S staining of the membrane prior to immunoblotting. Antisera raised against PR1 and PR5 proteins were used at a dilution of 1:250 and 1:1000 respectively. This experiment was repeated with similar results.

protein compared with wild type, both in the absence and presence of exogenous SA. Hence, SA responses appear to be compromised in *ups1* mutant plants, but the block in SA-mediated signalling is not as complete as that in *npr1* mutants.

ups1 responds normally to JA and ethylene

PDF1.2 expression during pathogenesis is reliant on both ethylene and JA signalling (Penninckx *et al.*, 1998) and these transduction pathways are also required for camalexin accumulation in Arabidopsis following *Psm* infection (Zhou *et al.*, 1999; K.J. Denby, unpublished data). Because camalexin accumulation and induction of *PDF1.2* mRNA were compromised in the *ups1* mutant during pathogenesis (Figures 3 and 5), we tested whether *ups1* was defective in responses downstream of ethylene and methyl jasmonate (MeJA). We employed an ethylene-inducible hevein-like (*HEL*) gene from Arabidopsis (Potter *et al.*, 1993). *HEL* mRNA expression was monitored in plants grown in increasing concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Figure 7a). *HEL* mRNA levels were comparable in *ups1* and wild-type AR208 plants whereas *HEL* expression was severely attenuated in the ethylene response mutant *ein2-1* (Guzman and Ecker, 1990). Consistent with a lack of impairment in ethylene signal transduction, the 'triple response' of etiolated seedlings grown in the presence of ACC (Bleecker *et al.*, 1988) was normal in *ups1* and wild-type AR208 plants, with thick short roots and hypocotyls, inhibition of hypocotyl elongation and

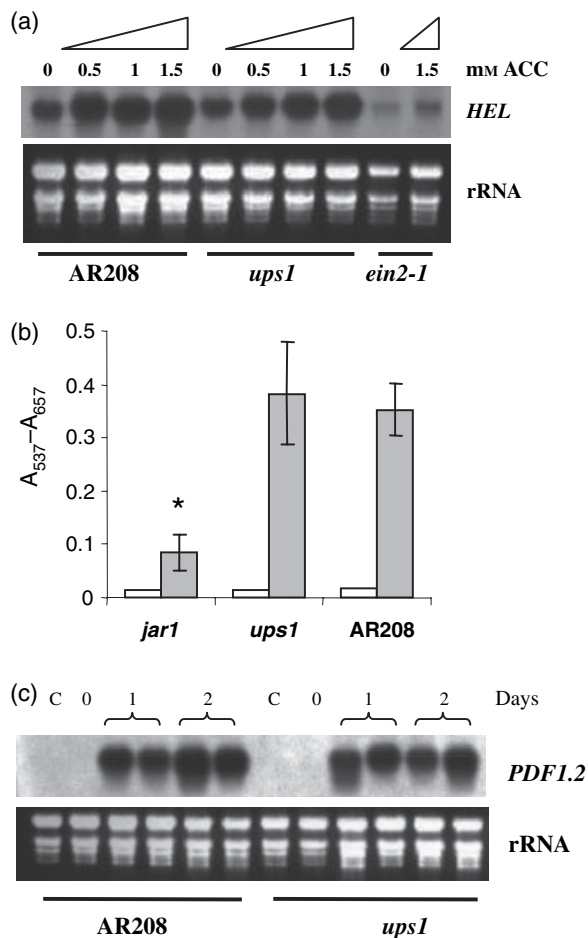


Figure 7. Ethylene and methyl jasmonate (MeJA)-mediated responses are unaffected in *ups1* plants. (a) Induction of *HEL* mRNA by 1-aminocyclopropane-1-carboxylate (ACC). Wild type (AR208), *ups1* and *ein2-1* plants were grown for 10 days on sterile nutrient agar plates containing 0, 0.5, 1 and 1.5 mM ACC. RNA blot hybridization analysis was performed with 20 μg total RNA in each lane and the rRNA panel indicates ethidium bromide staining of RNA as a loading control. The experiment was repeated with similar results. (b) Anthocyanin accumulation in response to MeJA. Plants were grown for 10 days on sterile nutrient agar plates prior to treatment with 45 μM MeJA (shaded bars) or 0.01% ethanol (clear bars). Anthocyanin content was determined 5 days after treatment. The data represent the mean and SD of six treated samples. Data sets marked with an asterisk are significantly different from wild type, as assessed by the Student's *t*-test ($P < 0.001$). (c) Accumulation of *PDF1.2* mRNA in response to MeJA. Plants were grown for 10 days on sterile nutrient agar plates prior to treatment with 45 μM MeJA. Samples were harvested at time 0 and up to 2 days after treatment, with biological replicates at 1 and 2 days. Control samples (C) were treated with 0.01% ethanol for 2 days. RNA blot hybridization analysis was carried out using 20 μg of total RNA and the rRNA panel indicates ethidium bromide staining of total RNA as a loading control. This experiment was repeated with similar results.

increased curvature of the apical hook being observed (data not shown).

The responses of MeJA were normal in *ups1* by several criteria. First, primary root growth of *ups1* and AR208 plants was inhibited by 10 μM MeJA, whereas *jar1* plants were

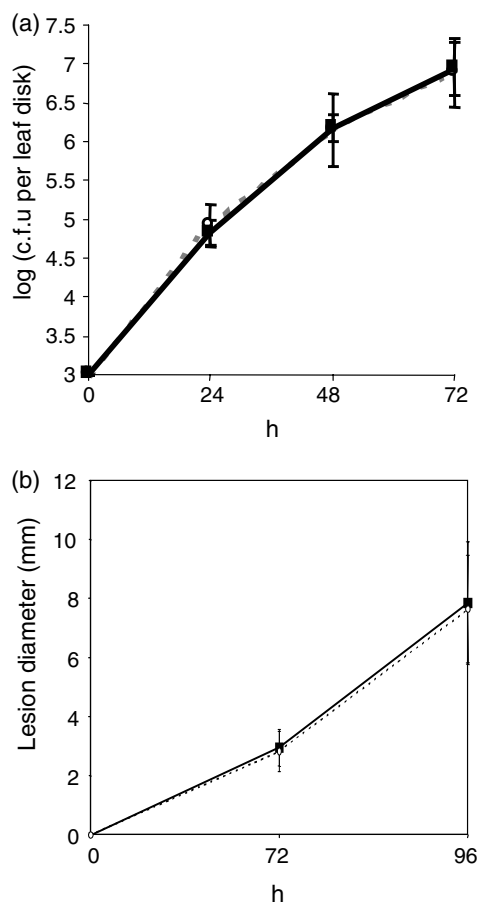


Figure 8. Wild type and *ups1* plants show similar disease resistance profiles. (a) Analysis of *Psm* growth in AR208 (filled squares, solid line) and *ups1* (open circles, dotted line) plants. Leaves were inoculated with *Psm* at an $OD_{600} = 0.0002$ [equivalent to 10^3 colony forming units (c.f.u.) cm^{-2} leaf area]. Leaf discs were taken 0, 1, 2 and 3 days after infection for each genotype and the number of c.f.u. per leaf disc determined. The data represent the mean and SD of eight replicate samples. (b) Analysis of *Botrytis cinerea* lesions in AR208 (filled squares, solid line) and *ups1* (open circles, dotted line) plants. Detached leaves were inoculated with a droplet of *B. cinerea* spores (1000 spores ml^{-1}) and the lesion diameter measured 3 and 4 days after infection. The data represent the mean and SD of 10–12 replicate samples and the experiment was repeated with similar results.

significantly less affected (data not shown) as previously reported by Staswick *et al.* (1992). Similarly, accumulation of anthocyanins in response to MeJA treatment was similar between wild type and *ups1* mutant plants (Figure 7b). Finally, the results shown in Figure 7(c) show that *PDF1.2* expression in *ups1* plants was comparable to that in wild-type plants after MeJA treatment (Manners *et al.*, 1998; Penninckx *et al.*, 1998). Taken together, these results suggest that *ups1* mutants are still capable of ethylene and MeJA perception and signalling, suggesting that defects in induction of defence responses after pathogen infection are possibly due to a mutation upstream of these signalling compounds.

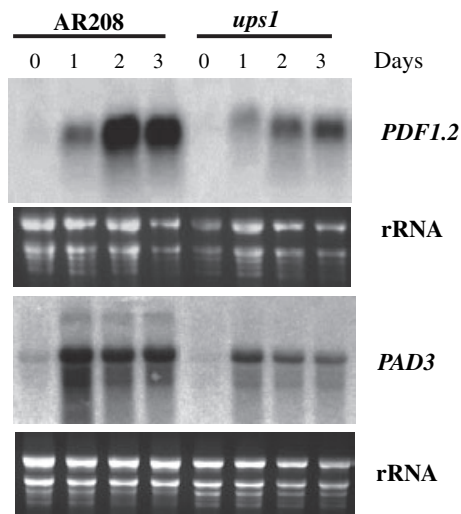


Figure 9. Reactive oxygen species-responsive gene expression in *ups1* plants is reduced compared with wild type. Wild type (AR208) and *ups1* seedlings were grown for 10 days on sterile nutrient agar plates prior to treatment with acifluorfen at a final concentration of 0.03 mM. Samples were harvested at time 0 and up to 3 days after treatment. RNA blot hybridization analysis of *PDF1.2* and *PAD3* mRNA expression was carried out using 15 μg of total RNA. The *rRNA* panel shows ethidium bromide staining of RNA as a loading control. Similar results were obtained when the experiment was repeated.

ups1 does not show increased pathogen susceptibility

As a number of defence responses are reduced in the *ups1* mutant, we hypothesized that the mutation would cause an increased susceptibility to pathogen infection. Contrary to this expectation, growth of the virulent bacterial pathogen *Psm* (Figure 8a) and leaf yellowing (data not shown) were comparable in *ups1* and wild-type plants throughout the infection period. Similarly, the diameter of lesions produced by inoculation with the necrotrophic fungal pathogen *B. cinerea* did not differ between wild type and *ups1* plants (Figure 8b).

Response of *ups1* to ROS

The involvement of ROS in camalexin regulation has been suggested from studies showing the accumulation of camalexin in response to compounds causing an increase in ROS (Tierens *et al.*, 2002; Zhao *et al.*, 1998). The production of ROS is also an early event in pathogenesis and may play an important role in defence signal transduction (Grant and Loake, 2000). This led us to further analyse *ups1* responses to ROS.

PDF1.2 expression was previously shown to be induced by oxidative stress via treatment with paraquat (Penninckx *et al.*, 1998). As shown in Figure 9(a), we found that acifluorfen treatment induced significant accumulation of *PDF1.2* mRNA in wild-type AR208 plants. However, *PDF1.2*

mRNA levels were lower in *ups1* seedlings (reduced 85% on average). Acifluorfen treatment also led to the accumulation of *PAD3* mRNA in wild-type plants, and again expression was markedly reduced in *ups1* mutants (50% on average) (Figure 9b). The observation that the induction of two genes is reduced in *ups1* plants in response to acifluorfen suggests that *ups1* mutants could be defective in their ability to sense and/or respond to ROS.

Genetic characterization of *ups1*

Camalexin levels were measured in the F₁ and F₃ generation of *ups1* × AR208 and *ups1* × Col crosses after *Psm* infection and acifluorfen treatment respectively. Segregants that resembled M₄ generation *ups1* plants in having <35% of wild-type camalexin accumulation were assigned a camalexin⁻ phenotype. Camalexin⁺ indicated levels similar to AR208. Controls were performed to ensure that homozygous mutant lines could be distinguished from heterozygous lines segregating wild type and mutant plants (data not shown).

The phenotype of the *ups1* mutation in response to abiotic or biotic stress is subtle, making genetic analysis difficult. However, two lines of evidence suggest that *ups1* is a monogenic, recessive mutation. First, F₁ plants from *ups1* crossed to the parental AR208 showed wild-type levels of camalexin following *Psm* infection consistent with *ups1* being recessive. Scoring of *Psm*-induced camalexin accumulation of F₂ progeny from this cross did not yield two clear phenotypic classes. However, one low camalexin accumulator was confirmed in subsequent generations and used for the experiments reported above. We had better success scoring acifluorfen-induced camalexin in 28 randomly chosen F₃ families from a Col × *ups1* cross. A segregation ratio of 22:6 WT:*ups1* was obtained, consistent with the hypothesis that that *ups1* is a monogenic recessive mutation ($\chi^2 = 0.19$; $P > 0.1$).

Discussion

The *ups1* mutant described in this paper was isolated on the basis of altered regulation of the trp biosynthetic gene *PAT1*. The fact that a single mutation causes reduced camalexin accumulation in response to several different stimuli suggests a common signalling mechanism. It is highly unlikely that the *ups1* mutation defines a camalexin biosynthetic locus as expression of defence genes is also compromised. All the experimental work detailed in this report has been carried out on backcrossed lines, which significantly reduces the possibility of multiple mutations contributing to the phenotype. *UPS1* is therefore likely to encode a signalling component involved in regulating camalexin accumulation and defence gene expression in response to both biotic and abiotic stress.

Camalexin has previously been shown to accumulate in response to oxidative stress (Thomma *et al.*, 1999; Zhao *et al.*, 1998) but in this report we demonstrate upregulation of *PAD3* mRNA levels after oxidative stress. As mentioned above, *PAD3* is a cytochrome P450 mono-oxygenase likely to be involved in camalexin biosynthesis. The expression of *PAD3* has been shown to correlate with camalexin synthesis following pathogen infection (Zhou *et al.*, 1999) and, in this report, oxidative stress. The reduced expression of *PAD3* in response to pathogen and acifluorfen treatment in *ups1* may be the cause of the reduced camalexin accumulation in this mutant, although *PAD3* expression is not sufficient for camalexin production as expression of *PAD3* in response to SA is not mirrored by camalexin accumulation (Zhou *et al.*, 1999).

Several mutants that show defects in camalexin production have been previously isolated, including the series *pad1* through *pad5* (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1997). In contrast to the three major signalling pathways disrupted by the *ups1* mutation, regulatory mutants *pad1*, 2, 4 and 5 each appear to affect a single signalling pathway. Although, *pad4* is defective in *Psm*-mediated activation of *PR1* transcription (Zhou *et al.*, 1998), it is not defective in SA-induced expression of *PR1* and has augmented JA responses (Gupta *et al.*, 2000). In a large-scale analysis of gene expression changes after *Psm* treatment, *pad1* clustered with mutations affecting JA signalling, and reduced JA responses were confirmed in this mutant (Glazebrook *et al.*, 2003). *pad2* clustered with mutations affecting SA signalling and, although *PR1* expression is completely blocked in *pad2* plants after infection with *Phytophthora porri* (Roetschi *et al.*, 2001), *PDF1.2* expression is unaffected indicating JA responses are intact. Published data indicate that *pad3* is likely to be a camalexin biosynthetic mutant (Zhou *et al.*, 1999). A recent paper using cDNA microarray analysis suggested that *PAD3* was also involved in regulating defence gene expression, however these experiments were unable to distinguish between genes directly affected by the *pad3* mutation, and those with altered expression due to the increased *A. brassicicola* infection in *pad3* mutant plants (Naruska *et al.*, 2003). Therefore, the *ups1* mutation appears unique in affecting camalexin accumulation as well as SA-, JA- and ethylene-dependent defence responses.

The only other published mutation shown to affect camalexin accumulation is *esa1* (enhanced susceptibility to *Alternaria*) (Tierens *et al.*, 2002), which has several similarities to *ups1*. Camalexin accumulation is reduced in *esa1* in response to pathogen infection (both necrotrophic and biotrophic), paraquat and acifluorfen. In addition, *PDF1.2* expression is delayed and/or reduced in *esa1* after infection. However, unlike *ups1*, *esa1* does not appear to impair SA-mediated responses and *PDF1.2* expression is significantly

lower in *esa1* compared with wild type after treatment with ethylene or MeJA (Tierens *et al.*, 2002).

ups1 is unique in its effects on both SA and JA/ethylene signalling after pathogen infection, and current models suggest that a mutation with this phenotype is likely to be affecting an early event in defence signalling. Our working hypothesis is that *ups1* is compromised in the ability to detect and/or respond to ROS signals. ROS are known to accumulate during incompatible plant–pathogen interactions (recently reviewed in Vranova *et al.*, 2002) and two Arabidopsis homologues of the mammalian NADPH oxidase catalytic subunit produce the majority of ROS in response to infection with avirulent *Pseudomonas* (Torres *et al.*, 2002). There is less information on whether ROS play a role in defence response induction during compatible interactions. However, levels of copper zinc superoxide dismutase (Cu/Zn-SOD) protein, one of the main antioxidant enzymes, increase dramatically during infection with virulent and avirulent *Psm* (Kliebenstein *et al.*, 1999). It is also highly likely that amino acid starvation leads to chloroplast damage and resulting ROS accumulation.

Reactive oxygen species can activate both SA- and JA/ethylene-mediated signalling in plants, indicating that a defect in responding to ROS could account for the breadth of phenotypes seen in *ups1*. *PDF1.2* is induced by ROS and this induction requires intact JA and ethylene signalling (Penninckx *et al.*, 1998). ROS accumulation has been shown to lead to SA synthesis and stimulation of the SA signalling pathway in several systems including Arabidopsis (Chamnongpol *et al.*, 1998; Eckey-Kaltenbach *et al.*, 1997; Sandermann *et al.*, 1998; Sharma *et al.*, 1996; Yalpani *et al.*, 1994). However, not only does ROS lead to an increase in SA levels, but SA also leads to accumulation of ROS (Chen *et al.*, 1993) and SA-dependent expression from the *as-1* cis element occurs via ROS, again indicating production of ROS in response to SA (Garretton *et al.*, 2002). If *ups1* were defective in the perception of, or response to, ROS then this ROS-SA amplification loop (McDowell and Dangl, 2000) would not function properly, potentially reducing or delaying SA responses as seen in *ups1*.

In contrast to *PAD3* and *PDF1.2*, the *GST1* gene has been shown to be expressed independently of SA or JA and to be a reliable marker of ROS production in Arabidopsis (Grant *et al.*, 2000). *ups1* mutants show reduced accumulation of GST1 protein after amino acid starvation, oxidative stress and pathogen infection consistent with this mutant having a defect in ROS perception or response.

An alternative model is that UPS1 has a role in synergistic interactions between the SA and JA/ethylene signalling pathways, downstream of SA production. Most documented interactions between SA and JA signalling are antagonistic and there is limited evidence for positive interactions (Kunkel and Brooks, 2002). However, the expression of

several genes can be induced by SA and ethylene or SA and JA (Schenk *et al.*, 2000) suggesting that co-regulation of gene expression is occurring. Interestingly, camalexin accumulation in response to *Psm* infection is an example of SA and JA/ethylene signalling working together, and both pathways are required for maximal synthesis (Zhao and Last, 1996). UPS1 may therefore define a signalling component that mediates positive cross talk between these pathways. The fact that defence responses in *ups1* are reduced or delayed, rather than absent, could indicate that UPS1 is involved in potentiating defence responses rather than initial activation, or is a partial loss-of-function mutation. This partial phenotype may also explain why we did not observe increased susceptibility to *Psm* or *B. cinerea* in *ups1* mutants.

In conclusion, *ups1* is a unique mutant in that it exhibits defects in all the three major defence signalling pathways (SA, JA and ethylene) suggesting the wild-type UPS1 protein is involved in early signalling events during pathogenesis. Future work will be aimed at identifying the *UPS1* gene and testing our hypothesis that *ups1* is compromised in the perception of and/or response to ROS.

Experimental procedures

Plant growth conditions and isolation of *ups1*

Arabidopsis thaliana plants were grown under constant under cool white fluorescent illumination (80–100 $\mu\text{mol photon sec}^{-1} \text{m}^{-2}$) on 'Cornell mix' (Landry *et al.*, 1995) in a controlled environment room at 24°C. Plate-grown plants were grown on sterile PN agar (Haughn and Somerville, 1986) under the same conditions. GUS reporter gene activity was assayed as described previously (Pruitt and Last, 1993).

Chemical treatments

As described by Zhao *et al.* (1998), 10 \times solutions were applied evenly to 2-week-old plants grown on sterile PN agar plates. Final concentrations were as follows: acifluorfen, 30 μM ; lysine and threonine, 2 mM each; MeJA, 45 μM in 0.01% (v/v) ethanol. Plants were returned to the growth room after treatment. Specified concentrations of ACC were incorporated directly into PN medium. Leaves from soil-grown plants treated with SA were infiltrated with a 1-mM solution.

Pathogen infection

Pseudomonas syringae pv *maculicola* ES4326 was grown overnight at 30°C in Luria-Bertoni medium with 100 $\mu\text{g ml}^{-1}$ streptomycin. The bacteria were washed twice in 10 mM MgSO_4 and diluted to the OD₆₀₀ specified in the text with 10 mM MgSO_4 . The bacterial solution was infiltrated into the rosette leaves of 3-week-old plants using a syringe as previously described (Glazebrook and Ausubel, 1994). Quantification of *Psm* growth in planta was carried out as described in Glazebrook and Ausubel (1994) except bacteria were grown on Luria-Bertoni agar rather than Kings B. A *B. cinerea* culture was obtained from infected grape vine and was maintained on apricot

halves. Spores were harvested in water and adjusted to a final concentration of $1-5 \times 10^3$ spores ml^{-1} using pure grape juice. Excised leaves were placed in a tissue culture jar containing 0.8% agar and 3 μl of the spore suspension was spotted in the middle of the leaf blade and allowed to dry for 1 h before closing the jars to maintain high humidity. The jars were kept at 25°C, in a 16-h light regime and monitored daily for lesion development. The diameter of the dark, grey lesions was measured at days 3, 4 and 5. Control plant samples were inoculated with the same volume of grape juice.

RNA and camalexin analysis

Samples were frozen in liquid nitrogen after collection and stored at -70°C. RNA was isolated using a guanidinium thiocyanate-phenol-chloroform protocol (Chomczynski and Sacchi, 1987). Fifteen micrograms of total RNA was separated by electrophoresis through a formaldehyde-agarose gel, blotted onto hybridization membrane (Hybond-N; Amersham Pharmacia Biotech, Little Chalfont, UK) by capillary transfer and cross-linked on the blots by UV illumination. ^{32}P -labelled probes were generated according to the manufacturer's instructions by random priming of gel-purified template DNA (Megaprime; Amersham Pharmacia Biotech). *PAD3* (AT3G26830) template DNA was prepared by PCR amplification of Arabidopsis genomic DNA using previously described primers (Zhou *et al.*, 1999). A 373-bp fragment of the *HEL* gene (At3g04720) was amplified from genomic DNA by PCR with the primers 5'-TAC-ACAGTGGCTACGGTGGC-3' and 5'-CCCTTAAACACTGAAGCA-3'. A 400-bp *PDF1.2* (AT5G44420) DNA fragment inserted in plasmid pZL1 (Penninckx *et al.*, 1996) was excised from amplified plasmid by digestion with *Sall* and *NotI*. The *PR-1* (AT2G14610) DNA template was prepared from genomic DNA using the primers 5'-GCTCTTGTCTTCCCTCG-3' and 5'-GTGTAGTGACCACAAACTC CA-3'. A 1.2-kb *PR2* (AT3G57260) DNA fragment inserted into pBlue-script (Uknes *et al.*, 1992) was amplified by PCR with T3 and T7 primers. Camalexin extraction and quantitation was performed as described previously (Zhao and Last, 1996).

Immunoblot analysis

Protein was extracted by homogenizing Arabidopsis tissue samples in protein extraction buffer (Zhao and Last, 1995). The homogenate was cleared of tissue debris by a 5-min centrifugation step at 9300 *g* and supernatants were transferred to clean tubes and kept at 4°C. Protein concentrations in the crude extracts were determined using the Bio-Rad protein assay, using BSA as a standard. Equal amounts of proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and blotted as described previously (Zhao and Last, 1995). Anti-PAT1, anti-ASA1, anti-GST1, anti-PR5 and anti-PR1 serum previously generated (Kliebenstein *et al.*, 1999; Zhao and Last, 1995; M.K. Pelletier and R.L. Last, unpublished data) were incubated at 1:1000, 1:1000, 1:1000 and 1:125 dilutions, respectively, for 4 h. The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit (Chemicon, Harrow, UK) at a 1:5000 dilution for 1 h. Chemiluminescent detection was carried out as previously described (Durrant and Fowler, 1994) and visualized with Kodak film or a GeneGnome system (Syngene, Cambridge, UK).

Anthocyanin quantification

Fresh weight samples (80 mg) were incubated for 2 h in 300 μl of 1% (v/v) HCl/methanol at 30°C. Samples were centrifuged for 2 min at

13 000 *g* and the supernatant extracted with 300 μl of chloroform. The chloroform phase was discarded and the total volume brought up to 1 ml with water. A_{537} minus A_{657} was used as a measure of anthocyanin content.

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Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2327/TPJ2327sm.htm>

Figure S1. Camalexin accumulation in wild type (AR208), *ups1* BC1 and *ups1* BC2 plants.

Figure S2. PR1 and GST1 protein accumulation in wild type (AR208), *ups1* BC1 and *ups1* BC2 plants after *Psm* infection.

Figure S3. *PDF1.2* mRNA levels in wild type (AR208), *ups1* BC1 and *ups1* BC2 plants 3 days after acifluorfen treatment.

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