Forward and reverse genetics to identify genes involved in the age-related resistance response in *Arabidopsis thaliana*

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SUMMARY

Age-related resistance (ARR) occurs in numerous plant species, often resulting in increased disease resistance as plants mature. ARR in *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* is associated with intercellular salicylic acid (SA) accumulation and the transition to flowering. Forward and reverse genetic screens were performed to identify genes required for ARR and to investigate the mechanism of the ARR response. Infiltration of SA into the intercellular space of the ARR-defective mutant *iap1-1* (important for the ARR pathway) partially restored ARR function. Inter- and intracellular SA accumulation was reduced in the mutant *iap1-1* compared with the wild-type, and the SA regulatory gene *EDS1* was also required for ARR. Combining microarray analysis with reverse genetics using T-DNA insertion lines, four additional ARR genes were identified as contributing to ARR: two plant-specific transcription factors of the NAC family [*ANAC055* (At3g15500) and *ANAC092* (At5g39610)], a UDP-glucose glucosyltransferase [*UGT85A1* (At1g22400)] and a cytidine deaminase [*CDA1* (At2g19570)]. These four genes and *IAP1* are also required for ARR to *Hyaloperonospora parasitica*. *IAP1* encodes a key component of ARR that acts upstream of SA accumulation and possibly downstream of *UGT85A1*, *CDA1* and the two NAC transcription factors (*ANAC055*, *ANAC092*).

INTRODUCTION

The interaction of plants and pathogens has led to the evolution of many complex defence pathways (Maor and Shirasu, 2005). An initial line of defence encountered by pathogens is referred to as 'basal resistance', in which the recognition of microbe-associated molecular patterns (MAMPs), such as bacterial flagellin, leads to basal defence induction (Eulgem, 2005; Felix *et al.*,

1999; Heath, 2000; Kunze *et al.*, 2004). Activation of the basal resistance pathway leads to callose deposition in the cell wall (Kim *et al.*, 2005) and the production of reactive oxygen species (Nurnberger *et al.*, 2004) and the expression of defence-related genes (Navarro *et al.*, 2004). Some pathogens have evolved the ability to suppress or elude plant basal resistance (Speth *et al.*, 2007) and plants have responded by evolving additional resistance responses, such as *R* gene-mediated resistance (Maor and Shirasu, 2005), in which a specific resistance (*R*) receptor interacts directly or indirectly with a specific pathogen-derived avirulence product, leading to defence and the hypersensitive response (HR) (Heath, 2000).

Age-related resistance (ARR) has been observed in diverse species, including rice, pepper, cowpea, tobacco and *Arabidopsis thaliana* (reviewed in Develey-Rivière and Galiana, 2008). As some plants mature, they become increasingly resistant to normally virulent pathogens. ARR has been associated with the transition to flowering in some cases or senescence in others, and with the accumulation of secondary metabolites or defence proteins. Whether ARR affords broad-spectrum resistance to different pathogens has not been addressed for most plants; however, mature tobacco plants become more resistant to the oomycetes *Peronospora tabacina* (Wyatt *et al.*, 1991) and *Phytophthora parasitica* (Hugot *et al.*, 1999) and to Tobacco mosaic virus (Fraser, 1981; Yalpani *et al.*, 1991, 1993). Moreover, recent studies have demonstrated that ARR is induced in mature *Arabidopsis* in response to virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) and pv. *maculicola* (Kus *et al.*, 2002), as well as *Hyaloperonospora parasitica* (Rusterucci *et al.*, 2005). During ARR in *Arabidopsis*, *Pst*-induced chlorotic disease symptoms are reduced and an HR-like response is not observed (Kus *et al.*, 2002). Virulent *Pst* growth is reduced 10- to 100-fold (Kus *et al.*, 2002), whereas *H. parasitica* conidiospore production is reduced five- to 50-fold, in mature ARR-competent relative to young plants. The absence of an HR-like response (Kus *et al.*, 2002) suggests that ARR is not a form of *R* gene-mediated resistance. Moreover, ARR provides resistance to two pathogen types (bacterium and

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oomycete), suggesting that it is not a form of basal resistance in which molecules common to a particular pathogen type are recognized by MAMP receptors (Eulgem, 2005). The ability to manifest ARR in *Arabidopsis* is associated with flowering in plants both delayed and accelerated in the transition to flowering (Rusterucci *et al.*, 2005). In standard ARR experiments, Col-0 plants become competent for ARR at the transition to flowering, which occurs at approximately 6 weeks of age in plants grown under short-day conditions (Kus *et al.*, 2002).

Salicylic acid (SA) accumulation is required during many *R* gene-mediated and basal resistance responses and during systemic acquired resistance (SAR) (Delaney *et al.*, 1994, 1995; Uknes *et al.*, 1993; Vernooij *et al.*, 1994). There is mounting evidence that, during SAR, SA acts as a signalling molecule by inducing the oxidation of key cysteines, leading to the translocation of reduced non-expressor of pathogenesis-related 1 (NPR1) into the nucleus, where it interacts with reduced TGA transcription factors, which subsequently up-regulate defence genes, such as *pathogenesis-related 1* (*PR1*) (reviewed in Fobert and Despres, 2005). SA accumulation is also required for the *Arabidopsis* ARR response (Cameron and Zaton, 2004; Kus *et al.*, 2002), as demonstrated by the ARR-defective phenotype of SA-deficient *NahG* (Gaffney *et al.*, 1993) *sid1* and *sid2* (Nawrath and Métraux, 1999). However, ARR occurs in the *npr1-1* (non-expressor of *PR1*) mutant, and *PR1* gene expression is reduced during ARR, suggesting that SA does not play its NPR1-dependent signalling role during ARR, as is seen in other resistance responses, such as SAR and basal resistance (Cameron and Zaton, 2004; Kus *et al.*, 2002).

During ARR, SA accumulation in the intercellular space has been associated with anti-*Pst* activity (Cameron and Zaton, 2004). In addition, the reduction of SA in the intercellular space using salicylate hydroxylase impairs ARR, whereas the addition of SA to the intercellular space enhances ARR in the wild-type and restores ARR in the *sid2* mutant (Cameron and Zaton, 2004). Taken together, these data suggest that intercellular SA may act as an antimicrobial agent against *Pst* during ARR in *Arabidopsis*. Our knowledge of the genes involved in ARR is limited. Therefore, a classical genetic screen for ARR-defective mutants and a microarray/reverse genetics approach were implemented to identify the genes required for ARR.

RESULTS

Identification of *iap1-1* (important for the ARR pathway), an ARR-defective mutant

Classical mutant screening was employed to identify the genes involved in ARR using the standard ARR assay, in which *Arabidopsis* grown under short-day conditions is delayed in the transition to flowering and becomes ARR competent at the transition

to flowering or mature stage at approximately 6 weeks of age (Kus *et al.*, 2002). Approximately 5000 M2 fast neutron-mutagenized Col-0 seeds (Lehle Seeds, Round Rock, TX, USA) were grown for 6 weeks and inoculated with virulent *Pst* [10^6 colony-forming units (cfu)/mL]. Plants displaying disease symptoms similar to those of the ARR-defective *NahG* transgenic line, as compared with wild-type Col-0 ARR-competent plants, were selected as potential mutants (M2 generation) and were re-screened in the M3 and M4 generations. The *iap1-1* mutant supported high levels of bacterial growth (approximately 10^7 cfu/leaf disc) and was therefore compromised in ARR. *iap1-1* was genetically characterized by backcrossing with Col-0 wild-type to produce the F1 and F2 generations. Mature F1 plants supported intermediate levels of bacterial growth and symptoms (approximately 4.0×10^6 cfu/leaf disc) compared with wild-type ARR-competent plants (approximately 6×10^5 cfu/leaf disc). F2 progeny segregated in a 1 : 2 : 1 ratio (chi-squared $P > 0.5$) of ARR-competent ($<1.0 \times 10^5$ cfu/leaf disc) to intermediate ARR [$(2.0\text{--}8.0) \times 10^5$ cfu/leaf disc] to ARR-defective (1.0×10^6 to 3.0×10^7 cfu/leaf disc) respectively, suggesting that *iap1-1* is a semi-dominant mutation. Typical disease symptoms are shown in Fig. 1. *iap1-1* mutant plants displayed no obvious developmental abnormalities. A homozygous *iap1-1* plant line was isolated in the F3 generation and was used in all subsequent experiments.

iap1-1 is not compromised in basal resistance

Pst suppresses basal resistance in young *Arabidopsis* plants (Speth *et al.*, 2007); however, this suppression is not complete, such that *Arabidopsis* exhibits some resistance to *Pst*. For example, basal defence mutants *sid2* and *eds1* (enhanced disease susceptibility 1) are more susceptible than the wild-type to *Pst* (Falk *et al.*, 1999; Nawrath and Métraux, 1999). To determine whether IAP1 is required not only for the ARR response in mature plants, but also for basal resistance in young plants, young [3 weeks post-germination (wpg)] and mature (6 wpg) *iap1-1* and Col-0 were inoculated with *Pst*. Young *iap1-1* and Col-0, plus mature *iap1-1*, supported high *Pst* levels ($>10^7$ cfu/leaf disc) compared with mature ARR-competent Col-0 (Fig. 2a). Bacterial levels in young *iap1-1* and Col-0 were not significantly different (Student's *t*-test), indicating that basal resistance is not affected by the *iap1-1* mutation.

In standard ARR assays, bacterial levels are measured at 3 days post-inoculation (dpi); therefore, bacterial growth was monitored over 3 days to determine when the ARR defect in *iap1-1* is first observed. Bacterial growth in Col-0 and *iap1-1* was similar at 0, 1 and 2 dpi, demonstrating that the number of bacteria infiltrated and *Pst* growth over 2 days was similar in both. At 3 dpi, Col-0 plants displayed ARR, whereas *Pst* levels were 10-fold higher ($P = 0.0042$, Student's *t*-test) in ARR-defective *iap1-1* (Fig. 2b).

Fig. 1 Disease symptoms in Col-0 and *iap1-1* (homozygous and heterozygous). Leaves were inoculated with 10^6 colony-forming units (cfu)/mL *Pseudomonas syringe* pv. *tomato* (*Pst*) and photographed at 3 days post-inoculation (dpi).

ARR to *H. parasitica* is compromised in *iap1-1*

Previous studies have demonstrated that ARR in *Arabidopsis* is also effective against the oomycete pathogen *H. parasitica*, virulent isolate Noco 2 (Rusterucci *et al.*, 2005). To determine whether *IAP1* function is also required for ARR to *H. parasitica*, ARR assays were performed using mature plants inoculated with *H. parasitica* isolate Noco 2 by spraying leaves with 10^6 sporangia/mL. Spore numbers per leaf were quantified at 7 dpi. Col-0 plants supported little *H. parasitica* growth ($\sim 10\,000$ spores/leaf) and therefore displayed ARR; however, $\sim 180\,000$ spores/leaf were observed on *iap1-1* plants, suggesting that functional *IAP1* is also required for the ARR response to *H. parasitica* (Fig. 3a,b).

Fig. 2 *In planta* bacterial levels in *iap1-1*, Col-0 and *NahG*. (a) Col-0, *iap1-1* and *NahG* were inoculated with 10^6 colony-forming units (cfu)/mL *Pseudomonas syringe* pv. *tomato* (*Pst*) at 3 and 6 weeks post-germination (wpg). *In planta* bacterial levels [cfu/leaf disc (ld)] were monitored at 3 days post-inoculation (dpi) and are presented as the mean $\pm$ standard deviation (SD) of three samples. This experiment was repeated four times with similar results. (b) *In planta* bacterial growth in mature *iap1-1* and Col-0 plants (6 wpg) was measured over 3 dpi (10^6 cfu/mL *Pst*) and is presented as the mean of three samples $\pm$ SD. The bacterial density was significantly lower (Student's *t*-test) in Col-0 than *iap1-1* by day 3. This experiment was repeated three times with similar results.

SA partially rescues the *iap1-1* ARR defect

Previous studies in our laboratory have suggested that the ARR pathway leads to an accumulation of SA in the intercellular space, where it may act as an antimicrobial agent during ARR (Cameron and Zaton, 2004; Kus *et al.*, 2002). If *IAP1* is upstream of SA accumulation in the ARR pathway and SA accumulation is a key component of ARR, then SA addition to the intercellular space prior to *Pst* inoculation should rescue the ARR defect in *iap1-1*, as previously observed in *sid2* (Cameron and Zaton, 2004). SA (0.1 mM) was infiltrated into mature *iap1-1* and Col-0 plants, 5 and 24 h prior to inoculation with *Pst* (10^6 cfu/mL). SA

Fig. 3 *Hyaloperonospora parasitica* infection of *iap1-1*. (a) Six-week-old Col-0 and *iap1-1* plants were sprayed with the virulent *H. parasitica* isolate Noco 2. Photographs were taken 7 days after infection. (b) The number of *H. parasitica* spores per infected leaf was quantified using a haemocytometer. This experiment was repeated with similar results.

is still present in the intercellular space at 5 h post-infiltration, whereas it has been absorbed into the cell by 24 h post-infiltration (Cameron and Zaton, 2004). ARR was enhanced 20-fold in Col-0 plants inoculated with *Pst* at 5 h post-SA infiltration (hpSAi) compared with those inoculated at 24 hpSAi or water-infiltrated controls (t -test <0.05 ; Fig. 4a). *Pst* levels were reduced two-fold in *iap1-1* plants challenged with *Pst* at 5 hpSAi compared with those inoculated at 24 hpSAi or water-infiltrated controls (t -test <0.05 ; Fig. 4a). In two additional experiments, *Pst* levels were reduced two- and four-fold in *iap1-1* inoculated with *Pst* at 5 hpSAi compared with water-infiltrated controls (data not shown). ARR was enhanced in Col-0 and *iap1-1* plants only when SA was still present in the intercellular space (5 hpSAi). *Pst* levels were reduced in *iap1-1* to a lesser extent compared with Col-0; therefore, the ARR defect in *iap1-1* was partially restored by intercellular SA application.

Intercellular SA accumulation is reduced in *iap1-1* in response to *Pst*

The ARR-defective phenotype of *iap1-1* was partially rescued by intercellular SA infiltration, suggesting that *IAP1* lies upstream of intercellular SA accumulation in the ARR pathway, leading to the prediction that *iap1-1* plants will accumulate little intercellular SA relative to wild-type plants. To address this question, intercellular washing fluids (IWFs) were collected from mature Col-0

Fig. 4 Salicylic acid (SA) application and SA levels in *iap1-1* and Col-0. (a) Leaves of 5-week-old plants were inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst*) at 5 or 24 h post-SA infiltration (hpi; with 0.1 mM). *In planta* bacterial levels [colony-forming units/leaf disc (cfu/ld)] were monitored 3 days after *Pst* inoculation (SA, *Pst*) and compared with control treatments (H, *Pst*), where water infiltration was followed by inoculation with *Pst* 24 h later. Asterisks indicate a significant decrease in bacterial density in SA-treated plants vs. the corresponding water-treated plants (Student's t -test). The mean $\pm$ standard deviation (SD) of three replicate samples is shown. Experiments in which *Pst* was inoculated at 5 hpSAi were repeated twice with similar results. (b) Intercellular washing fluids (IWFs) were collected from mature leaves that were either mock-inoculated or inoculated with *Pst* (10^6 cfu/mL). SA levels were determined and are presented as ng/mL of IWF collected. Asterisks indicate a significant difference in SA levels in mock-inoculated leaves compared with leaves inoculated with *Pst* (Student's t -test). The mean $\pm$ SD of three replicate samples is shown. This experiment was repeated twice with similar results. (c) Mature leaves were mock-inoculated or inoculated with *Pst* (10^6 cfu/mL). IWFs were removed and total SA levels (SA plus SA glucosides) were determined in leaves and presented as ng/g fresh weight. Asterisks indicate a significant difference in SA levels in mock-inoculated leaves compared with leaves inoculated with *Pst* (Student's t -test). The mean $\pm$ SD of three replicate samples is shown. This experiment was repeated with similar results.

and *iap1-1* plants at 24 h post mock-inoculation with 10 mM MgCl_2 or inoculation with *Pst* (10^6 cfu/mL). This time point was chosen, as previous work has demonstrated that intercellular SA levels increase in IWFs by 24 h post-inoculation (hpi) (Cameron and Zaton, 2004). Gas chromatography-mass spectrometry (GC-MS) analysis was employed to measure free SA levels in IWFs collected from ARR-competent Col-0 (*in planta Pst* $\sim 10^5$ cfu/leaf disc at 3 dpi) and ARR-incompetent *iap1-1* (*in planta Pst* $\sim 10^7$ cfu/leaf disc at 3 dpi) plants. Free SA was measured, as previous experiments support the hypothesis that unconjugated SA acts in the intercellular space as an antimicrobial agent during ARR (Cameron and Zaton, 2004; Kus *et al.*, 2002); moreover, experiments in tobacco indicate that conjugated SA is not biologically active (reviewed in Ryals *et al.*, 1994). IWFs collected from Col-0 leaves inoculated with *Pst* accumulated ~ 190 ng/mL SA, compared with ~ 60 ng/mL in IWFs collected from mock-inoculated leaves (*t*-test <0.05 ; Fig. 4b). No significant difference in intercellular SA accumulation was observed in IWFs collected from *iap1-1* leaves that were mock-inoculated or inoculated with *Pst* (Fig. 4b). These data demonstrate that *iap1-1* does not accumulate intercellular SA in response to *Pst*, and suggest that IAP1 is upstream of intercellular SA accumulation in the ARR pathway.

Intracellular SA accumulation is reduced in *iap1-1* in response to *Pst*

The *iap1-1* mutation results in a loss of accumulation of SA in the intercellular space in response to *Pst*, suggesting that IAP1 is involved in SA biosynthesis or perhaps in the transport of SA to the intercellular space. If *iap1-1* is involved in SA biosynthesis, intracellular SA accumulation in response to *Pst* should also be reduced in *iap1-1*. Mature Col-0 and *iap1-1* intracellular SA levels were determined in leaves from which the IWFs were removed at 24 h post mock-inoculation or inoculation with *Pst* (10^6 cfu/mL). This time point was chosen, as previous work has demonstrated that intercellular SA levels increase by 24 hpi during ARR (Cameron and Zaton, 2004). GC-MS analysis was employed to measure total SA levels (SA plus SA glucosides) in ARR-competent Col-0 (*Pst* $\sim 10^5$ cfu/leaf disc at 3 dpi) and ARR-incompetent *iap1-1* (*Pst* $\sim 10^7$ cfu/leaf disc at 3 dpi) plants. Col-0 leaves inoculated with *Pst* accumulated $\sim 10\,000$ ng/g fresh weight SA compared with ~ 3000 ng/g fresh weight from mock-inoculated leaves (Fig. 4c). Total intracellular SA levels were reduced to 1000 ng/g fresh weight in *iap1-1* leaves inoculated with *Pst* or mock-inoculated (Fig. 4c). These data demonstrate that *iap1-1* does not accumulate intracellular SA in response to *Pst*, and suggests that IAP1 is upstream of intracellular SA accumulation in the ARR pathway.

eds1-1 is ARR defective

EDS1 is an essential regulator necessary for the accumulation of SA, which, in turn, acts as a signalling molecule to up-regulate downstream defences (Falk *et al.*, 1999; Feys *et al.*, 2001, 2005). As *EDS1* is involved in SA accumulation, it was hypothesized that it may play a role in ARR. *eds1-1* and wild-type Ws plants were assayed for ARR by inoculating young and mature plants with *Pst*. A significant decrease in bacterial growth (*t*-test <0.05) was not observed in *eds1-1*, as the bacterial density remained high ($\sim 10^7$) in both young and mature plants (Fig. 5a). Conversely, *in planta* bacterial levels were reduced 17-fold in mature relative to young wild-type plants. These data suggest that *EDS1* is required for the ARR response.

As *EDS1* is involved in SA accumulation and intercellular SA infiltration partially rescues the *iap1-1* ARR defect, a similar experiment was performed with *eds1-1*. The addition of SA to the

Fig. 5 (a) *In planta* bacterial levels in *eds1-1*, *jar1-1*, *jin1-1*, Col-0 and Ws. Plants were inoculated with 10^6 colony-forming units (cfu)/mL *Pseudomonas syringae* pv. *tomato* (*Pst*) at 3 and 6 weeks post-germination (wpg). *In planta* bacterial levels [cfu/leaf disc (cfu/ld)] were monitored at 3 days post-inoculation (dpi) and are presented as the mean $\pm$ standard deviation (SD) of three samples. This experiment was repeated once for *jin1* and twice for *eds1-1* and *jar1-1* with similar results. (b) *eds1-1* and Col-0 plants (5-week-old) were infiltrated with water (H, *Pst*-5) or salicylic acid (SA) (0.1 mM) (SA, *Pst*-5), followed by inoculation with *Pst* 5 h later, as in Fig. 4. This experiment was repeated twice with similar results.

intercellular space of *eds1-1* prior to inoculation with *Pst* resulted in a two-fold reduction (*t*-test not statistically significant) in bacterial density relative to water-infiltrated control plants; however, these plants still supported high levels of *Pst* ($\sim 2 \times 10^7$ cfu/leaf disc) (Fig. 5b). It is interesting to note that, in one of two other experiments, there was a statistically significant reduction (*t*-test <0.05) in *Pst* growth in SA-infiltrated *eds1-1* plants; however, *Pst* levels remained high (10^7 cfu/leaf disc, data not shown). Thus, the addition of SA to the intercellular space of *eds1-1* plants did not result in the rescue of the *eds1-1* ARR defect.

The ARR response occurs in *jar1-1* and *jln1-1*

Jasmonic acid (JA) and related metabolites are lipid-derived compounds which act as signals in defence to some pathogens (often necrotrophic) and in the wound response to insects, as well as in plant growth and development (reviewed in Wasternack, 2007). Work to date suggests that SA acts as an anti-*Pst* agent in the intercellular space during ARR, rather than as a signal for the up-regulation of defence genes such as *PR1* (Cameron and Zaton, 2004; Kus *et al.*, 2002). Therefore, other signalling molecules, such as JA, may be required for ARR signalling. To test this hypothesis, two JA mutants, *jar1-1* (*jasmonate-resistant 1*) and *jln1-1* (*jasmonate-insensitive 1*), were assayed for ARR competence.

JAR1 encodes a JA-amino synthetase that activates JA for optimal signalling in *Arabidopsis* by conjugating it to amino acids, such as isoleucine (Ile) (Staswick and Tiryaki, 2004). Therefore, *jar1-1* mutants do not accumulate activated JA-Ile and are defective for downstream JA-Ile-induced defence signalling (Staswick and Tiryaki, 2004). Col-0 and *jar1-1* mutants were assayed for ARR competence by inoculating both young and mature plants with *Pst* (Fig. 5a). A 10-fold decrease in bacterial density was observed in mature relative to young Col-0 and *jar1-1* plants. This significant decrease in bacterial growth (*t*-test <0.05) in mature plants suggests that *jar1-1* mutants maintain a functional ARR pathway despite the disruption in JA signalling.

Studies have indicated that the phytotoxin coronatine, produced by the *P. syringae* group of pathovars (including *Pst*), acts as a molecular mimic of JA-Ile (Brooks *et al.*, 2005; Krumm *et al.*, 1995; Staswick and Tiryaki, 2004) to elicit the JA signalling pathway downstream of *JAR1* and *JIN1*, leading to the suppression of SA-mediated defences, and thereby favouring *Pst* growth (Laurie-Berry *et al.*, 2006). Therefore, although studies with *jar1-1* and *Pst* will reveal whether a JA-dependent response requires JA-Ile, it cannot establish whether an intact JA signalling pathway is required (Laurie-Berry *et al.*, 2006). Instead, studies with jasmonate-insensitive mutants, such as *jln1-1*, are necessary. *JIN1* is a MYC2 transcription factor (Lorenzo *et al.*, 2004) that functions downstream of *JAR1*-synthesized JA-Ile

and *Pst*-produced coronatine, and therefore is required for JA defence signalling, even in the presence of coronatine-producing pathogens (Laurie-Berry *et al.*, 2006). Young and mature Col-0 and *jln1-1* plants were tested for their ability to display ARR by inoculation with *Pst* (Fig. 5a). A significant 10-fold decrease (*t*-test <0.05) in bacterial levels in mature relative to young Col-0 and *jln1-1* plants confirms that *jln1-1* mutants have an intact ARR pathway, suggesting that *JIN1*-dependent JA signalling is not required for ARR.

Identification of ARR-associated genes using microarray analysis

A microarray experiment was conducted to identify genes that are differentially regulated during ARR. Gene expression was compared between mature Col-0 plants that were mock-inoculated or inoculated with *Pst*. It was possible to analyse only one time point after inoculation, and therefore samples were collected at 12 hpi on the basis of previous intercellular SA reduction experiments using salicylate hydroxylase, which suggested that intercellular SA negatively affects *Pst* growth during ARR between 5 and 24 h after inoculation (Cameron and Zaton, 2004). RNA extraction and cDNA synthesis were performed on three replicates of each treatment, and each replicate was analysed using an Affymetrix GeneChip microarray (see Supporting Information). The signal strength of each gene was averaged over the three replicates of each treatment, and the average signal strength of each gene was compared between samples that were mock-inoculated or inoculated with *Pst*. Significance analysis of microarrays (SAM; Tusher *et al.*, 2001) determined which genes were significantly up- or down-regulated. SAM identifies fewer false-positive up- or down-regulated genes than an average *t*-test when dealing with microarray data (Tusher *et al.*, 2001). A delta value of 1.483 resulted in the identification of 231 differentially expressed genes with a corresponding false discovery rate (*q* value) of $<15\%$. SAM identified 125 significantly up-regulated and 105 significantly down-regulated genes. The complete list of genes identified in this microarray is available at http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi. Genes up-regulated greater than two-fold are listed in Table 1 and significantly down-regulated genes are listed in Table S2 (see Supporting Information).

Three members of the CYTOCHROME P450 (*CYP*) gene family were differentially regulated in the microarray. The most highly up-regulated microarray gene, *CYP71A13* (~ 3.5 -fold up-regulation, Table 1), plays a role in camalexin production in *Arabidopsis* (Nafisi *et al.*, 2007). *CYP85A2* was also up-regulated (~ 1.4 -fold, http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi), and is involved in brassinosteroid (BR) biosynthesis, which is important for leaf and flower development, flowering time and defence to pathogens (Kim *et al.*, 2005; Nakashita

Table 1 Microarray of age-related resistance (ARR)-associated genes (up-regulated $\geq$ two-fold)*.

AGI no.†	Probe ID no. (affynames)‡	Transcript signal ratio§	q value (%)¶	Functional annotation**
At2g30770	267567_at	3.5	3.8	CYP71A13: cytochrome P450 71A13 may be involved in defence response, camalexin biosynthesis, indoleacetaldoxime dehydratase activity
At5g24780 At5g24770	245928_s_at	2.8	3.0	VSP1 and VSP2: vegetative storage protein 1 shows putative phosphatase activity, defence, jasmonic acid responses; vegetative storage protein 2 shows putative phosphatase activity, anti-insect activity, induced by abscisic acid, jasmonic acid, salt, water deficiency, senescence, insect feeding and wounding
At1g75750	262947_at	2.7	3.0	GASA1: GA-responsive GAST1 may be responsive to gibberellic acid, abscisic acid, brassinosteroids; may be localized to the cell wall
At3g47420	252414_at	2.4	3.0	Glycerol-3-phosphate transporter, putative cell membrane location, carbohydrate transport, sugar:hydrogen symporter activity
At3g01970	258975_at	2.3	3.8	WRKY45: putative WRKY transcription factor
At5g39610	249467_at	2.3	3.7	ANAC092: NAC transcription factor
At3g15500	258395_at	2.3	3.5	ANAC055: NAC transcription factor
At5g56870	247954_at	2.2	3.7	β -GAL4: β -galactosidase 4, putative carbohydrate metabolism
At5g13080	245976_at	2.1	3.8	WRKY75: putative WRKY transcription factor involved in nutrient import, lateral root development, response to stress, nutrients levels
At2g19570	265943_at	2.1	3.7	CDA1: cytidine deaminase 1
At1g22400	261934_at	2.1	3.8	UGT85A1: UDP-glucosyl transferase 85A1
At3g50770	252136_at	2.0	3.0	CML41: calmodulin-related protein 41, putative calcium ion binding
At1g70690	260179_at	2.0	3.7	Kinase receptor-like protein, putative function in response to pathogens

*Gene expression in mature plants was compared between mock- and *Pseudomonas syringae* pv. *tomato* (*Pst*)-inoculated plants at 12 h post-inoculation.

†Gene AGI number.

‡Probe ID number.

§Transcript signal ratio represents the fold change in transcript signal after *Pst* inoculation compared with mock inoculation.

¶q value represents the false discovery rate assigned by significance analysis of microarrays (SAM).

**Functional annotation according to The *Arabidopsis* Information Resource 8 (TAIR 8), May 16, 2008.

et al., 2003; Nomura *et al.*, 2005); little is known about the biochemical role of *CYP72A15* (~0.77-fold, http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi).

Members of the NAC (petunia *NO APICAL MERISTEM* and *Arabidopsis* *ATAF1*, and *CUP-SHAPED COTYLEDONS*) and *WRKY* plant transcription factor families were up-regulated in the microarray (*ANAC055* and *ANAC092*, ~2.3-fold; *ANAC029*, ~1.8-fold; *WRKY45*, ~2.3-fold; *WRKY75*, ~2.1-fold) (Table 1, http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi). The NAC and *WRKY* gene families are implicated in a number of biotic and abiotic stress responses (Olsen *et al.*, 2005; Rushton and Somssich, 1998). *ANAC055* and *ANAC092* are expressed in response to NaCl and abscisic acid (ABA) treatments (He *et al.*, 2005; Tran *et al.*, 2004). In addition, *ANAC055* gene expression is up-regulated in response to methyl jasmonate (MeJA) treatments in a COI1-dependent manner (Bu *et al.*, 2008; He *et al.*, 2005). *ANAC029* appears to be a positive regulator of the onset of leaf senescence (Guo and Gan, 2006). NAC and *WRKY* genes may contribute to ARR gene regulation.

A CYTIDINE DEAMINASE (*CDA*) gene family member (~2.1-fold) was up-regulated and two URIDINE DIPHOSPHATE-GLUCOSYLTRANSFERASE (*UGT*) gene family members were identified in the microarray (Table 1). *CDA* genes play a role in RNA editing by converting cytidine to uridine (Faivre-Nitschke *et al.*, 1999; Gott and Emeson, 2000; Vincenzetti *et al.*, 1999); therefore, post-transcriptional gene regulation may be important

during ARR. *UGT85A1* was up-regulated (~2.1-fold: Table 1), whereas *UGT73B2* was down-regulated (~0.8-fold: http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_browser.cgi). *UGT85A1* expression is observed in leaf, stem, flower and root tissue (Woo *et al.*, 2007), whereas *UGT73B* is expressed in response to some pathogens, oxidative stress, wounding and SA or MeJA application (Langlois-Meurinne *et al.*, 2005). *Arabidopsis* contains ~120 putative *UGTs* (Li *et al.*, 2001) that are involved in the conjugation of glucose to proteins and secondary metabolites, including SA (Jones *et al.*, 1999; Li *et al.*, 2001; Yalpani *et al.*, 1992). The ARR-associated *UGTs* may be involved in the modification of SA or another metabolite that is important for ARR.

Microarray/reverse genetics identifies genes required for ARR to *Pst* and *H. parasitica*

By combining the ability to identify ARR-associated genes using microarray analysis with reverse genetics using T-DNA insertion lines (*Arabidopsis* Biological Resource Center, ABRC), genes that are not only up-regulated during ARR, but whose function contributes to ARR, can be identified. A number of ARR microarray genes, *ANAC055* (SALK_014331), *ANAC092* (SALK_090154), *CDA1* (SALK_036597) and *UGT85A1* (SALK_085809), β -galactosidase (At5g56870, SALK_093071) and putative glycerol-3-phosphate permease (At3g47420, SALK_096228)

were listed as containing T-DNA insertions by ABRC. Plants containing homozygous T-DNA insertions in their respective genes were identified by polymerase chain reaction (PCR). Reverse transcriptase (RT)-PCR with gene-specific primers was performed to confirm that the T-DNA insertion reduced the expression of these genes (Fig. S1, see Supporting Information). RT-PCR analysis demonstrated that both *ANAC092* and *ANAC055* gene expression was abolished in the *anac092* and *anac055* mutants, confirming the study by He *et al.* (2005) with *anac092* (Fig. S1a). However, expression of *UGT85A1* and *CDA1* in the *ugt85A1* and *cda1* mutants was not completely abolished; rather expression was reduced compared with the wild-type (Fig. S1b,c). These T-DNA mutants were assayed for ARR by comparing *in planta* *Pst* levels in young and mature plants compared with Col-0 control plants. T-DNA insertion lines in β -galactosidase (SALK_093071) and the putative glycerol-3-phosphate permease (SALK_096228) displayed a wild-type ARR response (data not shown). In contrast, mature T-DNA insertion mutants *anac055*, *anac092*, *cda1* and *ugt85A1* were more susceptible to *Pst* compared with the wild-type ARR-competent Col-0 (Fig. 6a). Both *anac092* and *anac055* were compromised in ARR, as demonstrated by a modest 10- and 14-fold reduction in *Pst* levels in mature relative to young plants, respectively (Expt #1 and #2, Fig. 6a). In contrast, a full ARR response was observed in mature relative to young wild-type Col-0 (154-fold *Pst* reduction). The *cda1* mutant was compromised in ARR, but to a lesser extent, as it still displayed a 65-fold reduction in *Pst* density in mature relative to young plants (Expt #3, Fig. 6a). Although the ARR response was rather modest in Col-0 in the *ugt85A1* experiment, there was little reduction in *Pst* density in mature relative to young *ugt85A1* plants, indicating that *ugt85A1* is compromised in ARR (Expt #4, Fig. 6a).

Another way to quantify ARR is to compare *Pst* levels in mature ARR-competent Col-0 vs. each mutant (Fig. 6a, Expt #1–#4), such that higher *Pst* levels in the mutants equals greater susceptibility or compromised ARR. Compared with mature Col-0, mature *anac055* and *anac092* plants were seven-fold more susceptible, mature *cda1* plants were two-fold more susceptible and mature *ugt85A1* plants were five-fold more susceptible than their respective Col-0 controls (*t*-test, $P < 0.05$ between each Col-0/mutant pair in Fig. 6a). In at least two other experiments (data not shown), similar results were observed (*anac055* and *anac092*, two- to seven-fold more susceptible; *cda1*, two- to six-fold more susceptible; *ugt85A1*, three- to five-fold more susceptible). These data suggest that *anac055*, *anac092*, *cda1* and *ugt85A1* mutants are compromised in ARR. However, mature mutant plants still supported a 10–65-fold decrease in *Pst* density relative to young mutant plants (Fig. 6a), suggesting that ARR is not completely abolished in these plants.

Basal resistance to *Pst* in young plants was also monitored in these ARR mutants to determine whether these ARR genes are

Fig. 6 (a) *In planta* *Pseudomonas syringe* pv. *tomato* (*Pst*) levels in young and mature Col-0, *anac092*, *anac055*, *cda1*, *ugt85A1* and *sid2*. Plants were inoculated with 10⁶ colony-forming units (cfu)/mL *Pst* at 3, 5 or 6 weeks post-germination (wpg). *In planta* bacterial levels [cfu/leaf disc (cfu/ld)] were monitored at 3 days post-inoculation (dpi) and are presented as the mean $\pm$ standard deviation (SD) of three samples. Col-0 controls are presented for each independent experiment. Significant differences between mature Col-0 and mutants are indicated by an asterisk ($P < 0.05$ Student's *t*-test). Different letters represent significant differences between mature genotypes in Expt. 3 (Tukey's honest significant difference, $P < 0.05$). These experiments were repeated at least twice with similar results. (b) Mature (6 wpg) Col-0, *anac092*, *anac055*, *cda1* and *ugt85A1* were spray inoculated with *Hyaloperonospora parasitica* isolate Noco 2. The number of *H. parasitica* spores per infected leaf was quantified using a haemocytometer at 7 dpi. This experiment was repeated twice with similar results.

also required for basal defence. Young *anac055*, *anac092*, *cda1* and *ugt85A1* plants supported high *in planta* *Pst* levels, similar to Col-0 (~10⁷ cfu/leaf disc, Fig. 6a), suggesting that functional *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* are not required for basal resistance.

The ARR response in *Arabidopsis* is also effective in reducing the growth of *H. parasitica* (Rusterucci *et al.*, 2005). Therefore, to determine whether *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* are also required for ARR to *H. parasitica*, mature Col-0, *anac055*, *anac092*, *cda1* and *ugt85A1* plants were inoculated

Table 2 Growth of *Hyaloperonospora parasitica* in age-related resistance (ARR) mutants.

Genotype	No. of plants inoculated*	No. of leaves inoculated*	Infection (%)†
Col-0	10	168	50
<i>anac055</i>	11	181	81
<i>anac092</i>	10	164	100
<i>cda1</i>	8	118	71
<i>ugt85a1</i>	12	188	91

**Hyaloperonospora parasitica* isolate Noco 2 suspension (10^6 conidiosporangia/mL) was sprayed on to Col-0 and ARR mutant leaves [6 weeks post-germination (wpg)], and a plant was considered to be infected if sporangia were observed on most leaves (~20 leaves/plant).

†Infection (%) was calculated as the number of infected plants divided by the total number of plants inoculated. This experiment was repeated twice with similar results.

with *H. parasitica* isolate Noco 2, and the number of plants that supported *H. parasitica* sporulation at 8 dpi was determined (Table 2, Fig. 6b). Only 50% of mature Col-0 plants supported *H. parasitica* sporulation, whereas 100% of *anac092*, 91% of *ugt85a1*, 81% of *anac055* and 71% of *cda1* plants supported *H. parasitica* sporulation (Table 2), suggesting that *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* contribute to the ARR response to *H. parasitica*. Spores per infected leaf were also quantified for these plants. Mature Col-0 plants supported 4.0×10^3 spores per infected leaf, whereas spore levels were six-fold higher on *anac092*, 2.5-fold higher on *anac055* and five-fold higher on *cda1* plants (Fig. 6b, *t*-test $P < 0.05$). Spore levels were similar on *ugt85a1* and Col-0, suggesting that *ANAC055*, *ANAC092* and *CDA1* are more important for ARR to *H. parasitica* than is *UGT85A1*.

ARR gene expression analysis in Col-0 and *iap1-1*

The semidominant loss-of-function nature of *iap1-1* suggests that *IAP1* may act as a positive regulator of downstream genes in the ARR pathway. RT-PCR was used to determine whether SA-associated genes (*ICS1*, *SID1*, *EDS1*) and the ARR genes identified in this study were expressed differentially in *iap1-1* compared with Col-0. Leaves were collected after inoculation with *Pst* (10^6 cfu/mL, 7–24 hpi) or left untreated. Expression of *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, *ICS1*, *SID1* and *EDS1* was similar in *iap1-1* and Col-0 (Fig. S2a,b, Table S1, see Supporting Information), suggesting that *IAP1* is not upstream of these genes in the ARR pathway. *EDS1* was constitutively expressed in untreated leaves and its expression was not increased in response to virulent *Pst*, as observed previously (Falk *et al.*, 1999). It is interesting to note that *ICS1* (*SID2*), the penultimate enzyme in the chorismate biosynthesis pathway (Wildermuth *et al.*, 2001), and *SID1*, a putative chloroplast SA transporter (Nawrath *et al.*, 2002), were expressed early at 7 and 14 hpi

Fig. 7 Age-related resistance (ARR) gene expression analysis. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of leaves collected from 3 and 6 weeks post-germination (wpg) Col-0 at <5 min post-inoculation (mpi) or 12, 24 and 48 h post-inoculation (hpi) with 10^6 colony-forming units (cfu)/mL *Pseudomonas syringae* pv. *tomato* (*Pst*); 28 PCR cycles were used for *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1* and *ACTIN* primers. This experiment was replicated once with similar results.

(Fig. S2a,b), but these genes were not identified in the microarray, which represents ARR gene expression at 12 hpi. Plants used in the microarray experiment were grown in growth chambers without added humidity in the winter (60% humidity), whereas all other experiments presented in this paper were performed in chambers with added humidity (humidity maintained between 70 and 85%). *Pst* growth is enhanced under conditions of higher humidity (Agrios, 2005); therefore, in higher humidity chambers, *Pst* would begin the infection process quickly, leading to earlier initiation of ARR (Fig. S3a,b, see Supporting Information).

As *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* contribute to ARR in mature plants and do not appear to be required for basal resistance in young plants (Fig. 6a), ARR gene expression patterns may differ in young ARR-incompetent plants relative to mature ARR-competent plants. RT-PCR was performed on leaves collected from young (*in planta Pst* levels $\sim 10^7$ cfu/leaf disc) and mature (*in planta Pst* levels $\sim 10^5$ cfu/leaf disc) Col-0 plants at <5 min post-inoculation (mpi) and at 12, 24 and 48 hpi with *Pst* (Figs 7, S3). *CDA1* and *UGT85A1* were similarly expressed in young and mature plants. *ANAC055* was highly expressed at 48 hpi in young relative to mature plants (Figs 7, S3). Weak expression of *ANAC092* was observed in young plants until 48 hpi; however, *ANAC092* was expressed to similar levels throughout ARR in mature plants (5 mpi, 12, 24 and 48 hpi).

DISCUSSION

Classical mutant screening identified the ARR-defective mutant *iap1-1*. Young and mature *iap1-1* plants are equally susceptible to virulent *Pst*, unlike ARR-competent wild-type plants. In addition, *iap1-1* is defective in ARR to *H. parasitica*. Together, these

data indicate that *IAP1* is required for the ARR response to both a bacterial and oomycete pathogen, suggesting that ARR may provide broad-spectrum resistance, as has been documented for ARR in tobacco (Fraser, 1981; Hugot *et al.*, 1999; Wyatt *et al.*, 1991; Yalpani *et al.*, 1991, 1993).

SA infiltration prior to the *Pst* inoculation of *iap1-1* resulted in a two-fold decrease in bacterial growth, partially restoring ARR, suggesting that SA accumulation may be impaired in *iap1-1* and that *IAP1* may be upstream of SA accumulation. Subsequent analysis indicated that *iap1-1* is deficient in both intra- and intercellular SA accumulation during ARR, suggesting that *IAP1* functions upstream of SA biosynthesis. During SA infiltration experiments, ARR was enhanced to a greater extent in Col-0 than in *iap1-1*, perhaps because *iap1-1* has lower basal intracellular SA levels than Col-0, creating a large concentration gradient between inter- and intracellular compartments. Therefore, infiltrated SA remained in the intercellular space for an insufficient length of time to rescue the *iap1-1* ARR defect. This phenomenon has been observed previously in *NahG* plants (Cameron and Zaton, 2004) and may explain why the *eds1-1* ARR defect was not rescued by SA infiltration. Alternatively, it is possible that infiltration of SA into the intercellular space via a needle-less syringe is not as effective as the natural SA delivery method that is hypothesized to include SA transport to the cell wall in the vicinity of cell wall-associated bacterial colonies (Cameron and Zaton, 2004). In addition, the fact that SA addition alone did not rescue the *iap1-1* defect suggests that intercellular accumulation of other antimicrobial compounds may be required for a successful ARR response.

EDS1 was tested for its involvement in ARR because it is required for SA accumulation in response to virulent *Pst* (Feys *et al.*, 2001). Mature *eds1-1* plants did not exhibit ARR, indicating that functional *EDS1* is required for ARR. Phytoalexin-deficient 4 (*PAD4*) function is also required for ARR (Cameron and Zaton, 2004), and *EDS1* and *PAD4* are thought to act together in a complex that leads to SA accumulation and defence signalling during basal resistance to virulent *Pseudomonas* (Feys *et al.*, 2001; Wiermer *et al.*, 2005). Therefore, it is possible that the *EDS1*–*PAD4* complex is a component of both basal resistance and ARR in *Arabidopsis*.

ARR microarray analysis at 12 hpi identified over 200 differentially expressed genes. The most highly expressed gene in the ARR microarray analysis is a P450, *CYP71A13*, which has recently been shown to be involved in the biosynthesis of the phytoalexin camalexin (Nafisi *et al.*, 2007). However, ARR occurs in the *pad3-1* camalexin mutant (Kus *et al.*, 2002), suggesting that the phytoalexin camalexin is not required for ARR. A putative T-DNA insertion mutant in *CYP71A13* is currently being characterized to determine whether this P450 produces a secondary metabolite that is required during ARR. Another cytochrome P450 gene, *CYP85A2*, was also up-regulated in the microarray and has been implicated

in the BR signalling pathway, suggesting that BR signalling may be involved in ARR. BR signalling has been implicated in defence, as demonstrated by BR-induced resistance in potato and tomato to a variety of pathogens (reviewed in Krishna, 2003). Increased resistance in potato was associated with elevated levels of ABA and ethylene, but not SA, suggesting that BR-induced defence may be independent of SA (Krishna, 2003). As our accumulated work suggests that intercellular SA accumulation, rather than NPR1/SA signalling, is required for ARR, it seems probable that other signalling molecules, such as BR, may be involved in the ARR pathway.

A number of genes that contribute to the ARR response have been identified, indicating that a single time point (12 hpi) snapshot of ARR gene expression is an inexpensive yet effective method of gene discovery. ARR gene discovery is ongoing in our laboratory as new T-DNA insertions in additional ARR microarray genes become available. T-DNA insertion mutants in *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* were compromised in ARR to *Pst*. However, ARR was not completely abolished, suggesting that other members of each respective gene family may be compensating for the mutations in *anac055*, *anac092*, *cda1* and *ugt85A1*, or there may be other signalling pathways that contribute to ARR. *ANAC092* is expressed at early time points after the inoculation of mature ARR-competent relative to young ARR-incompetent plants, suggesting that early expression of *ANAC092* is required for a successful ARR response.

ANAC055 and *ANAC092* belong to the large plant-specific NAC gene family, consisting of 105 and 75 putative members in *Arabidopsis* and rice, respectively (Ooka *et al.*, 2003). These genes encode transcription factors with a conserved N-terminal DNA-binding NAC domain (Aida *et al.*, 1997, 1999; Duval *et al.*, 2002; Hegedus *et al.*, 2003; Taoka *et al.*, 2004; Vroemen *et al.*, 2003; Xie *et al.*, 2000), and a variable C-terminal transactivation domain (Duval *et al.*, 2002; Hegedus *et al.*, 2003; Taoka *et al.*, 2004; Xie *et al.*, 2000). Studies have indicated that *ANAC055* and *ANAC092* regulate the expression of genes during abiotic stress responses (He *et al.*, 2005; Tran *et al.*, 2004) and *ANAC055* regulates the expression of JA-related genes during fungal infection (Bu *et al.*, 2008). The identification of targets of the putative *ANAC055* and *ANAC092* transcription factors will provide insights into the ARR signalling pathway. In addition, *ANAC055* and *ANAC092* are members of the same gene family and may play redundant roles during ARR; therefore, the analysis of *anac055anac092* double mutants is currently underway (Fig. S1a).

The *jar1-1* and *jin1-1* JA pathway mutants were competent for the ARR response, strongly suggesting that this branch of the JA signalling pathway is not required for the ARR response. This is interesting in light of the fact that a number of JA-associated genes were up-regulated in the ARR microarray (*VSP1*, *ANAC055*), and suggests that the *Pst*-produced JA mimic, coronatine (Staswick and Tiryaki, 2004), may be acting to up-regulate

the expression of these genes. Alternatively, it is possible that *VSP1* and *ANAC055* are expressed during ARR via a *JAR1*- and *JIN1*-independent branch of the plant JA pathway.

The ARR-defective phenotypes of *eds1* and *iap1-1* indicate that these genes are necessary for a successful ARR response, whereas the partially ARR-defective phenotypes of *anac055*, *anac092*, *cda1* and *ugt85A1* suggest that these genes contribute to differing degrees to the ARR response to *Pst*. In addition, ARR to *H. parasitica* was also compromised in *anac055*, *anac092*, *cda1* and, to a lesser extent, *ugt85a1*, suggesting that these genes, together with *IAP1*, are important for ARR to both pathogens. Our expression analysis indicates that *IAP1* may lie downstream of *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1*, *ICS1* and *EDS1*, but upstream of SA accumulation in the ARR pathway. Mapping of *IAP1* and *iap1-1* is ongoing and will provide insight into the role of *IAP1* during the ARR response. The absence of the HR (Kus *et al.*, 2002) suggests that ARR is not a form of *R* gene-mediated resistance. Moreover, ARR provides resistance to two pathogen types (bacterium and oomycete), suggesting that it is not a form of basal resistance in which molecules common to a particular pathogen type are recognized by MAMP receptors (Eulgem, 2005). We speculate that ARR is a developmentally controlled response that occurs at the transition to flowering, allowing mature *Arabidopsis* to recognize the presence of *Pst* and *H. parasitica* to elicit ARR signalling and intercellular antimicrobial SA accumulation.

EXPERIMENTAL METHODS

Plant growth conditions

The *A. thaliana* mutant *iap1-1* [*IAP1* registered on The *Arabidopsis* Information Resource (TAIR) gene symbol/name web page] was used in conjunction with controls, wild-type Columbia (Col-0) and Wassilewskija (Ws), transgenic *NahG* (K. Lawton, Syngenta, Research Triangle Park, NC, USA) and *sid2* (C. Nawrath, University of Fribourg, Fribourg, Switzerland). Additional mutants examined included *eds1-1* (TAIR), *jar1-1* and *jln1-1* (B. Kunkel, Washington University, St. Louis, MO, USA), *anac055* (SALK_014331), *anac092* (SALK_090154), *cda1* (SALK_036597), *ugt85A1* (SALK_085809), *glycerol-3-PO₄ permease* (SALK_096228) and *β-galactosidase* (SALK_093071) (*Arabidopsis* Biological Resource Center, Ohio State University, Columbus, OH, USA; Alonso *et al.* (2003). Fast neutron-mutagenized M2 Col-0 (glabrous) seeds were obtained from Lehle Seeds and used in the classical ARR mutant screen. Seeds were surface sterilized, germinated and grown as described previously (Rusterucci *et al.*, 2005). The humidity ranged between 75% (winter) and 85% (summer). Plants were grown under a short-day 9-h photoperiod at 22–24 °C. *Arabidopsis* is delayed in the transition to flowering when grown under short-day con-

ditions (Piñero and Coupland, 1998). In standard ARR experiments, Col-0 plants become competent for ARR at the transition to flowering stage, which occurs at approximately 6 weeks of age (Kus *et al.*, 2002).

Pst and *H. parasitica* ARR assays

Arabidopsis plants were inoculated with virulent *Pst* strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr Andrew Bent (University of Wisconsin, Madison, WI, USA) (Whalen *et al.*, 1991). *Pst* was grown to mid-logarithmic phase in King's B medium and kanamycin (50 µg/mL) overnight, shaken at room temperature (22–25 °C) and then diluted to 10⁶ cfu/mL in 10 mM MgCl₂. Three to four leaves of young (3 wp) and four to six leaves of mature plants (5 or 6 wp) on 7 to 12 plants were inoculated with virulent *Pst*, and *in planta* bacterial levels were determined as described previously (Kus *et al.*, 2002; Wolfe *et al.*, 2000). Mutants tested for ARR competency included *iap1-1*, *eds1-1*, *jar1-1*, *jln1-1*, *anac055*, *anac092*, *cda1*, *ugt85A1*, *glycerol-3-PO₄ permease* and *β-galactosidase*. Photographs of leaves were taken with a Hewlett Packard (Palo Alto, CA, USA) 5.6 MP 56X zoom digital camera.

Infection with *H. parasitica* isolate Noco 2 was performed by spraying an asexual suspension (10⁶ conidiosporangia/mL) onto leaves of mature plants (6 wp). Mature plants possessed approximately 20 leaves and were scored as susceptible if most leaves displayed sporangiophores at 7 or 8 dpi. In addition, spores from the leaves of 8–12 plants (110–180 total leaves) were collected, and the spore number per leaf was quantified using a haemocytometer.

SA infiltration experiments, IWF collection and SA determination

SA infiltration experiments and IWF collections were performed as described previously (Cameron and Zaton, 2004; Kus *et al.*, 2002). IWFs were filter sterilized to remove *Pst*. SA levels were also determined in leaves after IWF removal. SA levels were measured using GC-MS (Schmelz *et al.*, 2004; Supporting Information).

Microarray analysis and gene expression using RT-PCR

See Supporting Information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Age-related resistance (ARR) gene expression analysis in T-DNA insertion mutant plants. (a) Reverse transcriptase-polymerase chain reaction (RT-PCR) with primers for *ANAC055* and *ANAC092* was performed on untreated Col-0 and *anac055anac092* leaf samples and leaves collected from mature [6 weeks post-germination (wpg)] *anac055* and *anac092* plants

at 12 hpi with *Pseudomonas syringae* pv. *tomato* (*Pst*). (b) RT-PCR with primers for *CDA1* was performed on untreated Col-0 and *cda1* leaf samples (left panel), leaves collected from untreated (un) mature (6 wpg) Col-0 and *cda1* plants and leaves collected at 48 hpi with *Pst* (right panel). (c) RT-PCR with primers for *UGT85A1* was performed on untreated Col-0 and *ugt85a1* leaf samples (left panel), leaves collected from untreated (un) mature (5 wpg) Col-0 and *ugt85a1* plants and samples collected at 48 hpi with *Pst* (right panel). *ACTIN* was used as a loading control. The number of PCR cycles used is shown.

Fig. S2 Age-related resistance (ARR) gene expression analysis in *iap1-1* and Col-0. (a) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed to monitor *ICS1*, *SID1*, *EDS1*, *ANAC055*, *ANAC092*, *CDA1* and *UGT85A1* gene expression in Col-0 and *iap1-1*. Mature [6 weeks post-germination (wpg)] Col-0 and *iap1-1* leaves were collected from 7 to 24 h post-inoculation (hpi) with *Pseudomonas syringae* pv. *tomato* (*Pst*) [10^6 colony-forming units (cfu)/mL] and from untreated controls (UN). *ACTIN* was used as a loading control; 28 cycles of RT-PCR were used for all primers. This experiment was repeated twice more with similar results. (b) One replicate is shown; 28 cycles of RT-PCR were used for all primers in this replicate.

Fig. S3 Replicate age-related resistance (ARR) gene expression analysis in young and mature Col-0. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis on leaves collected from (a) young [3 weeks post-germination (wpg)] and (b) mature (6 wpg) Col-0 plants at 5 min post-inoculation (mpi) and 12, 24 and 48 h post-inoculation (hpi) with 10^6 colony-forming units (cfu)/mL *Pseudomonas syringae* pv. *tomato* (*Pst*). *ANAC055*, *ANAC092*, *CDA1*, *UGT85A1* and *ACTIN* primers were used. The number of PCR cycles used is indicated.

Table S1 Forward and reverse primer sequences in reverse transcriptase-polymerase chain reaction (RT-PCR).

Table S2 Most down-regulated genes in the age-related resistance (ARR) microarray.

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