Both the Jasmonic Acid and the Salicylic Acid Pathways Contribute to Resistance to the Biotrophic Clubroot Agent Plasmodiophora brassicae in Arabidopsis

Séverine Lemarié1, Alexandre Robert-Seilaniantz1, Christine Lariagón1, Jocelyne Lemoine1, Nathalie Marnet2, Mélanie Jubault3, Maria J. Manzanares-Dauleux3 and Antoine Gravot4,*

1INRA, UMR1349 IGEPP, F-35653 Le Rheu, France
2Plateau de Profilage Métabolique et Métabolomique (P2M2) Centre de Recherche Angers Nantes BIA, INRA de Rennes, F-35653 Le Rheu, France
3AGROCAMPUS OUEST, UMR1349 IGEPP, F-35000 Rennes, France
4Université Rennes 1, UMR1349 IGEPP, F-35000 Rennes, France
*Corresponding author: E-mail, antoine.gravot@univ-rennes1.fr; Fax, +33(0) 223485150.

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The role of salicylic acid (SA) and jasmonic acid (JA) signaling in resistance to root pathogens has been poorly documented. We assessed the contribution of SA and JA to basal and partial resistance of Arabidopsis to the biotrophic clubroot agent Plasmodiophora brassicae. SA and JA levels as well as the expression of the SA-responsive genes PR2 and PR5 and the JA-responsive genes ARGAH2 and THI2.1 were monitored in infected roots of the accessions Col-0 (susceptible) and Bur-0 (partially resistant). SA signaling was activated in Bur-0 but not in Col-0. The JA pathway was weakly activated in Bur-0 but was strongly induced in Col-0. The contribution of both pathways to clubroot resistance was then assessed using exogenous phytohormone application and mutants affected in SA or JA signaling. Exogenous SA treatment decreased clubroot symptoms in the two Arabidopsis accessions, whereas JA treatment reduced clubroot symptoms only in Col-0. The cpr5-2 mutant, in which SA responses are constitutively induced, was more resistant to clubroot than the corresponding wild type, and the JA signaling-deficient mutant jar1 was more susceptible. Finally, we showed that the JA-mediated induction of NATA1 drove N(δ)-acetylornithine biosynthesis in infected Col-0 roots. The 35S::NATA1 and nata1 lines displayed reduced or enhanced clubroot symptoms, respectively, thus suggesting that in Col-0 this pathway was involved in the JA-mediated basal clubroot resistance. Overall, our data support the idea that, depending on the Arabidopsis accession, both SA and JA signaling can play a role in partial inhibition of clubroot development in compatible interactions with P. brassicae.

Keywords: Arabidopsis thaliana • Clubroot • Jasmonic acid • Partial resistance • Plasmodiophora brassicae • Salicylic acid.

Abbreviations: ARGAH2, ARGINASE 2; cpr5-2, constitutive expressor of PR genes 5; dpi, days post-inoculation; edr5-1, enhanced disease susceptibility 5; EMS, ethylmethane sulfonate; ESI, electrospray ionization; ET, ethylene; JA, jasmonic acid; jar1, jasmonate resistant 1; LOX2, LIPOXGENASE 2; MelA, methyl jasmonate; NATA1, N-ACETYLTRANSFERASE ACTIVITY 1; npr1-2, non-expresser of PR genes; PR2, PATHOGENESIS RELATED 2; PRS, PATHOGENESIS RELATED S; qPCR, quantitative PCR; SA, salicylic acid; THI2.1, THIONIN 2.1; WT, wild type.

Introduction

A large body of genetic and cellular studies on plant defense signaling have led to the general conceptual model in which different hormonal pathways tightly control specific plant responses toward pathogens with different lifestyles (Thomma et al. 1998, Oliver and Ipcho 2004, Glazebrook 2005). This model suggests that two distinct hormonal pathways are solicited differentially in response to biotrophs/hemibiotrophs and necrotrophs: salicylic acid (SA)-mediated defenses generally act against biotrophs/hemibiotrophs such as powdery and downy mildew, whereas jasmonic acid (JA)/ethylene (ET)-dependent defenses generally inhibit necrotrophs including Alternaria, Pythium and Botrytis (Thomma et al. 1998, Glazebrook 2005). Even if at present this model is still being actively studied and was confirmed in some pathosystems (Pieterse et al. 2012, Thaler et al. 2012, Derksen et al. 2013, Caarls et al. 2015), this viewpoint has become nuanced by reports which highlighted the many positive cross-talks between SA, JA and other hormones during pathogenesis (Robert-Seilaniantz et al. 2011, Derksen et al. 2013). For example, Glazebrook (2005) provided evidence that the JA pathway may help in defense against some biotrophs such as Peronospora parasitica and Erysiphe spp. However, in contrast to the abundant literature on foliar pathogens, the biology of root defense against telluric pathogens has been less well studied (Okubara and Paulitz 2005, Balmer and Mauch-Mani 2013, Chen et al. 2014). In Arabidopsis thaliana, JA- and SA-controlled responses in roots and their role in the orchestration of defenses have been described for only a few pathosystems. Thus, it is not clear whether the SA- and JA-responsive genes commonly used to investigate defense signaling in leaves can be extrapolated to root defense studies, or whether the classical JA/SA antagonistic model is valuable to study root–pathogen interactions.
Clubroot is a worldwide root disease affecting *Brassicaeae* species and caused by the obligate biotrophic soilborne *Plasmodiophora brassicae*. The life cycle of this pathogen comprises a primary phase restricted to the root hairs and a secondary phase of several weeks in cortical and stele cells. During this secondary phase, the intracellular development of pathogen plasmodia causes cell hyperplasia and hypertrophy, and results in the formation of root galls (Kageyama and Asano 2009). In the *A. thaliana* Col-0 accession, JA responses contributed to a weak basal resistance toward some virulent strains of *P. brassicae*. In this accession, JA accumulated in developing clubs (Gravot et al. 2012), and several JA-responsive genes were induced in infected root tissues (Siemens et al. 2006, Gravot et al. 2012). Moreover, the *jasmonate resistant 1* (*jar1*) mutant, impaired in JA-ile accumulation, exhibited heightened susceptibility to clubroot (Agranval et al. 2011, Gravot et al. 2012) suggesting that the JA response participated in basal defense in Col-0. This JA response also included both the *JAR1*-dependent induction of the arginase-encoding gene *ARGININE AMINO-HYDROLASE2* (*ARGAH2*) and arginase biochemical activity in infected roots (Jubault et al. 2008a, Gravot et al. 2012). Abolition of the induction of arginase activity in the *argh2* mutant line led to enhanced clubroot symptom development, thus providing further evidence for the role of the JA-dependent response in weak Col-0 basal resistance (Gravot et al. 2012). One hypothesis for this phenomenon is that the induction of *ARGAH2* could provide ornithine to the methyl jasmonate (MeJA)-induced product of the *N-ACYTYLTRANSFERASE ACTIVITY 1* (*NATA1*) gene, an enzyme involved in the biosynthesis of the biologically active N(\(\delta\))-acylamidomethyl compound. This atypical amino acid has been reported to exert a negative effect on the reproduction of aphids (Adio et al. 2011).

In addition to the above-described responses in Col-0, results from transcriptome analysis in Arabidopsis (Jubault et al. 2013) suggested that SA responses were triggered in the accession Bur-0 when infected by the compatible eH strain of *P. brassicae*. These data suggested a paradoxical situation where infection by the same single isolate, virulent on the two genotypes Bur-0 and Col-0, would induce two different defense responses depending on the plant genotype. Bur-0 was described as partially resistant to eH (Alix et al. 2007, Jubault et al. 2008b), i.e. exhibited a lower level (compared with Col-0) of clubroot symptom development. We thus hypothesized that SA- and JA-triggered defenses could drive contrasted levels of resistance in Bur-0 and Col-0, respectively. This assumption was, however, only based on the comparison of transcriptomic fingerprints and needed further investigation.

The aim of the present study was therefore to obtain a more comprehensive view of SA- and JA-dependent root cellular responses to *P. brassicae* infection, and to clarify their respective role in the partial inhibition of clubroot development. First, we monitored the expression of JA and SA responses in Col-0 and Bur-0 during the secondary phase of infection. SA- and JA-responsive gene expression was paralleled with SA and JA quantification. Then, the role of SA and JA in clubroot resistance was evaluated by (i) treating clubroot-infected plants with SA and JA; and (ii) phenotyping a set of mutants affected in SA or JA signaling. Finally, to gain an improved understanding of clubroot-induced JA-controlled responses, we tested the hypothesis that JAR1-dependent *ARGAH2* induction is associated with the downstream induction of N(\(\delta\))-acylamidomethyl biosynthesis, thus contributing to clubroot resistance. We therefore quantified *NATA1* expression in both genotypes, determined the N(\(\delta\))-acylamidomethyl content in clubroot-infected roots and evaluated clubroot resistance in N(\(\delta\))-acylamidomethyl-deficient and overaccumulating mutants.

**Results**

*P. brassicae* induced large SA and weak JA responses in partially resistant Bur-0, contrasting with large JA responses in susceptible Col-0

The expression of the SA-responsive genes PATHOGENESIS RELATED PR1, PR2 and PR5 was monitored in Col-0 and Bur-0 infected roots during the secondary phase of infection (at 10, 14 and 17 days post-inoculation (dpi)). PR1 expression remained at very low levels (data not shown) under all conditions. PR2 (Fig. 1A) and PR5 (Fig. 1B) were not induced in the susceptible accession Col-0 during the infection time course. In the Bur-0 accession, however, infection induced a 10- and 15-fold increase in PR2 and PR5 expression, respectively, at 14 dpi. Thereafter, at least for PR5, the induction level was sustained at 17 dpi.

SA accumulation was then paralleled with expression of these SA-responsive genes at 10, 14 and 17 dpi. SA accumulation was not observed in Col-0 following infection at any time point considered (Fig. 1C). In contrast, Bur-0 accumulated more SA at 14 dpi, consistent with the kinetics of SA-responsive gene induction (Fig. 1A–C). However, at 10 and 17 dpi, no statistically significant differences in SA accumulation were detected between inoculated and non-inoculated Bur-0 plants (*P* = 0.81 and *P* = 0.44, respectively). Thus, these results suggested that *P. brassicae* infection triggered an SA signaling pathway during the secondary phase of infection in Bur-0, but not in Col-0.

We then monitored the expression of the JA-responsive genes *THIONIN2.1* (*THI2.1*) and *ARGAH2*. The expression of *THI2.1* and *ARGAH2* remained at relatively low levels in non-inoculated roots. The infection induced the expression of *THI2.1* (Fig. 2A) and *ARGAH2* (Fig. 2B) in Col-0 at 10 dpi, and in both genotypes at 14 and 17 dpi. *THI2.1* expression in Col-0 was 4-fold higher at 14 dpi and 2-fold higher at 17 dpi than in Bur-0. The same pattern of expression was observed for *ARGAH2*; the expression in Col-0 was four times higher at 14 dpi and twice as high at 17 dpi than in Bur-0. Infection induced JA accumulation at 10, 14 and 17 dpi in Col-0 and at 14 and 17 dpi in Bur-0 (Fig. 2C). However, JA accumulation was 2–3 times higher in Col-0 than in Bur-0 infected roots at each time point (Fig. 2C).

These results indicated that *P. brassicae* infection preferentially induced the JA pathway during the secondary phase of infection in the susceptible accession Col-0. In contrast, in the partially resistant Bur-0, clubroot infection induced the SA
pathway in the middle of the secondary phase of infection and then the JA pathway, but to a lesser extent at later stages.

Exogenous hormone treatments and analyses of hormone-deficient mutants indicated a positive role for both JA and SA signaling in the control of clubroot infection

The involvement of SA and JA in the response to clubroot was evaluated by exogenous application of these hormones. Col-0 and Bur-0 clubroot-inoculated plants were treated every 2 d with SA (500 μM) or MeJA (50 μM) starting from 10 dpi. Clubroot symptoms were then quantified at 21 dpi. Non-treated inoculated Bur-0 plants exhibited fewer clubroot symptoms than Col-0, consistent with the previously reported partial resistance of Bur-0 (Jubault et al. 2008b) (Fig. 3A, B). Exogenous SA treatment led to the decrease of clubroot symptoms in both Col-0 and Bur-0 genotypes. In contrast, JA treatment reduced clubroot symptoms only in Col-0 (Fig. 3B). The relative amount of P. brassicae DNA in total root-extracted DNA was evaluated through quantitative PCR (qPCR). The results indicated that JA treatment reduced root pathogen density within infected roots of Col-0 and Bur-0 (Fig. 3C). Despite the above-described effect of exogenous SA on symptom development, this treatment had no impact on pathogen density within root samples.

SA- and JA-responsive genes and clubroot symptoms were then evaluated in a set of mutants affected in SA and JA signaling during clubroot infection. The expression of the SA marker PR2 and the JA marker THI2.1 and clubroot symptoms were evaluated in the mutant lines constitutive expresser of PR genes 5 (cpr5-2), jar1, enhanced disease susceptibility 5 (eds5-1), nahG and non-expresser of PR genes 1 (npr1-2).

The SA marker PR2 was expressed 3-fold more in infected roots of the cpr5-2 mutant than in the wild type (WT) Col-0 (Fig. 4A). In parallel, expression of the JA marker THI2.1 (Fig. 4B) was abolished, suggesting that activation of the SA pathway could repress the JA pathway in the roots of this mutant. Clubroot symptoms in the cpr5-2 mutant were 2-fold less severe than those of WT Col-0 (Fig. 4C, D), suggesting that the constitutive activation of SA responses may inhibit clubroot symptom development.

In the mutant line jar1, which is defective in the biosynthesis of JA-Ile, no difference in PR2 expression was detected between this mutant and WT Col-0 (Fig. 4A). In parallel, expression of the JA marker THI2.1 (Fig. 4B) was abolished, suggesting that activation of the SA pathway could repress the JA pathway in the roots of this mutant. Clubroot symptoms in the cpr5-2 mutant were 2-fold less severe than those of WT Col-0 (Fig. 4C, D), suggesting that the constitutive activation of SA responses may inhibit clubroot symptom development.

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JA responses induced by *P. brassicae* infection. Interestingly, clubroot symptoms were slightly reduced by the *eds5* mutation (Fig. 4C), supporting the idea that overexpression of JA responses could be responsible for enhanced resistance in this genotype.

In contrast, in the *nahG* line, which is deficient in SA accumulation, and in the SA signaling-deficient mutant *npr1*, the expression of *PR2* (Fig. 4A) and *THI2.1* (Fig. 4B) and clubroot symptoms (Fig. 4C) were not significantly different compared with the WT Col-0.

### The JA-dependent responses to clubroot in Col-0 induced the biosynthesis of N(\(\delta\))-acetylornithine via NATA1

We then investigated whether the JA-mediated induction of ARGAH2, which is involved in l-ornithine biosynthesis and induced during club development, could be associated with the induction of NATA1. The expression of NATA1 and the accumulation of N(\(\delta\))-acetylornithine were monitored in Col-0 and Bur-0 at 10, 14 and 17 dpi. NATA1 expression was highly induced by clubroot infection, but only in Col-0, where its expression was 40 times higher in inoculated vs. non-inoculated roots (Fig. 5A). In contrast, NATA1 was expressed at very low levels in both non-inoculated and inoculated Bur-0 roots. These results suggested that clubroot infection in Col-0 triggered the induction of a JA-mediated response module including ARGAH2 and NATA1, which could contribute to the biosynthesis of N(\(\delta\))-acetylornithine. Indeed, N(\(\delta\))-acetylornithine accumulated 100 times more in the infected roots of Col-0 compared with clubs of Bur-0 at 14 and 17 dpi (Fig. 5B).

We then evaluated whether the JA-mediated induction of N(\(\delta\))-acetylornithine contributes to clubroot resistance in Col-0 (Fig. 6). We therefore quantified the development of clubroot symptoms on the N(\(\delta\))-acetylornithine-deficient mutant *nata1* and the N(\(\delta\))-acetylornithine-overaccumulating mutant 35S::NATA1 (Adio et al. 2011). Compared with the WT (Col-0 background), *nata1* was one-third more susceptible to clubroot, in contrast to 35S::NATA1 which was one-third more resistant to clubroot (Fig. 6A). The density of *P. brassicae* within infected roots, evaluated by qPCR, was approximately a quarter less in the roots of the 35S::NATA1 overexpressor in comparison with the WT (Fig. 6B).

Thus, these results suggested that the JA-induced accumulation of N(\(\delta\))-acetylornithine does appear to contribute to basal resistance in the susceptible accession Col-0.

### Discussion

In this study, we aimed to investigate the involvement of SA and JA pathways in the resistance of Arabidopsis to one...
compatible *P. brassicae* isolate. Overall, our findings give a more detailed view of JA- and SA-triggered defenses induced by this pathogen in Arabidopsis roots. This study also suggests that both hormonal pathways contribute to the inhibition of the post-invasive development of clubroot. The contribution of the SA and JA pathways to the resistance response appears to depend on the Arabidopsis accession considered.

Indeed, differences in hormonal responses among Arabidopsis accessions, notably between Bur-0 and Col-0 (Koornneef et al. 2008, Ahmad et al. 2011), were previously detected with exogenous hormone treatments (Rao et al. 2000, Kliebenstein et al. 2002, van Leeuwen et al. 2007) or after pathogen inoculation (Perchepied et al. 2006). However, these observations were mostly made in leaves, and few data are available concerning the response at the root level.

SA accumulation and the expression of the SA-responsive genes during clubroot infection are poorly documented. Ludwig-Müller et al. (2015) reported low levels of SA accumulation in Col-0 clubroot-infected roots at the end of the secondary phase of infection (21 and 28 dpi). At the transcriptional level, Siemens et al. (2006) obtained similar results, reporting low induction of SA marker genes in Col-0. In the Bur-0 accession, the strong induction of *PR2* and *PR5* observed in this study agrees with the microarray results obtained by...

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**Fig. 3** Clubroot symptoms (A and B) and quantification of *Plasmodiophora brassicae* DNA (C) in infected roots of the partially resistant accession Bur-0 and the susceptible accession Col-0 treated or not with SA or MeJA. (A) Illustration of clubroot symptoms of non-treated and treated Col-0 and Bur-0 accessions at 21 dpi. (B) At 10 dpi, Col-0 and Bur-0 accessions were treated with 1 ml of SA (500 μM) or MeJA (50 μM) at the crown of the plant every 2 d to 21 dpi. Clubroot symptoms were evaluated using the GA/LA disease index. Error bars represent the SE (12 plants per block, four randomized blocks). (C) Pathogen DNA quantification (Pb) by qPCR, expressed as the percentage of the mean Pb content in inoculated roots of Col-0 without chemical treatment. Error bars represent the SE (pool of 12 plants per block, four randomized blocks). Asterisks indicate statistically significant differences according to the Wald tests applied on a linear mixed model, *P* < 0.05. Scale bars = 1.6 cm.
Jubault et al. (2013). The absence of PR1 expression in both Col-0 and Bur-0 clubroot-infected roots agrees with the microarray results of Siemens et al. (2006) and Jubault et al. (2013). Interestingly, low levels of PR1 expression were reported in other root pathosystems, notably after challenging with *Heterodera schachtii* (Wubben et al. 2008) and *Fusarium oxysporum* (Edgar et al. 2006).

The time-course induction of SA-related defenses in Bur-0 is consistent with a possible role for the SA pathway in limiting clubroot development during the secondary phase of infection. The idea that SA responses can contribute to partial resistance was also supported by our hormone treatments and genetic approaches. Indeed, we showed that SA treatment had a protective effect against clubroot symptoms in both Arabidopsis accessions. Agarwal et al. (2011) and Lovelock et al. (2013) also reported a similar positive effect of exogenous SA treatments on clubroot resistance, thus suggesting that this phenomenon can be robustly observed independently of specific laboratory conditions or growth culture parameters. In our work, however, the SA treatment did not modulate *P. brassicae* density within infected roots. This result may suggest that symptom extent and pathogen density in infected tissues can be controlled by different factors.

The possible role of SA signaling in clubroot partial resistance was furthermore supported by the reduction of clubroot symptom development in the *cpr5-2* mutant. Previous works (Bowling et al. 1997, Clarke et al. 2000) highlighted that the *cpr5-2* mutation leads (in the leaves) to the constitutive accumulation of SA, and to the SA-dependent induction of both NPr1-dependent and NPr1-independent resistance responses (including the induction of the JA/ET marker PDF1.2). In our work, *cpr5-2* infected by *P. brassicae* displayed a large induction of PR2, thus supporting that SA responses are activated in the roots of this mutant. In contrast, the very low expression of THI2.1 suggests that the *cpr5-2* mutation does not trigger JA responses in the roots infected by *P. brassicae*.

Clubroot did not induce SA responses in Col-0 under our conditions; thus, we did not expect an SA-accumulating/signaling mutants to show a specific phenotype upon clubroot infection. Accordingly, clubroot symptoms were similar in Col-0, *npr1-2* and *nahG*. However, it was more difficult to explain the observed phenotype of the SA-deficient mutant *eds5-1*, which exhibited slightly less symptoms than Col-0. In this mutant, the low levels of PR2 expression were consistent with a defect in SA signaling (Rogers and Ausubel 1997, Volko et al. 1998, Nawrath

![Fig. 4](https://academic.oup.com/pcp/article-abstract/56/11/2158/2755899)
and Métraux 1999, Nawrath et al. 2002). However, the JA response biomarker THI2.1 was highly induced (compared with Col-0), suggesting that the intensity of JA responses was higher in eds5-1 than in Col-0. The eds5-1 mutant has been mainly characterized at the foliar level and showed reduced SA accumulation and no differences in the expression of PR2 and PR5 following P. syringae pv. tomato inoculation compared with the WT Col-0 (Rogers and Ausubel 1997, Volko et al. 1998, Nawrath and Métraux 1999). To our knowledge, induction of THI2.1 in the inoculated leaves of this mutant has never been reported. Thus the slight increase in clubroot resistance observed in the eds5-1 mutant could be associated with high expression of the JA-responsive gene THI2.1. These results would also suggest that EDS5 is involved in the partial down-regulation of the JA pathway in Arabidopsis roots.

MeJA treatment did not reduce clubroot symptoms in Bur-0. We can therefore speculate that the endogenous clubroot-induced SA had an antagonistic effect on the exogenously applied MeJA. The antagonism between the two hormone pathways has been highlighted in several host–pathogen interactions (Mur et al. 2006, Leon-Reyes et al. 2010, Derksen et al. 2013, Caarls et al. 2015).

JA accumulation during clubroot infection was previously reported by Grsic et al. (1999) in the susceptible Chinese cabbage (Brassica rapa ssp pekinensis) and in Col-0 by Gravot et al. (2012). The high JA levels observed during the secondary phase of infection in Chinese cabbage roots were similar to those we obtained in the susceptible Col-0 accession. In our experimental conditions, we demonstrated that JA accumulation in Col-0 coincided with the induction of the JA-responsive genes THI2.1 and ARGAH2. This is consistent with the microarray results of Siemens et al. (2006) in clubroot-infected Col-0 roots. Siemens et al. (2006) reported that LOX2 (LIPOXYGENASE2) and THI2.1 genes were induced 60- to 90-fold at 23 dpi when galls are well developed. In contrast, ARGAH2 was less induced compared with LOX2 and THI2.1. The JA-dependent genes LOX2, THI2.1 and VEGETATIVE STORAGE PROTEINS (VSP), and the JA- and ET-dependent PDF1.2 genes have been widely used as JA transcriptional marker genes in leaf defense responses (Epple et al. 1995, Lorenzo et al. 2004, Memelink 2009, Derksen et al. 2013). Under our experimental conditions, induction of VSP2 and
PDF1.2 was not observed in clubroot-infected Col-0 roots. These results suggested that root defense responses can diverge from leaf defense responses, as previously highlighted in different reports on hormone-treated or pathogen-challenged roots (Edgar et al. 2006, Badri et al. 2008, Attard et al. 2010, Balmer and Mauch-Mani 2013).

The lack of induced THI2.1 and ARGAH2 expression in the jar1 mutant confirmed that these two genes are valuable JA response markers in the Arabidopsis–clubroot pathosystem. Thionins are cysteine-rich, basic proteins with toxic activity in vitro against plant pathogenic fungi and bacteria (reviewed by Sels et al. 2008) and can have thioredoxin activity (Johnson et al. 1987). ARGAH2 activity was reported to be induced during clubroot infection and was JA dependent (Jubault et al. 2008a, 2013) and can have thioredoxin activity (Johnson et al. 1987). THION1 and THION2 are JA and SA-dependent and SA-dependent defenses act against necrotrophs (Thomma et al. 2005). The application of MeJA was also shown to decrease PR1 expression in the Col-0 background (Kuiper et al. 2000). The ethylmethane sulfonate (EMS)-generated jar1 mutant (mutation in the cellulose synthase CEV1), which constitutively activates JA and ET signaling, a salicylate hydroxylase (which converts SA to catechol; You et al. 1991) and may not trigger equivalent resistance levels. At the foliar level, antagonistic rather than synergistic effects between SA and JA pathways have been reported more often: SA-dependent defenses act against biotrophs, and JA- and ET-dependent defenses act against necrotrophs (Thomma et al. 1998, Glazebrook 2005). The role of JA against biotrophs is poorly documented (reviewed by Antico et al. 2012). Interestingly, the cev1 mutant (mutation in the cellulose synthase CEV1), which constitutively activates JA and ET signaling, is more resistant to the biotrophs E. cichoracearum and P. syringae pv. maculicola (Ellis et al. 2002), pathogens which are known to induce SA responses (reviewed by Glazebrook 2005). The application of MeJA was also shown to decrease susceptibility to the virulent pathogen P. syringae DC3000 (van Wees et al. 1999), the powdery mildew E. cichoracearum and the downy mildew P. parasitica (Zimmerli et al. 2004). Interestingly, Pieterse et al. (1998) provided experimental evidence that basal resistance against P. syringae DC3000 is controlled by the combined action of SA, JA and ET in Arabidopsis. Ellis et al. (2002) suggested that both the JA and SA signaling pathways are able to increase resistance to P. syringae but SA appeared to be more effective than JA. These results suggest that JA activation in Arabidopsis can protect plants against some biotrophic pathogens but not all. In tomato, Thaler et al. (2004) showed that jasmonate-deficient mutant (def1) plants are more susceptible to the biotrophs P. syringae and Xanthomonas campestris and to the oomycete Phytophthora infestans but not to the biotrophs Oidium spp. and Cladosporium fulvum. Thus, these studies again suggest that JA can protect against pathogens with different lifestyles.

In the case of clubroot infection, the SA pathway appears to be more efficient than the JA pathway in clubroot resistance because: (i) Bur-0 is more resistant to clubroot disease than Col-0 and (ii) the cpr5-2 mutant (which constitutively activates the SA signaling) is more resistant to clubroot disease than eds5-1 (in which JA responses are highly activated).

This study provides new insights concerning the role of both JA and SA pathways in resistance of Arabidopsis against the biotrophic root pathogen P. brassicae. In addition, we highlighted the fact that two different hormonal responses may be induced in response to the same isolate of P. brassicae in different Arabidopsis accessions.

**Materials and Methods**

**Inoculum and plant material**

The inoculum used in all the clubroot tests was the ‘selected’ eH isolate (Fahling et al. 2003) which belongs to the P1 pathotype according to Somé et al. (1996). The host differential set described by Somé et al. (1996) was included in each test as a control.

The Bur-0 (172AV) and Col-0 (186AV) Arabidopsis seeds were provided by the Versailles Arabidopsis Stock Center (http://publiclines.versailles.inra.fr/). The Bur-0 (172AV) and Col-0 (186AV) accesses were described previously as partially resistant and susceptible to the P. brassicae eH isolate, respectively, by Alix et al. (2007).

The ethylmethane sulfonate (EMS)-generated jar1 mutant (JASMONATE RESISTANT) mutant (Col-0 background) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). In this mutant, the biosynthesis of the biologically active JA-Ile conjugate is significantly abolished (Staswick and Tiryaki 2004).

The transgenic NahG Arabidopsis plants express the bacterial gene encoding a salicylate hydroxylase (which converts SA to catechol; You et al. 1991) and consequently cannot accumulate SA (Delaney et al. 1994). In the EMS recessive mutant, npr1-2 (for nonexpressor of PR genes) [Col-0(fah1-2) background], SAR and PR1 expression following treatments with SA or 2,6-dichloroisonicotinic acid (INA) or bacterial infection is impaired (Cao et al. 1994, Glazebrook et al. 1996, Cao et al. 1997). The At4g39030 gene, which encodes a MATE (multidrug and toxin extrusion) transporter family, is deleted in the EMS recessive mutant eds5-1 (for enhanced disease susceptibility5 allele) to sid1 and eds5-2 [Col-0(fah1-2) background]. This mutant is characterized by reduced SA accumulation and PR1 expression after bacterial infection (Rogers and Ausubel, 1997, Volko et al. 1998, Nawrath and Métraux 1999, Nawrath et al. 2002). The cpr5-2 mutant (for constitutive expresser of PR genes 5) is a constitutive expresser of PR1, PR2 and PRS genes and accumulates 30 times higher levels of SA than the WT (Bowling et al. 1997, Boc et al. 1998). Professor Jonathan D.G. Jones (Sainsbury Laboratory, Norwich, UK) kindly provided nahG, npr1-2 and
eds5-1 mutant seeds. The cpr5-2 mutant was kindly provided by Dr. Xinnian Dong (Duke University, USA).

The seeds of the nata1 (for N-acetyltransferase activity 1) and 35S::NATA1 mutants, which are respectively deficient in and overaccumulators of N(0)-acetylornithine (Adio et al. 2011), were kindly provided by Professor Georg Jander (Cornell University, USA).

Experimental design

Clubroot resistance tests were performed in a randomized block design with four replicates, each containing 12 plants per genotype. Clubroot symptoms were quantified at 21 dpi.

Gene expression and metabolite accumulation in Col-0 and Bur-0 were quantified at 10, 14 and 17 dpi, which corresponds to the secondary phase of infection under our experimental conditions. The test was conducted in a randomized block design with six replicates, each containing 24 inoculated and non-inoculated plants per genotype and per kinetic time point.

For all the mutants analyzed, the effect of the mutation on the expression of SA- and JA-responsive genes was verified at 21 dpi in inoculated plants (four replicates each containing 12 plants).

Symptom quantification

Symptoms of Col-0, Bur-0 and the mutants were sown individually in pots containing a mix of compost: vermiculite (2:1, v/v). Seedlings were grown in a growth chamber (16 h of light at 200 μmol m⁻² s⁻¹ at 22°C and 8 h of dark at 19°C) and were inoculated at the crown 10 d after germination with 1 ml of the E. coli spore suspension (10⁷ spores ml⁻¹) (Manzanares-Dauleux et al. 2000) or distilled water for non-inoculated plants. Susceptibility to clubroot of the mutants and SA- and MeJA-treated plants was evaluated at 21 dpi by symptom quantification using image analysis. Inoculated plants were washed and photographed next to a scale. Clubroot symptoms were quantified using the GA/LA pathologic index. Briefly, this index was calculated as the ratio between the gall area (GA in cm²) and the square of the longest leaf length of the rosette (LA in cm²), determined by ImageJ software, which was then multiplied by 5,000 (Gravot et al. 2012).

For molecular and biochemical analysis, 3 cm of washed inoculated and non-inoculated roots were sampled, pooled, frozen in liquid nitrogen and stored at -80°C.

Phytohormone applications

Solutions of SA (Sigma Aldrich) and MeJA (Oltchemin Ltd.) were prepared in distilled water at the final concentrations of 500 or 50 μM, respectively. At 10 dpi (the beginning of the secondary phase of infection), Col-0 and Bur-0 were treated with 1 ml of SA or MeJA at the crown of the plant every 2 d to 21 dpi. Control plants were treated with an equivalent volume of distilled water.

Quantification of P. brassicae DNA content in infected roots

DNA was extracted from root samples using the NuclEaseSpin Plant Kit II (Macherey-Nagel) following the manufacturer’s instructions. PCR reactions were performed using a LightCycler 480 thermocycler (Roche) for the quantification of the P. brassicae target gene AF231027. Each reaction was performed with 2.5 ng of total DNA as template, and the following primers: Pb F, 5'-AGAGCTTGGCTCCTGCG-3'; Pb R, 5'-AGACTTGGCCTGGCGAT-3' as previously described in Lemarie et al. (2015). Four biological replicates were analyzed. Standard curves were performed using serial dilutions of DNA extracted from the roots of Col-0 at 21 dpi without chemical treatment, which was defined as a reference condition. Quantitative results were then expressed as the percentage of the P. brassicae mean DNA content in this reference condition.

RNA extraction and real-time (RT)-qPCR

Expression of the SA-responsive genes PR2 and PR5 and the JA-responsive genes THI2.1 and ARG12 was assessed in Col-0 and Bur-0 roots at 10, 14 and 17 dpi in the six replicates. The expression of these genes was also determined in all mutants at 21 dpi in the six replicates. Total RNA from infected and non-infected roots was extracted using the 'SV Total RNA Isolation System' kit (Promega) according to the manufacturer's instructions, with an additional step of DNase using the 'Ambion DNA-free' kit (Ambion). First-strand cDNA was then synthesized in a 20 μl reaction mixture containing 1.6 μg of total RNA with the 'Superscript II Reverse Transcriptase' kit (Invitrogen) with oligo(dT)₁₄ primers following the manufacturer’s instructions. Semi-quantitative RT-PCRs were performed as follows in a 12.5 μl final volume: 4 μl of diluted cDNA, 6.25 μl of 2× LightCycler 480 Syber Green I Master (Roche), 1.25 μl of nuclelease-free water and 4 μl of forward and reverse primers. The primer sets used to analyze the expression of the SA and JA biosynthesis/signaling marker genes were as follows: PR2 F (At3G57260), 5'-GCTCTTGGCCGCTTCCGT-3'; PR2 R, 5'-ACCTA TCATGTTGGTGTCAT-3'; PRS (At1G57040) F, 5'-TCGAGTTGATACAGT CGGTTT-3'; PRS R, 5'-GCTTTAAAGTTGACAGCTGA-3'; THI2.1 (At1G72260) F, 5'-GCAAATGAAATTGGATG-3'; THI2.1 R, 5'-CACACAC ACACAACACACAA-3'; ARG12 (At2G08870) F, 5'-TGTGACTTTGGAGT TAATCGTGTC-3'; ARG12 R, 5'-TGCTATAGACACACGACCTGTC-3'; NAT1 F (At2G9030) F, 5'-CCCCCCTTCTTGACAGCAT-3'; NAT1 R, 5'-CACACAC ACACAACACACAA-3'; PPA2 F (At1G13320) F, 5'-TAAA GTGTCGGAATGATCC-3'; PPA2 R, 5'-GATTCCAAACGTAACAGAACAC- AA-3'. Amplification reactions were carried out with 50 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 30 s and 72°C for 30 s, respectively, followed by melt curve analysis. A standard curve was established for each of the genes, using a dilution series from a cDNA pool of all samples. Gene expression levels were normalized to the expression of the housekeeping gene PPA2. Two independent technical replicates were analyzed.

SA and JA quantification

The phytohormones SA and JA were quantified in Col-0 and Bur-0 roots at 10, 14 and 17 dpi in the six replicates. For each time point and genotype, the two compounds were extracted from approximately 200 mg of freshly ground roots in 1.5 ml tubes. After addition of 1 ml of a methanol:water:formic acid (80:19:1 by vol.) mixture solvent, tubes were ultrasonicated and agitated at room temperature for 30 min. The tubes were then centrifuged at 1200 × g for 10 min and the supernatants were removed into new 1.5 ml tubes. The pellets were re-extracted with 1 ml of the extraction solvent and the supernatants were pooled and dried in a speed vacuum centrifuge. Dried residues were then resuspended in 100 μl of acidified methanol.

Phytohormones were analyzed by injecting 5 μl of resuspended samples in an Acquity UPLC system (Waters) coupled to a triple quadrupole detector equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed on an Acquity HSS T3 1.8 μm (2.1 × 150 mm) column using a gradient with solution A (0.1% of formic acid in water) and solution B (0.1% of formic acid in methanol). The elution gradient started with 99% of A and 1% of B, then 20 min later 100% of B and returned to the initial conditions within 5 min. The column temperature was maintained at 40°C and the flow rate at 0.35 ml min⁻¹. SA and JA were detected in Multi Reaction Monitoring (MRM) negative mode in the ESI source of the mass spectrometer applying 29 V and 25 V to the extraction cone respectively, and were fragmented at 13 V. The transitions obtained were: 136.6 → 92.8 for SA; and 209 → 59 for JA. Data were acquired with Masslynx software. Authentic SA and JA (Sigma Aldrich and Oltchemin Ltd.) were used as external standards.

N(δ)-acetylornithine quantification

N(δ)-acetylornithine was quantified in Col-0 and Bur-0 roots at 10, 14 and 17 dpi in the six replicates from 100 μl of resuspended acidified methanol hormone extracts (see above). N(δ)-acetylornithine quantification was performed by the evaporation of 10 μl of resuspended samples, and dried residues were solubilized with 50 μl of a 100 μM γ-aminoxybutyric acid (GABA) internal standard solution. Then, 5 μl of samples were derivatized with the AccQTag derivatization kit (Waters) by adding 35 μl of borate solution and 10 μl of AOQ (6 aminooxyximino-N-hydroxysuccinimidy carbamate) mixture solvent. The mix was then incubated for 10 min at 55°C, centrifuged and injected into the UPLC-TQD system. To determine the elution time and detection parameters of the genes, using a dilution series from a cDNA pool of all samples. Gene expression levels were normalized to the expression of the housekeeping gene PPA2. Two independent technical replicates were analyzed.

N(δ)-acetylornithine was quantified in Col-0 and Bur-0 roots at 10, 14 and 17 dpi in the six replicates from 100 μl of resuspended acidified methanol hormone extracts (see above). N(δ)-acetylornithine quantification was performed by the evaporation of 10 μl of resuspended samples, and dried residues were solubilized with 50 μl of a 100 μM γ-aminoxybutyric acid (GABA) internal standard solution. Then, 5 μl of samples were derivatized with the AccQTag derivatization kit (Waters) by adding 35 μl of borate solution and 10 μl of AOQ (6 aminooxyximino-N-hydroxysuccinimidy carbamate) mixture solvent. The mix was then incubated for 10 min at 55°C, centrifuged and injected into the UPLC-TQD system. To determine the elution time and detection parameters of the genes, using a dilution series from a cDNA pool of all samples. Gene expression levels were normalized to the expression of the housekeeping gene PPA2. Two independent technical replicates were analyzed.
Ultra eluent A, Waters) and solution B (acetonitrile gradient quality, Waters).

The elution gradient was applied as follows: 99.9% of A and 0.1% of B (0.5 min), 99.9–90.0% of A and 0.1–9.1% of B (5.96 min), 90.9–78.8% of A and 9.1–21.2% of B (2 min), 78.8–40.6% of A and 21.2–59.6% of B (0.4 min), 69.6% of B (0.5 min), and 99.9% of A and 0.1% of B (0.5 min). The column temperature was maintained at 53 °C and the flow rate at 0.7 ml min⁻¹. The retention time of the (d₆)-acetylornithine was 5.60 min. The ionization was performed in a positive mode at the ESI source of the mass spectrophotometer and the collision voltage applied for fragmentation was 23 V. N(d₆)-acetylornithine was detected in MRM mode, using the transition 345 > 171. The parent ion was the product of AccQTag derivatization of N(d₆)-acetylornithine (m/z = 174 + 171), and the daughter ion (m/z = 171) is a diagnostic ion generated by tandem mass spectrometry (MS/MS) for all amino acids derivatized with AccQTag. Data were acquired with Masslynx software. Results were normalized by using the BABA internal standard. The purity level of our home-made (d₆)-acetylornithine-equivalent (Sigma Aldrich) and then expressed in N(d₆)-acetylornithine equivalent.

**Statistical analysis**

Statistical analyses were performed with RGUI software using Wald tests applied on Linear Mixed Models (function ‘lsmeans’, package lsmeans) of all amino acids derivatized with AccQTag. Data were acquired with Masslynx software. Results were normalized by using the BABA internal standard. The purity level of our home-made (d₆)-acetylornithine-equivalent (Sigma Aldrich) and then expressed in N(d₆)-acetylornithine equivalent.

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**Disclosures**

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