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# Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of *Arabidopsis thaliana*

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## Abstract

**Plants often respond to attack by insect herbivores and necrotrophic pathogens with induction of jasmonate-dependent resistance traits, but respond to attack by biotrophic pathogens with induction of salicylate-dependent resistance traits. To assess the degree to which the jasmonate- and salicylate-dependent pathways interact, we compared pathogenesis-related protein activity and bacterial performance in four mutant *Arabidopsis thaliana* lines relative to their wild-type backgrounds. We found that two salicylate-dependent pathway mutants (*cep1*, *nim1-1*) exhibited strong effects on the growth of the generalist biotrophic pathogen, *Pseudomonas syringae* pv. *tomato*, whereas two jasmonate-dependent pathway mutants (*fad3-2fad7-2fad8*, *jar1-1*) did not. Leaf peroxidase and exochitinase activity were negatively correlated with bacterial growth, whereas leaf polyphenol oxidase activity and trypsin inhibitor concentration were not. Interestingly, leaf total glucosinolate concentration was positively correlated with bacterial growth. In the same experiment, we also found that application of jasmonic acid generally increased leaf peroxidase activity and trypsin inhibitor concentration in the mutant lines. However, the *cep1* mutant, shown previously to overexpress salicylic acid, exhibited no detectable biological or chemical responses to jasmonic acid, suggesting that high levels of salicylic acid may have inhibited a plant response. In a second experiment, we compared the effect of jasmonic acid and/or salicylic acid on two ecotypes of *A. thaliana*. Application of salicylic acid to the Wassilewskija ecotype decreased bacterial growth. However, this effect was not observed when both salicylic acid and jasmonic acid were applied, suggesting that jasmonic acid negated the beneficial effect of salicylic acid. Collectively, our results confirm that the salicylate-dependent pathway is more important than the jasmonate-dependent pathway in determining growth of *P. syringae* pv. *tomato* in *A. thaliana*, and suggest important negative interactions between these two major defensive pathways in the Wassilewskija ecotype. In contrast, the Columbia ecotype exhibited little evidence of negative interactions between the two pathways, suggesting intraspecific variability in how these pathways interact in *A. thaliana*.**

*Keywords*: DC3000, pathogenesis-related protein, phenotypic plasticity, plant defence, *Pseudomonas syringae*

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## Introduction

Plants must endure a variety of biotic stresses throughout their lifetime, including attack by insect herbivores and microbial pathogens. Because encounters with these enemies are unpredictable, plants generally express low constitutive levels of resistance, but then rapidly divert resources to resistance following the onset of damage (Karban & Baldwin 1997). An understanding of how plants induce resistance is therefore critical for interpreting ecological interactions between plants and their enemies, and for engineering greater protection of crops from agricultural pests (Baker *et al*. 1997; Thaler *et al*. 1999).

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Traits that protect plants from herbivores are not necessarily effective against pathogens, and vice versa. Physical defences, such as spines and trichomes (Mauricio & Rausher 1997; Traw & Dawson 2002), would obviously be more effective against herbivores than pathogens, because of differences in scale. Similarly, proteinase inhibitors, which slow digestion of plant tissue in the insect gut (Wolfson 1991; Broadway & Colvin 1992), and glucosinolate compounds, which are only activated following the rupture of their specialized cells in plant tissue (Chew 1988), are unlikely to be effective against biotrophic pathogens. On the other hand, peroxidase, which strengthens plant cell walls (Baker & Orlandi 1995), and exochitinase, which degrades chitin in fungal and bacterial cell walls (Punja & Zhang 1993; Bishop *et al*. 2000), would be likely to provide more resistance to pathogens than insect herbivores.

Furthermore, resistance traits that protect plants from biotrophic pathogens are not likely to be effective against necrotrophic pathogens (Maleck & Dietrich 1999; Thomma *et al*. 2001). Biotrophs require living cells and can be effectively suppressed through a hypersensitive response by the plant, whereby cells near the infection experience rapid death (Sticher *et al*. 1997). In contrast, necrotrophs require cell death to obtain nutrients and may actually harness the plant's own hypersensitive response to kill cells. For example, the *dnd* mutant of *Arabidopsis thaliana*, which exhibits reduced hypersensitive cell death (Yu *et al*. 2000), was also found to be significantly less susceptible to infection by a necrotrophic pathogen, *Botrytis cinerea* (Govrin & Levine 2000).

Given the differential efficacy of resistance traits, it is not surprising that plants respond differently to attack by herbivores, necrotrophs and biotrophs. For example, proteinase inhibitors (Fidantsef *et al*. 1999) and glucosinolates (Bennett & Wallsgrove 1994) are more strongly induced by insect feeding than by biotrophic pathogens. Conversely, PR-1, PR-2 and PR-5 are pathogenesis-related proteins that are induced following infection by biotrophic pathogens but not following insect feeding or attack by necrotrophic pathogens (Kunkel & Brooks 2002). Other pathogenesis-related proteins, such as peroxidase and exochitinase, appear to respond to both biotrophic pathogens (Summermatter *et al*. 1995) and necrotrophic pathogens (Norman-Setterblad *et al*. 2000; Schenk *et al*. 2000; Kunkel & Brooks 2002).

Jasmonic acid and salicylic acid are two chemicals that appear to underlie these differential plant induction responses (Fig. 1; Glazebrook 2001; Kunkel & Brooks 2002). Herbivore damage (Reymond *et al*. 2000) and necrotrophic pathogen infection (Penninckx *et al*. 1996) cause rapid increases in jasmonic acid, whereas biotrophic pathogen infection causes rapid increases in salicylic acid (Gaffney *et al*. 1993; Ryals *et al*. 1994; Ton *et al*. 2002). Leaf glucosinolate concentration (Kliebenstein *et al*. 2002), polyphenol oxidase activity (Constable & Ryan 1998; Thaler *et al*. 2002) and trypsin inhibitor activity (Cipollini 2002) are up-regulated by exogenous application of jasmonic acid, but not by a similar application of salicylic acid. In contrast, PR-1, PR-2 and PR-5 are up-regulated by salicylic acid, but not jasmonic acid (Glazebrook 2001; Kunkel & Brooks 2002). Interestingly, peroxidase and exochitinase have been shown to respond to both salicylic acid and jasmonic acid (Samac & Shah 1991; Norman-Setterblad *et al*. 2000; Schenk *et al*. 2000; Davis *et al*. 2002).

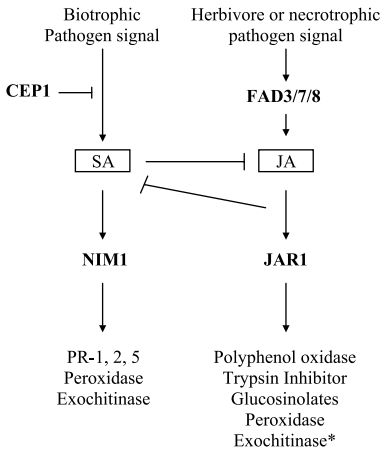


**Fig. 1** Diagram of the salicylate (SA) and jasmonate (JA)-dependent pathways in *Arabidopsis thaliana* showing relative positions of the four genes studied and putative defences produced (after Glazebrook 2001; Kunkel & Brooks 2002). The *CEP1* gene codes for a protein that down-regulates salicylic acid production (Clarke *et al*. 1998). The *NIM1* gene codes for a nuclear protein that regulates transcription of pathogenesis related proteins (Cao *et al*. 1997). The *FAD3*, *FAD7* and *FAD8* genes code for proteins that produce linolenic acid, the initial substrate for jasmonate biosynthesis (McConn & Browse 1996). The *JAR1* gene positively regulates jasmonate signalling by adenylating jasmonic acid (Staswick *et al*. 2002). *Exochitinase only responds to jasmonic acid in conjunction with ethylene (Norman-Setterblad *et al*. 2000).

There is increasing evidence that the jasmonate and salicylate-dependent pathways interact in plants. For example, exogenous application of salicylic acid and related compounds has been shown to inhibit both jasmonic acid synthesis and downstream activity in tomato (Doherty *et al*. 1988; Pena-Cortes *et al*. 1993; Doares *et al*. 1995; Nishiuchi *et al*. 1997). In addition, Felton *et al*. (1999) observed an inverse relationship between endogenous concentrations of salicylic acid and jasmonic acid in tobacco. In *A. thaliana*, the two pathways share an important gene for some responses, designated *NIM1* and *NPR1* from the ecotypes Wassilewskija and Columbia, respectively (Cao *et al*. 1997; Pieterse *et al*. 1998). Second, there is recent evidence that the *MAPK4* gene of the jasmonate-dependent pathway negatively regulates expression of the

salicylate-dependent pathway (Petersen *et al*. 2000). Finally, salicylic acid has been shown to inhibit expression of a protein specifically induced by the jasmonate-dependent pathway (van Wees *et al*. 1999). Despite clear potential in terms of expression of chemical resistance, negative cross-talk on biological resistance of *A. thaliana* has only recently been demonstrated (Kloek *et al*. 2001; Ellis *et al*. 2002; Stotz *et al*. 2002) and its importance is questioned (Schenk *et al*. 2000; Devadas *et al*. 2002; van Wees *et al*. 2000).

Negative cross-talk between defence pathways is a topic of great ecological interest for several reasons. First, plants may use negative cross-talk to suppress expression of inappropriate resistance when attacked, thereby avoiding the associated costs (Baldwin 1998; Purrington 2000; Cipollini *et al*. 2003). Second, herbivores and pathogens may manipulate plant defence by secreting salicylic acid, jasmonic acid, or their analogues during attack to prevent plants from activating the appropriate defence pathway (Maleck & Dietrich 1999). Finally, negative cross-talk may also cause the attack by one pest to alter the acceptability of that same host for other enemies (Bostock 1999; Maleck & Dietrich 1999; Bostock *et al*. 2001; Cui *et al*. 2002; Thaler *et al*. 2002). An understanding of the interactions between defence pathways will furthermore be important for determining how to manipulate resistance appropriately in crop plants (Thaler *et al*. 1999; Pieterse & van Loon 1999; Yun & Loake 2002).

In this study, we report the results of two experiments that assessed interactions between the jasmonate- and salicylate-dependent pathways in *A. thaliana*. In the first experiment, we compared chemical and biological resistance of the following four mutants: *nim1-1* (lacks response to salicylic acid), *cep1* (overexpresses salicylic acid), *fad3-2fad7-2fad8* (lacks jasmonic acid), *jar1-1* (deficient in response to jasmonic acid). In the same experiment, we also assessed responses of these four mutant lines to an application of jasmonic acid. In the second experiment, we compared chemical and biological resistance responses of two wild-types, Columbia and Wassilewskija, to an application of jasmonic acid alone, salicylic acid alone, or both compounds.

We predicted that the *cep1* mutant would have the highest activity of peroxidase and exochitinase and thus the lowest growth of *Pseudomonas syringae* pv. *tomato*, whereas the *nim1-1* mutant would have only constitutive activity of peroxidase and exochitinase, and therefore the highest bacterial growth. If cross-talk is not biologically important in *A. thaliana*, then the two jasmonate-dependent pathway mutants (*fad3-2fad7-2fad8* and *jar1-1*) should not differ from wild-type plants in peroxidase, exochitinase, or bacterial performance. This prediction assumes that jasmonic acid induces trypsin inhibitor activity, polyphenol oxidase activity, and glucosinolate concentration, but that these traits do not provide resistance against *P. syringae* pv. *tomato*. In the second experiment, we predicted that salicylic acid would trigger salicylate-dependent resistance, enabling plants to inhibit bacterial growth, but that an additional application of jasmonic acid would interfere with this inhibition if cross-talk is biologically important.

## Materials and methods

### *Lines with altered salicylate-dependent resistance*

The constitutive expressor of pathogenesis-related proteins (*cep1*) mutant lacks regulation of the expression of salicylic acid and therefore accumulates high levels of salicylic acid in plant tissues, as well as constitutive expression of pathogenesis-related proteins (Fig. 1, Silva *et al*. 1999). The noninducible immunity (*nim1-1*) mutant produces wild-type levels of salicylic acid but does not induce pathogenesis-related proteins following elicitation with bacteria or an application of salicylic acid (Delaney *et al*. 1995). Both the *cep1* and *nim1-1* mutants are from the Wassilewskija (Ws) ecotype background.

### *Lines with altered jasmonate-dependent resistance*

The fatty acid desaturase triple mutant (*fad3-2*, *fad7-2*, *fad8*), hereafter '*fad*', does not synthesize linolenic acid, a precursor to jasmonic acid, but can be rescued with exogenous application of jasmonic acid (Fig. 1, McConn & Browse 1996). The jasmonic acid response mutant (*jar1-1*) produces jasmonic acid, but does not adenylate it (Staswick *et al*. 2002), and therefore lacks induction of some jasmonate-mediated resistance to necrotic pathogens (Staswick *et al*. 1992, 1998). Both the *fad* and *jar1-1* mutants have the Columbia (Col) ecotype background.

### *Plant growth conditions*

Seeds were sown in wet Promix–BX (Premier Horticulture, Red Hill, PA) in 36-celled trays. Trays were first placed in the dark at 4 °C for 3 days, and then moved to an environmental growth chamber with a constant temperature of 20 °C and 12 h of artificial light at 500 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation from halogen arc lamps. Plants were watered daily and fertilized twice per week with 30 mL of 200 p.p.m. Peter's 15 : 16 : 17 solution. Trays were thinned to one plant per cell on the 10th day following germination.

### *Experimental design*

Both experiments were conducted using a completely randomized design. In the first experiment, there were six lines (four mutants plus the Ws and Col ecotypes), each represented by 60 plants, for a total of 360 plants. Half of

the replicates (30 plants per line) received 0.6 mL of 0.45 mM jasmonic acid in 4% ethanol (v/v water) on day 21 following germination as in Cipollini (2002), while the other half received a control solution consisting of 0.6 mL of 4% ethanol (v/v water). These solutions were applied to the leaf surface with a pipette. In the second experiment, there were two lines (Ws and Col), each represented by 60 plants, for a total of 120 plants. These plants received jasmonic acid or the control solution on day 21 as in the first experiment. Plants in the second experiment then received either 0.6 mL of 0.45 mM salicylic acid in water or a water control on day 22 as in Cipollini (2002). In both experiments, the four newest fully expanded leaves on day 26 were marked on each plant.

### *Preparation of leaf material*

Ten and five replicate plants per treatment were harvested for determination of protein activity and glucosinolate concentration, respectively. On days 27 and 28, the four marked leaves of each assigned plant were removed with razor blades, flash frozen in liquid nitrogen, and stored at −80 °C for 2 days. Leaves were then freeze-dried in a lyophilizer for 24 h and stored at room temperature prior to the analyses. Soluble leaf proteins were extracted by homogenizing 15 mg of lyophilized leaf material in 0.25 mL 0.05 M sodium phosphate buffer, pH 7.0. Leaf homogenates were centrifuged for 10 min at 8160 *g* at 4 °C, and the cleared supernatants (protein extracts) were used for all protein analyses. Total protein content per sample was determined according to Bradford (1976) using Bio-Rad protein dye reagent standardized with bovine serum albumin.

### *Measurement of protein activity*

Peroxidase activity in protein extracts was determined by following the oxidation of guaiacol for 1 min at 470 nm as in Moran & Cipollini (1998). Reaction mixtures (set up in 96-well plates) each contained 0.015 mL extract and 0.15 mL 0.25% guaiacol in 0.01 M sodium phosphate buffer, pH 6.0, containing 0.375% hydrogen peroxide. Peroxidase activity was expressed as $\Delta Abs_{470\,nm}$/min/mg total protein.

Polyphenol oxidase activity in protein extracts was determined in an identical fashion to peroxidase activity, except that reaction mixtures each contained 0.03 mL protein extract and 0.15 mL 0.00294 M caffeic acid in 0.05 M sodium phosphate buffer, pH 8.0.

Exochitinase activity in soluble protein extracts was assessed using hydrolysis of *p*-nitrophenyl-β-*N*-acetylglucosaminide in plate reader assays as in dal Soglio *et al*. (1998). Each reaction contained 0.02 mL protein extract, 0.2 mL 0.05 M potassium phosphate plus 0.15 M sodium sulphate (PPSS) buffer pH 6.0, and 0.1 mL substrate (2 mg/mL) dissolved in PPSS buffer. After 6 h of incubation at 40 °C, reactions were stopped and final absorbance was assessed at 405 nm. Exochitinase activity was expressed as $Abs_{405\,nm}$/mg total protein.

Trypsin inhibitor activity in soluble protein extracts was determined as in Cipollini & Bergelson (2000) by examining diffusion of protein extracts through a trypsin-containing agar followed by staining with *N*-acetylphenylalanine naphthyl ester and *O*-dianisidine. Trypsin inhibitor activity was expressed as μg trypsin inhibitor/mg extract protein and determined from a standard curve made with soybean trypsin inhibitor.

### *Measurement of total glucosinolate concentration*

Leaf glucosinolate concentrations were determined from high-performance liquid chromatography (HPLC) of the desulphated compounds (Bjerg & Sorensen 1987). Lyophilized leaf material was extracted in boiling 70% methanol using the method of Agerbirk *et al*. (2001). Glucosinolates were desulphated in open columns packed with 0.1 g DEAE Sephadex A-25 (Pharmacia Inc.) as described in Hugentobler & Renwick (1995).

HPLC analysis of desulphated glucosinolates was performed using a Hewlett-Packard Model 1100 system equipped with an autosampler, a 4.5 × 15-cm C-18 column (Luna, Phenomenex Corp.), and diode-array detector. The solvent programme (1 mL/min) started with 100% water for 2 min, followed by a linear change to 20% acetonitrile at 5 min, 35% acetonitrile at 15 min, and 100% acetonitrile at 18 min. Identities of peaks were determined from retention times relative to a sample for which glucosinolate compounds were identified by mass spectroscopy. Peak areas were measured at 229 nm and concentrations were determined relative to the peak area of a known concentration of internal standard, desulphobenzylglucosinolate, corrected using response factors provided by Buchner (1987). Total glucosinolate concentration was calculated as the sum of individual compound concentrations.

### *Assay of bacterial growth*

Fifteen replicate plants per treatment were challenged with *Pseudomonas syringae* pv. *tomato* DC 3000, a strain widely used for the study of resistance genetics in *Arabidopsis thaliana* (Preston 2000). Recent evidence suggests that *P. syringae* occurs in natural populations of *A. thaliana* (Jakob *et al*. 2002). Bacteria were streaked on King's B medium (King *et al*. 1954) to obtain a single colony. The colony was then transferred to a test tube containing 5 mL of liquid King's broth media, which was incubated at 28 °C and shaken for 24 h. A 1 : 10 dilution of this solution was incubated and shaken under the same conditions for 8 h. A 1.5-mL aliquot of this solution was then spun in an

Eppendorf tube at 2040 *g* and the liquid supernatant was discarded. The pellet of bacteria was resuspended in 10 mM $MgSO_4$ to obtain the inoculation solution as in Jakob *et al*. (2002). Four marked leaves per plant were each infiltrated with 0.02 mL of the solution on day 26 using a blunt-tipped syringe. Plating of the inoculation solutions revealed concentrations of $10^4$ and $10^5$ colony-forming units in the first and second experiments, respectively. Bacterial titres were measured 5 days later from a disk punched from one marked leaf per plant. Each disk was washed in 70% ethanol for 10 s, dried with a sterile paper towel, ground in an Eppendorf tube containing 200 µL of 10 mM $MgSO_4$, diluted to 1 : 10 000 in 10 mM $MgSO_4$, and plated on King's B medium using a spiral plater. Colonies were counted using a video camera/image analysis program (PROTOCOL 3.13) and checked by hand counts of representative plates.

### *Statistical analysis*

Protein activity, glucosinolate concentration and bacterial concentration were analysed for each experiment by two-way analysis of variance, with plant line and chemical application treated as fixed effects, and including the interaction term. Bacterial concentration was log transformed for analysis to conform to the assumption of normality of residuals. Roughly 5% of plates had no bacteria and these values were distributed evenly among the lines and treatments. To improve normality, these null values were excluded from the final analysis, but their inclusion did not affect the general patterns. The statistical analysis program, STATISTICA (1995), identified 11 additional outliers and these were also removed from the final analysis, but once again did not qualitatively affect the overall patterns.

A multiple regression of log bacterial concentration on activity of the four proteins and glucosinolate concentration was performed using the 12 treatment means (six plant lines × two jasmonic acid treatments) from the first experiment, or the eight treatment means (two plant lines × four chemical treatments) from the second experiment.

## Results

Resistance expression differed substantially between the salicylate-dependent pathway mutants and their wild-type background, the Wassilewskija ecotype. The *cep1* mutant, which is known to have high internal concentrations of salicylic acid, had five-fold higher activity of peroxidase ($F_{1,102} = 147.2$, $P < 0.001$; Fig. 2C) and two-fold higher activity of trypsin inhibitor ($F_{1,108} = 9.1$, $P = 0.003$; Fig. 2D) than did the wild-type plants. Additionally, the *cep1* mutant was highly resistant to bacterial colonization, containing less than a fiftieth of the concentration of bacteria present in wild-type leaves ($F_{1,159} = 149.9$, $P < 0.001$; Fig. 2F). The *nim1-1* mutant, which previous studies have shown does not induce chemical or biological resistance following salicylic acid application, exhibited similar levels of protein activity relative to the wild-type (Fig. 2A–E), but higher bacterial concentrations ($F_{1,159} = 13.8$, $P < 0.001$; Fig. 2F). Both the *nim1-1* mutant and Ws wild-type responded to jasmonic acid application by a two- to three-fold increase in peroxidase (Fig. 2C) and trypsin inhibitor (Fig. 2D) activity. Consistent with the presence of pathway cross-talk, the *nim1-1* plants treated with jasmonic acid supported a higher bacterial concentration than did the untreated *nim1-1* plants ($F_{1,159} = 5.2$, $P = 0.023$; Fig. 2F), and the *cep1* mutant did not respond significantly to jasmonic acid application (Fig. 2A–F).

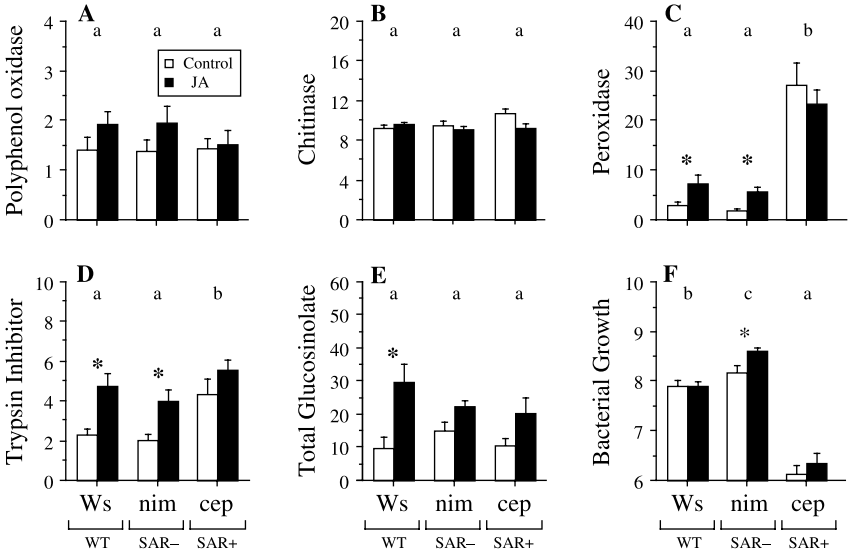


**Fig. 2** Comparison of *nim1-1* and *cep1* mutants relative to their wild-type background, following application of jasmonic acid (JA) or a control solution. Polyphenol oxidase and peroxidase (both $\Delta Abs_{470\,nm}$/min/mg total protein), exochitinase ($\Delta Abs_{405\,nm}$/mg total protein), and trypsin inhibitor (µg/mg total protein) were all averages of 10 samples. Total glucosinolate concentration (mM glucosinolate/mg dry leaf) was an average of five samples. Bacterial growth (log colony-forming units) was an average of 15 samples. Lines with different lowercase letters above were significantly different at $\alpha = 0.05$. An asterisk (*) indicates a significant effect of jasmonic acid at $\alpha = 0.05$ by planned comparison. WT = wild-type, SAR = systemic acquired resistance.

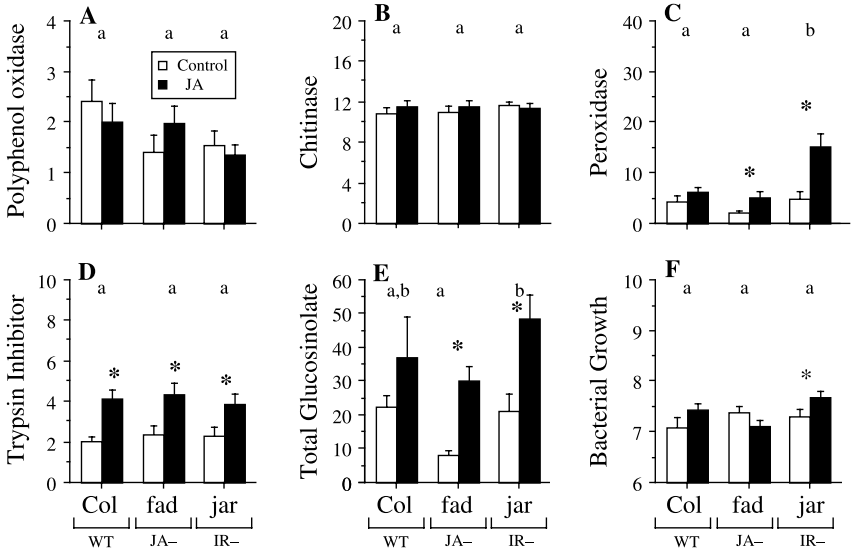


**Fig. 3** Comparison of *fad* and *jar1-1* mutants relative to their wild-type background, following application of jasmonic acid (JA) or a control solution. Units, sample sizes, and statistical analysis are as in Fig. 2. WT = wild-type, IR = jasmonate–induced resistance.

Resistance was somewhat altered in jasmonate-dependent pathway mutants relative to their wild-type background, the Columbia ecotype. The *jar1-1* mutant, which lacks some jasmonate-dependent responses, had higher constitutive concentrations of peroxidase than did wild-type plants ($F_{1,102} = 5.9$, $P = 0.017$; Fig. 3C). The *fad* mutant, which does not produce jasmonic acid, did not exhibit any significant constitutive differences in resistance relative to the wild-type background (Fig. 3A–F). Application of jasmonic acid increased peroxidase activity (Fig. 3C), trypsin inhibitor activity (Fig. 3D) and glucosinolate concentrations (Fig. 3E) in both mutants. Consistent with the presence of pathway cross-talk, the *jar1-1* plants treated with jasmonic acid exhibited a significantly higher bacterial concentration than was found in control plants ($F_{1,159} = 3.8$, $P = 0.054$; Fig. 3F).

A multiple regression of bacterial growth on the five chemical resistance traits in experiment no. 1 (Table 1) showed that leaf peroxidase ($P = 0.020$) and exochitinase activity ($P = 0.016$) were negatively correlated with bacterial growth, whereas leaf glucosinolate concentration ($P = 0.023$) was positively correlated with bacterial growth. In contrast, a multiple regression using data from experiment no. 2 revealed no significant relationships between leaf resistance traits and bacterial growth (Table 2).

For the Ws wild-type, application of salicylic acid alone had no effect on expression of proteins or total glucosinolate concentration (Fig. 4A–E). However, application of salicylic acid did reduce susceptibility of plants to *Pseudomonas syringae* by over 50% ($F_{1,41} = 8.1$, $P = 0.007$; Fig. 4F). In contrast, application of jasmonic acid alone tripled expression of peroxidase ($F_{1,33} = 4.7$, $P = 0.037$; Fig. 4C) and increased glucosinolate concentrations by 40% ($F_{1,16} = 12.8$, $P = 0.002$; Fig. 4E). Despite these chemical changes,

**Table 1** Standardized slope coefficients, *t*-values and *P*-values for experiment no. 1 from a multiple regression of leaf bacterial concentration on leaf exochitinase activity, peroxidase activity, polyphenol oxidase activity, trypsin inhibitor activity and total glucosinolate concentration

| Variable | Coefficient | *t*-value | *P*-value |
|---|---|---|---|
| Exochitinase | **–0.60** | –3.29 | **0.0164** |
| Peroxidase | **–0.76** | –3.13 | **0.0203** |
| Polyphenol oxidase | –0.28 | –1.53 | 0.1766 |
| Trypsin Inhibitor | –0.14 | –0.57 | 0.5864 |
| Glucosinolate | **0.60** | 3.00 | **0.0239** |

The data for the regression consisted of the 12 treatment means. Together, the predictor variables explained 86% of variation in leaf bacterial concentration ($F_{5,11} = 7.4$, $P = 0.015$). Significant coefficients and *P*-values at $\alpha < 0.05$ are indicated in bold.

**Table 2** Standardized slope coefficients, *t*-values and *P*-values for experiment no. 2 based on a multiple regression of leaf bacterial concentration on leaf exochitinase activity, peroxidase activity, polyphenol oxidase activity, trypsin inhibitor activity and total glucosinolate concentration

| Variable | Coefficient | *t*-value | *P*-value |
|---|---|---|---|
| Exochitinase | 2.85 | 1.23 | 0.3414 |
| Peroxidase | –9.98 | –1.92 | 0.1936 |
| Polyphenol oxidase | –0.50 | –0.51 | 0.6605 |
| Trypsin Inhibitor | 5.04 | 1.74 | 0.2227 |
| Glucosinolate | 4.18 | 1.71 | 0.2286 |

The data for the regression consisted of the eight treatment means. Together, the predictor variables explained 69% of variation in leaf bacterial concentration ($F_{5,7} = 0.9$, $P = 0.597$).

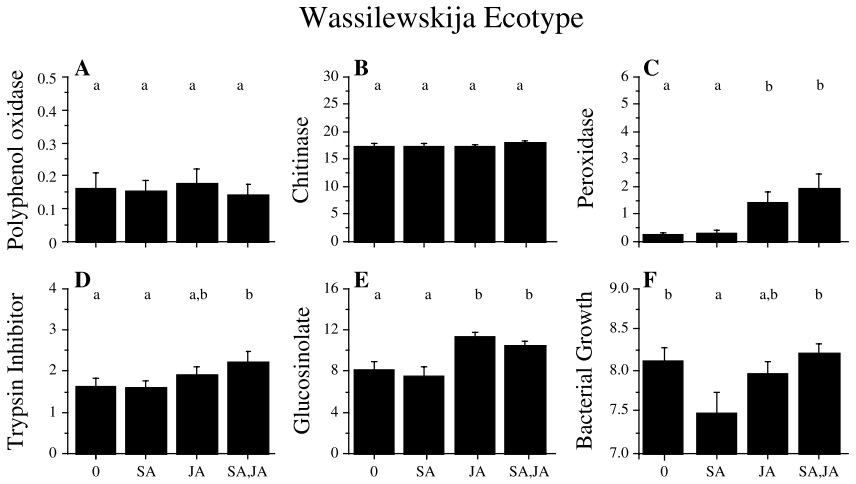


**Fig. 4** Effect of jasmonic acid (JA), salicylic acid (SA), or both (SA, JA) on protein activity, glucosinolate concentration, and bacterial growth of the Wassilewskija ecotype. Units and sample sizes are as in Fig. 2. Lines with different lowercase letters are significantly different at $\alpha = 0.05$ by planned comparison.

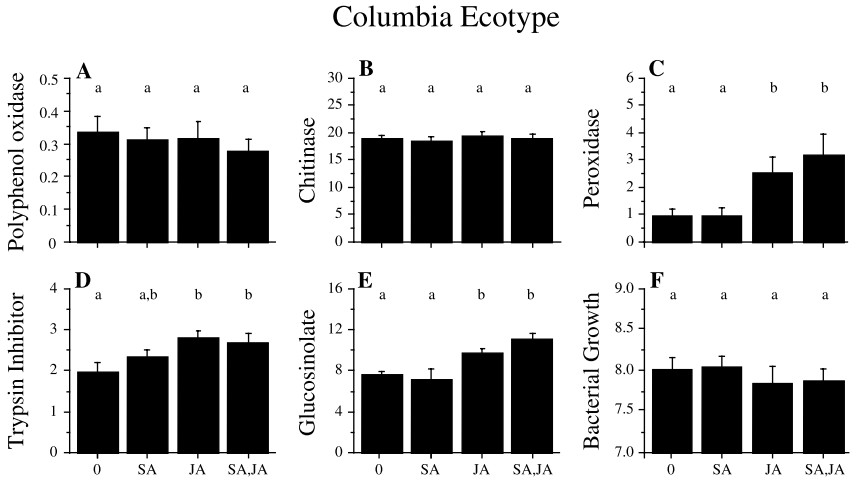


**Fig. 5** Effect of jasmonic acid (JA), salicylic acid (SA), or both (SA, JA) on protein activity, glucosinolate concentration, and bacterial growth of the Columbia ecotype. Units and sample sizes are as in Fig. 2. Lines with different lowercase letters are significantly different at $\alpha = 0.05$ by planned comparison.

application of jasmonic acid did not alter susceptibility of the Ws wild-type to *P. syringae* ($F_{1,41} = 0.51$, $P = 0.476$; Fig. 4F). Furthermore, plants that received both jasmonic acid and salicylic acid were just as susceptible to *P. syringae* as control plants ($F_{1,41} = 0.12$, $P = 0.728$; Fig. 4F).

For the Col wild-type, application of salicylic acid alone had no effect on expression of proteins (Fig. 5A–D), total glucosinolate concentration (Fig. 5E) or bacterial performance (Fig. 5F). In contrast, treatment with jasmonic acid caused a two- to three-fold increase in activity of peroxidase ($F_{1,36} = 4.6$, $P = 0.038$; Fig. 5C), trypsin inhibitor ($F_{1,34} = 9.5$, $P = 0.004$; Fig. 5D), and total glucosinolate concentration ($F_{1,12} = 8.3$, $P = 0.013$; Fig. 5E). As for the Wassilewskija wild-type, these changes did not translate into a significant reduction in bacterial performance on plants treated with jasmonic acid ($F_{1,42} = 0.56$, $P = 0.456$; Fig. 5F). There were also no significant interactions between the jasmonic acid and salicylic acid treatments in their effects on the Col wild-type (Fig. 5A–F).

## Discussion

In this study, we asked whether the jasmonate-dependent pathway, which increases plant resistance to herbivores and necrotrophic bacteria (McConn *et al*. 1997; Pieterse *et al*. 1998), would also affect plant resistance to a biotrophic bacterial pathogen. To address this question, we exogenously applied jasmonic acid to mutant *Arabidopsis thaliana* lines with characterized genetic abnormalities in their expression of chemical resistance to either herbivores or pathogens. Our results indicate that activation of the jasmonate-dependent pathway does not increase plant resistance to *Pseudomonas syringae*, and can actually decrease resistance to this pathogen under some circumstances. This assessment is consistent with recent research on tomato (Doherty *et al*. 1988; Felton *et al*. 1999; Thaler *et al*. 2002). It is also consistent with predictions from the recent discovery of genetic cross-regulation of these two resistance pathways in *A. thaliana* (Clarke *et al*.

1998; van Wees *et al*. 1999; Petersen *et al*. 2000; Kliebenstein *et al*. 2002), but differs from the conclusion of another recent study of *A. thaliana* (van Wees *et al*. 2000).

Our conclusion of negative cross-talk between jasmonate- and salicylate-dependent pathways is based on three findings. First, we found that *nim1-1* and *jar1-1* mutants treated with jasmonic acid supported higher growth of *P. syringae* DC3000 than control plants that were not treated with jasmonic acid (Figs 2F, 3F, respectively). In a subsequent experiment, we have confirmed this result (Traw & Bergelson, unpublished data). Other recent studies have obtained contrasting results with these same mutants. For example, application of methyl jasmonate to *A. thaliana* had no effect on the subsequent growth of *P. syringae* DC3000 in either *jar1-1* or the *npr* mutant, which is allelic to *nim1-1* (Pieterse *et al*. 1998), and reduced *P. syringae* growth in the Columbia ecotype (Pieterse *et al*. 1998; van Wees *et al*. 1999). It is possible that the conflicting results may reflect the fact that we used jasmonic acid, whereas Pieterse *et al*. (1998) and van Wees *et al*. (1999) used methyl jasmonate. Indeed, it now appears that some jasmonic acid is methylated (Seo *et al*. 2001) and some is adenylated (Staswick *et al*. 2002) during jasmonate-dependent induction, with different consequences for downstream activity. Thus, it is possible that application of methyl jasmonate may bypass a subset of responses that are only triggered by adenylated jasmonic acid. Also, our concentration of jasmonic acid (0.45 mM) was double the highest concentration of methyl jasmonate applied by van Wees *et al*. (1999), perhaps increasing the likelihood that we would see inhibitory effects. However, it is difficult to compare our concentrations in terms of biological activity because jasmonic acid is less soluble by plants than methyl jasmonate, therefore it takes more exogenous jasmonic acid to equal the effect of a given amount of methyl jasmonate. Last, we applied our chemical treatments 2 weeks earlier during plant growth than did Pieterse *et al*. (1998) and van Wees *et al*. (1999); evidence suggests that effects of pathway induction are more likely to be observed on younger plants than older plants (Summermatter *et al*. 1995; Kus *et al*. 2002).

Second, we found that the *cep1* mutant was the only line that failed to exhibit any induction of chemical resistance following application of jasmonic acid (Figs 2, 3), and this line is the only one that constitutively expresses high concentrations of salicylic acid. These results suggest that high endogenous concentrations of salicylic acid in the *cep1* mutant may have inhibited expression of the jasmonate-dependent pathway, as has previously been shown in other contexts by Clarke *et al*. (1998) and Petersen *et al*. (2000).

Third, we found that jasmonic acid negated the positive effect of salicylic acid on resistance of the Wassilewskija wild-type to *P. syringae* (Fig. 4F). Plants that received salicylic acid alone had a 50% reduction in bacterial performance relative to control plants, but plants that received both salicylic acid and jasmonic acid had no reduction in bacterial performance relative to control plants. Addition of salicylic acid or analogue compounds to Wassilewskija has been previously shown to reduce bacterial growth (Delaney *et al*. 1995; Dong *et al*. 1999), but negation of this effect by jasmonic acid has not been previously reported.

It is notable that the Columbia ecotype responded to the addition of salicylic acid in a qualitatively different manner than the Wassilewskija ecotype. For Wassilewskija plants, the addition of salicylic acid led to a decrease in bacteria performance in leaf tissue (Fig. 4F). For Columbia plants, the addition of salicylic acid had no effect on bacterial performance in leaf tissue (Fig. 5F). Differences in the type and magnitude of induced resistance are widespread among ecotypes of *A. thaliana* (Ton *et al*. 2001) and would have important implications for the evolutionary interactions between this plant and its natural pathogens.

Our lack of an observed effect of salicylic acid on pathogen resistance in Columbia was unexpected. Other studies have documented a negative effect of salicylic acid on bacterial performance in Columbia (van Wees *et al*. 1999; Ton *et al*. 2002), indicating that the salicylic acid-dependent pathway is clearly functional in this ecotype. One possible explanation for the contradictory results is that by dipping plants in a solution containing salicylic acid plus a surfactant, van Wees *et al*. (1999) and Ton *et al*. (2002) may have delivered a higher dose of salicylic acid to plants than we achieved by dripping the solution on leaves with a pipette, even though the concentrations of salicylic acid used were similar. Additionally, van Wees *et al*. (1999) and Ton *et al*. (2002) challenged plants with the bacterial pathogen 3 days after applying salicylic acid. We waited 5 days after applying salicylic acid before challenging with the bacterial pathogen, which may have reduced our likelihood of observing an effect.

This study is perhaps the first to quantify the relationship between chemical resistance traits and bacterial growth in *A. thaliana* (Tables 1, 2). Our results from experiment no. 1 support the general notion that peroxidase and exochitinase defend plants against the growth of *P. syringae* pv. *tomato* (Table 1). Both of these proteins have been shown to increase following infection of *A. thaliana* by *P. syringae* (Summermatter *et al*. 1995). Trypsin inhibitor and polyphenol oxidase activity, which are generally associated with the jasmonate-dependent pathway (Constable & Ryan 1998; Cipollini 2002; Thaler *et al*. 2002), were not correlated with the growth of *P. syringae*. Our finding that leaf total glucosinolate concentration was positively correlated with bacterial growth (Table 1) is interesting, given that the glucosinolate derivatives have been shown to inhibit bacterial growth in liquid media (Tierens *et al*. 2001). Because *P. syringae* pv. *tomato* does not rupture the plant cells (Preston

2000), it may prevent myrosinase, the associated enzyme, from catalysing the formation of toxic products. It is possible that the pathogen then benefits from the glucosinolates by converting them to nutritionally useful molecules.

Although leaf chemistry was strongly correlated with bacterial growth in experiment no. 1 (Table 1), much of that pattern was driven by constitutive differences among the mutant lines, and the *cep1* mutant in particular, rather than being an effect of induction by jasmonic acid. Experiment no. 2, which examined the induction by salicylic acid and jasmonic acid of wild-type plants, revealed no relationship between leaf chemistry and bacterial growth (Table 2). There are several possible explanations for why induction of leaf resistance traits did not correlate well with bacterial growth, whereas constitutive differences among mutants did. For one thing, there may be important components of the induction response to jasmonic acid or salicylic acid that we did not measure. For example, *A. thaliana* plants have numerous chitinase (Bishop *et al*. 2000) and peroxidase (Ostergaard *et al*. 1998) proteins, of which we measured only a subset. Alternatively, those proteins induced by salicylic acid or jasmonic acid may have less biological activity than those responsible for the constitutive differences among the mutant lines. In addition, it is possible that early expression of resistance is very important and therefore the constitutive up-regulation of resistance in the mutants may have had a disproportionately greater reduction in bacterial growth relative to the application of the exogenous chemical elicitors.

It was interesting that peroxidase activity was generally induced by jasmonic acid (Figs 2C, 3C), and in fact was induced more strongly by jasmonic acid than by an equivalent concentration of salicylic acid (Figs 4C, 5C; D. Cipollini and C. Slemmons, unpublished data). Peroxidase activity has been previously associated with the salicylate-dependent pathway in *A. thaliana* (Summermatter *et al*. 1995), but this no longer appears appropriate (Schenk *et al*. 2000). It is possible that peroxidase activity is partly induced by oxidative stress resulting from jasmonate or salicylate treatment and might therefore be a hallmark indicator of 'stress' independent of pathway regulation.

In summary, our results confirmed that the salicylate-dependent pathway is more important than the jasmonate-dependent pathway in determining growth of the biotrophic pathogen, *P. syringae* pv. *tomato* in *A. thaliana*, and suggested important negative interactions between these two major defensive pathways in the Wassilewskija ecotype. Difference in responses of the two wild-types (Wassilewskija and Columbia) hinted at important intraspecific variability in pathway induction in *A. thaliana*. This paper is perhaps the first to quantitatively link increased peroxidase and exochitinase activity with increased resistance against growth of *P. syringae* pv. *tomato* in *A. thaliana*. Our results suggest that further study of the interaction of the salicylate- and jasmonate-dependent pathways in the induction of these two proteins is warranted.

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Research in Joy Bergelson's laboratory addresses evolutionary aspects of the interactions between plants and pathogenic bacteria. Brian Traw is a postdoctoral researcher working with Joy Bergelson on the molecular basis of plant induction responses. Jed Kim assisted with this research as part of his undergraduate thesis at the University of Chicago. This research was conducted in collaboration with Don Cipollini, whose laboratory focuses on the chemical ecology of plant–insect interactions. Stephanie Enright is a technician in the Cipollini laboratory at Wright State University.

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