Original citation:

Permanent WRAP url:
http://wrap.warwick.ac.uk/78045

Copyright and reuse:
The Warwick Research Archive Portal (WRAP) makes this work of researchers of the University of Warwick available open access under the following conditions.

This article is made available under the Creative Commons Attribution 4.0 International license (CC BY 4.0) and may be reused according to the conditions of the license. For more details see: http://creativecommons.org/licenses/by/4.0/

A note on versions:
The version presented in WRAP is the published version, or, version of record, and may be cited as it appears here.

For more information, please contact the WRAP Team at: publications@warwick.ac.uk
Novel JAZ co-operativity and unexpected JA dynamics underpin *Arabidopsis* defence responses to *Pseudomonas syringae* infection

Marta de Torres Zabala1*, Bing Zhai2*, Siddharth Jayaraman1, Garoufalia Eleftheriadou1, Rebecca Winsbury1, Ron Yang1, William Truman3, Saijung Tang2, Nicholas Smirnoff1 and Murray Grant1

1Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK; 2College of Biological Sciences, China Agricultural University, Beijing 100093, China; 3Department of Plant Biology, University of Minnesota, Saint Paul, MN 55108, USA

**Summary**

- Pathogens target phytohormone signalling pathways to promote disease. Plants deploy salicylic acid (SA)-mediated defences against biotrophs. Pathogens antagonize SA immunity by activating jasmonate signalling, for example *Pseudomonas syringae* pv. tomato DC3000 produces coronatine (COR), a jasmonic acid (JA) mimic. This study found unexpected dynamics between SA, JA and COR and co-operation between JAZ jasmonate repressor proteins during DC3000 infection.
- We used a systems-based approach involving targeted hormone profiling, high-temporal-resolution micro-array analysis, reverse genetics and mRNA-seq.
- Unexpectedly, foliar JA did not accumulate until late in the infection process and was higher in leaves challenged with COR-deficient *P. syringae* or in the more resistant JA receptor mutant coi1. JAZ regulation was complex and COR alone was insufficient to sustainably induce JAZs.
- JAZs contribute to early basal and subsequent secondary plant defence responses. We showed that JAZ5 and JAZ10 specifically co-operate to restrict COR cytotoxicity and pathogen growth through a complex transcriptional reprogramming that does not involve the basic helix-loop-helix transcription factors MYC2 and related MYC3 and MYC4 previously shown to restrict pathogen growth. mRNA-seq predicts compromised SA signalling in a jaz5/10 mutant and rapid suppression of JA-related components on bacterial infection.

**Introduction**

Successful infection of plants by bacterial pathogens is mediated by bacterial ‘effector’ proteins targeted to the host cell via a specialized type III secretion apparatus and by small-molecule virulence factors. These virulence components function collectively to suppress innate immunity activated by plant pattern recognition receptor recognition of microbe-associated molecular patterns (MAMPs; reviewed in Macho & Zipfel, 2014; Zipfel, 2014) and modify host metabolism to enhance pathogen fitness. Pathogen modulation of phytohormones by effectors has emerged as a key pathogen virulence strategy. The hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have been associated with plant immunity. SA is generally considered to be singly or collectively important for immunity to a range of necrotophs (reviewed in Glazebrook, 2005). Recently, other hormones, such as abscisic acid (ABA), auxin and gibberellins, have been implicated in moulding defence responses (Grant & Jones, 2009; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

Genetic studies have revealed that jasmonates antagonize SA-mediated biotrophic plant defences (Kloek *et al.*, 2001; Brooks *et al.*, 2005), although jasmonates have also been reported to synergize SA defence (Mur *et al.*, 2006; Truman *et al.*, 2007). The virulent hemibiotroph *P. syringae* pv. tomato strain DC3000 (DC3000) rapidly induces ABA biosynthesis in *Arabidopsis thaliana*, which also antagonizes SA accumulation (de Torres Zabala *et al.*, 2007, 2009).

Some pathogens have evolved the capacity to produce hormones or hormone mimics (Grant & Jones, 2009; Robert-Seilaniantz *et al.*, 2011). The non-host-specific chlorosis inducing the polyketide toxin coronatine (COR) is synthesized by some (tomato, glycinea, atropurpurea, morsprunorum and maculicola) *P. syringae* pathovars (Ichihara *et al.*, 1977; Bender *et al.*, 1999). COR, formed by the ligation of two distinct structural compounds, coronamic acid (CMA) and coronafacic acid (CFA)

*These authors contributed equally to this work.*
(Bender et al., 1998), mimics the bioactive plant jasmonate (3R,7S)-jasmonoyl-1-isoleucine (JA-Ile; Katsir et al., 2008; Fonseca et al., 2009b). It promotes opening of stomata, aiding bacterial entry to the apoplast, facilitates apoplastic bacterial multiplication, contributes to disease symptoms and promotes systemic susceptibility (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006).

COR has been instrumental in the genetic dissection of jasmonate signalling pathways, including the discovery of the coronatine insensitive 1 (COI1) jasmonate receptor. However, our understanding of the extent to which COR mimics jasmonates in plant defence and its in planta targets remains rudimentary. COR is a multifunctional defence suppressor. COR’s ability to suppress host defences is partly associated with antagonism of SA signalling via COI1 activation (Kloek et al., 2001; Zhao et al., 2003); however, COR also suppresses callose deposition and promotes DC3000 multiplication in a COI1-independent manner (Geng et al., 2012).

The best-characterized JA response pathway in A. thaliana is mediated by the transcription factor MYC2 (Lorenzo et al., 2004; Chini et al., 2007). MYC2 is held in a transcriptionally inactive state through binding of JAZ (jasmonate–ZIM domain-containing) repressor proteins. In the presence of JA-Ile or COR, JAZs are ubiquitinated by the E3 ubiquitin ligase complex (SCF<sup>COI1</sup>) and then degraded by the 26S proteasome, freeing MYC2 to activate jasmonate signalling networks. MYC2 also activates the transcription of JAZs, leading to a negative feedback loop that represses transcriptional repression (Lorenzo et al., 2004; Melotto et al., 2006; Chini et al., 2007; Thines et al., 2007; Fonseca et al., 2009a). Arabidopsis thaliana contains at least 12 JAZs, many encoding splice variants (Chung et al., 2010) and most capable of homo- and heterodimerization (Chini et al., 2009; Chung & Howe, 2009). This combinatorial complexity may well provide the specificity that underpins the diversity of processes regulated by jasmonates (Pauwels & Goossens, 2011; Kazan & Manners, 2012). Although MYC2 was originally identified as the target of JAZs, the number of transcription factors interacting with JAZ proteins has expanded significantly (Wasternack, 2014). However, our understanding of how individual JAZs collectively contribute to the myriad of jasmonate-regulated responses remains limited.

This study explores the dynamics, interaction and contribution of JA, COR and JAZs to DC3000 disease progression. Inconsistent with a scenario in which JA antagonizes SA signalling, JA accumulated very late in the infected leaf, suggesting that host defence mechanisms temper JA antagonism of basal defence. JAZ transcripts showed a complex regulatory pattern, differentially contributing to induced basal immunity to MAMPs and a rapid and sustained induction in response to DC3000. Detailed genetic analyses revealed that JAZ5 and JAZ10 function co-operatively to attenuate phytoxocity mediated by COR and to moderately restrict bacterial growth. Collectively, the induction of JAZs concomitant with in planta COR production and enhanced JA in the absence of COR suggest that plants may actively respond to bacterial COR via the sustained activation of JAZ-based defences.

**Materials and Methods**

**Arabidopsis thaliana growth**

*Arabidopsis thaliana* (L.) Heynh. genotypes were sown in Levington F2 compost with sand and stratified for 2 d at 4°C. Plants were grown under short days at 65% humidity in a controlled environment chamber (10 h light, 120 μmol m<sup>–2</sup> s<sup>–1</sup>, at 22°C day, 20°C night) for 5 wk before use. The Arabidopsis genotypes studied in this work were Col-0, sid2-1 (Wildermuth et al., 2001), pad4-1 (Jirage et al., 1999), myc2 and 35S::MYC2 (Lorenzo et al., 2004) and a set of single, double and triple jaz knockout lines, the parental lines derived from the Nottingham Arabidopsis Stock Centre. Details of the lines and genotyping primers are provided in Supporting Information Table S1.

**Pseudomonas infections**

Bacterial cultures were maintained, prepared and inoculated in Kings B medium as described previously (de Torres et al., 2006). For RNA and metabolite extractions and growth curves, leaves were inoculated with a 1-ml needleless syringe on their abaxial surface with a bacterial suspension adjusted with 10 mM MgCl<sub>2</sub> to a final optical density at 600 nm (OD<sub>600</sub>) of 0.15 or as indicated in the figure legends. All bacterial growth measurements were determined from a minimum of five independent replicates, each comprising three challenged leaves per plant. Significant growth differences between treatments were determined by Students t-test (P<0.5), error bars representing the standard deviation (SD) of the mean. All experiments were repeated at least three times.

**Hormone measurements**

Each hormone determination was measured in triplicate with each replicate consisting of a minimum of six infected leaves from two plants. Inoculum densities were as described in the figure legends. Samples were collected at appropriate times, frozen immediately in liquid nitrogen and subsequently freeze-dried. Hormone extractions were performed on 10 mg of powdered, freeze-dried tissue, exactly as detailed in Forcat et al. (2008). Figures are representative of at least two replicated experiments. Significant differences between treatments were determined by one-way ANOVA using the least-significant difference *post-hoc* test, error bars representing the SD of the mean. Methyl jasmonate (MeJA) and JA-Ile were purchased from OLCHE MIME (Olomouc, Czech Republic). CFA was a gift from Robin Mitchel (HortResearch Auckland, New Zealand).

**RNA extraction and quantitative PCR analysis**

Total RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were performed as described by de Torres Zabala et al. (2009). *Act2* (At3g18780) was used as internal standard to normalize cDNA abundance between samples. Relative expression levels are expressed in
For RNA expression studies, bacterial inoculum densities were adjusted to OD 600 = 0.15. For each treatment, mock, DC3000 or DC3000hrpA, four biological replicates comprising leaf 8 of 4-wk-old plants were syringe infiltrated. Samples were collected at 0, 2, 3, 4, 6, 7, 8, 10, 12, 14 and 17.5 h post-inoculation (hpi) and snap frozen in liquid nitrogen. RNA was extracted and hybridized to CATMA arrays (Allemear Sch et al., 2005) and processed as described previously (Breeze et al., 2011). The data comprise the means from four single-leaf biological replicates and two technical replicates per time point. Blue and yellow matrices indicate that differential expression is significantly (Student’s t-test P = 0.01, n = 8) induced or suppressed, respectively, between treatments. The full infection time course microarray analysis data are deposited at Gene Expression Omnibus (GEO) under the accession number GSE56094.

Root assays

Surface-sterilized wild-type and various combinations of jaz mutants were germinated on 25-cm² plates containing half-strength Murashige and Skoog (MS) medium supplemented with either COR (Sigma) at 0.02 or 0.2 μM, or MeJA (Sigma) at 10 or 50 μM. Plates were incubated in an upright position under short days (see the ‘Arabidopsis thaliana’ growth subsection above) and the root length of 8-d-old seedlings was measured using ImageJ software (http://imagej.nih.gov/ij/).

mRNA-seq

Total RNA from naïve or DC3000-challenged leaves (OD 600 = 0.15) of Col-0 or jaz5/10 plants was isolated at 6, 8, 12 or 16 hpi. Three leaves from four plants were collected at each time point and RNA was prepared as described earlier. mRNA-seq libraries were prepared using Poly(A)-RNA, pooled from three biological replicates for each time point. Directional RNA libraries were prepared using Illumina’s (San Diego, CA, USA) ScriptSeq v.2 protocol, and library size and concentrations were calculated using a Bioanalyser DNA7500 chip. Libraries were pooled in equimolar amounts, denatured and diluted to 6.5 pM, clustered and 100-bp paired-end sequenced on an Illumina HiSeq 2500 using Illumina SBS reagents. Data were analysed by gFOLD (Feng et al., 2012) using a cutoff of log2 1.6. Data are available from GEO under the submission number GSE72461.

Results

Hormone and COR accumulation during DC3000 infection

We measured JA, ABA, SA and COR in wild-type A. thaliana (Col-5) and the JA receptor mutant coi1-16 (cleared of the pen2 mutation; Westphal et al., 2008), which is compromised in most jasmonate responses (Turner et al., 2002), following infection with either P. syringae DC3000 or strain DC3000Δcfa6ΔcmaA, which is deficient in the production of both COR precursors, CFA and CMA (Brooks et al., 2004). JA rather than JA-Ile was measured because DC3000 responses are wild-type in jar1 mutants (Staswick & Tiryaki, 2004; Laurie-Berry et al., 2006), although JA acts as a proxy for many active jasmonates and JA-Ile dynamics often mirror JA dynamics (Balcke et al., 2012). The DC3000hrpA mutant, which activates plant basal defence, but is unable to deliver effectors or to synthesize appreciable quantities of COR (de Torres Zabala et al., 2009), did not induce appreciable JA accumulation (Fig. 1a) compared with virulent DC3000 (Fig. 1a). Indeed, JA did not accumulate until 18 hpi in all challenges (Fig. 1a), whereas COR first accumulated between 6 and 10 hpi in DC3000-inoculated leaves (Fig. 1b). Unexpectedly, at 18 hpi, JA accumulated to twice the level in leaves challenged with DC3000Δcfa6ΔcmaA compared with DC3000 (Fig. 1a). Rather than stimulating (Cui et al., 2005; Laurie-Berry et al., 2006), COR appears to directly or indirectly compromise JA accumulation (Fig. 1a).

In coi1-16, JA levels were c. 50% of wild-type levels (Fig. 1a), indicating that COI1-independent processes contribute to JA accumulation. By contrast, JA levels in DC3000Δcfa6ΔcmaA-challenged coi1-16 leaves were similar to those in DC3000Δcfa6ΔcmaA-challenged Col-5. Together, these results suggest that JA accumulation following DC3000Δcfa6ΔcmaA challenge is largely COI1 independent. JA levels in the 2-oxophytodienoic acid reductase opr3 JA biosynthetic mutant (Stintzi & Browse, 2000) were c. 80-fold lower than in the parental Ws-0 ecotype. Thus, DC3000-induced JA is most probably derived from de novo synthesis (Fig. S2).

SA levels were maximal at 6 hpi in DC3000-challenged Col-5 and decreased thereafter, consistent with effector-mediated suppression of SA (Fig. 1c). At 12 hpi, SA levels were significantly higher in Col-5 leaves challenged with DC3000Δcfa6ΔcmaA (c. 2.5-fold) or coi1-16/DC3000Δcfa6ΔcmaA (c. three-fold) compared with Col-5/DC3000 inoculation. The marked decrease in SA levels between 12 and 18 h in leaves challenged with DC3000Δcfa6ΔcmaA (Fig. 1c) parallels the concomitant increase in JA seen in both genotypes at this time (Fig. 1a). However, in DC3000/coi1-16-infected leaves, SA levels only differed significantly from DC3000/Col-5 challenge at 18 hpi. These data suggest that COR-dependent suppression of SA during DC3000
infection is not fully COI1 dependent. Neither COR nor COI1 markedly affected the rapid (6 hpi) ABA accumulation (de Torres Zabala et al., 2009), which reached maximal levels at 12 hpi (Fig. 1d). ABA levels only differed from the wild-type in coi1-16/DC3000Δcfa6:Δcma (Δcfa6/Δcma) strain (OD600 = 0.15) and foliar levels of (a) jasmonic acid (JA), (b) coronatine, (c) salicylic acid (SA) and (d) abscisic acid (ABA) were determined at 6, 12 and 18 h post-inoculation (hpi), as well as non-inoculated tissue (ni). Different letters indicate significant differences (P < 0.05) between the corresponding treatments at that time point as assessed by one-way ANOVA using the least-significant difference (LSD) post-hoc test (means ± SD; n = 3). Experiments were repeated at least three times and all showed the same trend.

In summary, foliar JA accumulates unexpectedly late in the infection process, well after bacterial multiplication occurs. A significant proportion of this JA accumulation is COI1 independent and, counter-intuitively, antagonized by COR production. This pattern of antagonism is consistent with COR triggering a host defence response.

**Induction of transcripts encoding JA biosynthetic components during a compatible interaction**

We next examined the expression of genes related to jasmonate signalling in a high-resolution microarray experiment, reporting *A. thaliana* responses to virulent DC3000, DC3000hrpA and mock (MgCl₂) challenges (Fig. 2a; Table S2). We compared JA biosynthetic genes induced during basal defence (DC3000hrpA vs MgCl₂; Fig. 2ai) with a compatible interaction (DC3000 vs MgCl₂; Fig. 2aii), using mock challenge to remove any wound response induced by syringe infiltration (de Torres Zabala et al., 2009; Fig. S1). JA biosynthesis pathway genes were induced within 7 hpi by DC3000 and were also significantly, but more weakly, induced by DC3000hrpA challenge, which does not lead to the accumulation of appreciable amounts of JA (Figs 2aiii, S1). Concomitant suppression of *CYP74B2* (At4g15440), encoding hydroperoxide lyase 1 involved in the production of hexanal and 12-oxo-cis-9-dodecenoic acid from 13-hydroperoxide (Duan et al., 2005), potentially increases substrate availability for JA synthesis following DC3000 challenge.

**Induction of JAZ transcriptional repressors during bacterial challenge**

The disparity between early (7 hpi) transcriptional activation of JA biosynthetic genes and late (18 hpi) accumulation of *de novo* JA is striking. This result implies that active host defences alleviate JA antagonism of SA signalling and desensitize the host to COR. One possibility is that specific sustained induction of JAZs may lock JAZ targets into a constitutively repressed state. The analysis of JAZ and MYC2 expression profiles (Fig. 2b; Table S2) revealed that MYC2 and most JAZs, with the exception of JAZs 4, 11 and 12, were rapidly induced by DC3000 between 6 and 7 hpi (Fig. 2bi), albeit with contrasting dynamics. Generally JAZs (and MYC2) were more strongly induced by DC3000. JAZ1, JAZ5 and JAZ6 (but not MYC2) most rapidly responded to
JAZ5 and JAZ10 collaborate to restrict COR phytotoxicity

This almost synchronous induction of JAZs coincided with the first detectable levels of COR (Fig. 1b; de Torres Zabala et al., 2009). We used a reverse genetic approach to investigate the possible role of JAZs in the enhanced JA accumulation in the absence of COR production (Fig. 1a,b). Arabidopsis T-DNA insertion mutants in JAZs 1–7, JAZ10 and JAZ12 showed wild-type symptoms following DC3000 challenge, with the exception of mildly enhanced chlorosis in jaz10 leaves (Fig. 3aii), as reported previously (Demianski et al., 2012). To address possible functional co-operation between JAZs, we generated a collection of double mutants (Table S1) and screened for altered symptoms. Strikingly, the jaz5/10 double mutant (see Fig. S3 for transcript accumulation in mutants) showed strong chlorosis at 5 d post-inoculation (dpi) following either syringe infiltration with DC3000 (Fig. 3aiii) or dip inoculation, which was not evident in either single mutant (Fig. 3ai,aii) or in any other double mutant combination tested (Table S1). The incorporation of additional knockouts of JAZ2, 3, 6 or 7 into the jaz5/10 background did not further enhance leaf chlorosis (Table S1). Remarkably, chlorotic symptoms were completely abolished in jaz5/10 plants challenged with DC3000 cor mutants (Fig. 3av,avi). The COR toxin induces chlorosis and jaz5/10 leaves co-inoculated with COR (0.25 μM) and DC3000 (Fig. 3b) showed stronger symptom development compared with Col-0 (Fig. 3biii,biv), indicating enhanced COR sensitivity. Co-infiltration of DC3000 Δcfa6:ΔcmaA with COR (0.25 μM) partly restored the chlorosis phenotype in jaz5/10, but not Col-0 (Fig. 3biii,bvii), whereas DC3000Δcfa6:ΔcmaA challenge alone failed to cause symptoms in either genotype (Fig. 3bvi,bvii). Collectively, these results demonstrate a co-operative role for JAZ5 and JAZ10 in suppressing the phytotoxic effects of COR.

We compared jasmonate sensitivity of jaz5/10 to various other double mutant combinations using root growth assays on medium supplemented with MeJA (5.0 μM) or COR (0.2 μM). jaz5/10 showed no significant difference in root growth compared with most other double mutant combinations (Figs 4a, S4), yet only jaz5/10 developed the strong chlorotic symptoms following DC3000 challenge (Fig. 4b). Chlorosis was dependent on bioactive COR, as co-infiltration with DC3000Δcfa6:ΔcmaA and CFA did not induce enhanced chlorosis (Fig. 4c). These results show that the co-operative activities of JAZ5 and JAZ10 abrogate COR phytotoxicity in DC3000-infected leaves. We next tested whether JAZ5/10 reduced sensitivity to COR by enhancing COR degradation or preventing the synthesis/transport of COR by the bacteria. We discounted the possibility that jaz5/10 mutants influence COR production, as levels of expression of the bacterial Cfa6 polyketide synthase or the CorR regulator (Fig. 4d) were similar. Indeed, CorS levels were actually lower in jaz5/10. We excluded hyperaccumulation of COR, as COR was lower in jaz5/10 mutants than in enhanced susceptibility...
pad4 or sid2 mutants at 16 hpi with DC3000 (Fig. 4e). Finally, exogenous application of COR did not enhance COR stability in jaz5/10 (Fig. 4f). Thus, JAZ5 and JAZ10 can function in planta to restrict COR cytotoxicity.

jaz5/10 is moderately more susceptible and shows altered hormone levels

jaz5/10 supported significantly enhanced growth of DC3000 at 3 dpi at low inoculum (OD$_{600} = 0.0002$) compared with Col-0, jaz5 and jaz10, corroborating the observation of moderately enhanced symptoms, but not enhanced bacterial growth, in jaz10 (Demianski et al., 2012). However, the increased susceptibility of jaz5/10 was modest compared with the highly susceptible pad4 mutant (Jirage et al., 1999) (Fig. 5a). This result suggests that JAZ5 and JAZ10 make a small, but significant, contribution to host defence against DC3000. Interestingly, at higher inoculum (OD$_{600} = 0.002$), both jaz5/10 and jaz10 supported more bacterial growth (Fig. 5b), suggesting that JAZ10 may restrict bacterial growth at high titre, such as experienced later in disease development. Furthermore, jaz5/10 was more susceptible than Col-0 to co-infiltration of DC3000Δcfa with COR (Fig. 5c), indicating that JAZ5 and JAZ10 moderate COR virulence.

Hormone profiles revealed complex responses. Compared with Col-0, ABA was higher at 10 hpi and SA was lower at 20 hpi in DC3000-challenged jaz5/10, possibly explaining its moderately increased susceptibility (Fig. 5a). As in Fig. 1, JA levels were significantly higher in DC3000ΔcmaA than in DC3000-challenged Col-0 leaves at 20 hpi (Fig. 5f). Notably, JA was even higher in COR-deficient DC3000ΔcmaA-challenged jaz5/10 leaves compared with Col-0 (Fig. 5g). This result indicates that JAZ5 and JAZ10 help to restrict late JA accumulation. Although COR levels were correspondingly higher in jaz5/10 leaves challenged with DC3000 compared with Col-0 (Fig. 5g), JA was moderately, but significantly, lower in DC3000-challenged jaz5/10 leaves. Collectively, these results reveal a complex interaction in which JAZ5/10 contributes not only to reduced cytotoxicity, but to enhanced foliar JA in the absence of COR, but restricts JA accumulation in the presence of COR.

Bacterial COR alone is insufficient for sustained induction of JAZs

The strong chlorosis induced by DC3000 on jaz5/10 leaves is reminiscent of symptoms on DC3000-infected tomato (Ishiga
et al., 2009). The sustained JAZ induction following DC3000 challenge is coincident with COR production. To investigate whether COR alone activates JAZ transcription, we measured the expression of JAZ5, JAZ10 and MYC2 in Col-0 following mock, DC3000 or pure COR challenge. We tested high (5 μg ml⁻¹; 16 μM) and low (0.5 μg ml⁻¹; 1.6 μM) COR concentrations.
Low represents the maximal levels experimentally detected in DC3000-challenged Col-0 leaves (de Torres Zabala et al., 2009) (Fig. 6).

MYC2 was not transcriptionally responsive to DC3000, only transiently increasing at 6 hpi, despite significant COR accumulation in infected leaves at 16 hpi (Fig. 6d). By
contrast, MYC2 showed sustained dynamics at both COR concentrations, notably being highest at 16–20 hpi with [COR]low – despite COR being only a fraction of that found in DC3000-challenged leaves at that time point (compare Fig. 6a and d).

JAZ5 and JAZ10 transcript dynamics differed between COR and DC3000 challenges (Fig. 6b–d). As in Fig. 2, JAZs were strongly induced at 6 hpi by DC3000, despite COR being virtually undetectable. JAZ10 accumulation was maximal at 16 hpi (Fig. 6b), whereas JAZ5 was maximal at 8 hpi (Fig. 6c). In contrast with the sustained MYC2 induction in response to COR application (Fig. 6a), JAZ5 and JAZ10 were induced transiently, peaking at 2 hpi before rapidly declining. Despite COR levels being four-fold lower, JAZ5 and JAZ10 transcript accumulation at 8 hpi with DC3000 was similar to [COR]low at 2 hpi (Fig. 6d). Thus, although JAZs/10 expression was transiently induced in response to initial COR application, additional co-stimulatory factors, such as MAMPs (Fig. 2b), appear to be necessary to sustain or synergistically enhance JAZ5/10 expression.

The jaz5/10 chlorosis phenotype is not dependent on MYC2 and cannot be replicated by exogenous application of JA-Ile or by HopX

Both JAZ5 and JAZ10 interact with MYC2 in yeast two-hybrid assay. If the release of MYC2 induces the jaz5/10 chlorotic infection phenotype, overexpression of MYC2 should reproduce this phenotype. DC3000-challenged 35S::MYC2 overexpression lines (Lorenzo et al., 2004) were no more chlorotic than wild-type plants (Fig. S5a), yet jaz5/10 and 35S::MYC2 supported moderately increased growth of DC3000 (Fig. S5b). Furthermore, no discernible difference in chlorosis between DC3000-challenged jaz5/10 and myc2jaz5/jaz10 (Fig. S5c) indicated that the loss of myc2 does not attenuate the chlorotic phenotype. Taken together, these data indicate that the jaz5/10 response to DC3000 expressing COR is elaborated independently of MYC2 function. Moreover, only DC3000 cor co-infiltration with COR (0.2 μM), but not MeJA (50 mM) or JA-L-Ile (50 mM), induced strong chlorotic symptoms (Fig. S5d).

Pseudomonas syringae pv. tabaci 11528 does not produce COR, but encodes the cysteine protease HopX1 which interacts with and promotes the degradation of JAZ proteins in a COI1-independent manner (Gimenez-Ibanez et al., 2014). Although DEX-inducible HopX1 induces chlorotic symptoms and strongly upregulates JAZ5, JAZ10 and JAZ12 (Gimenez-Ibanez et al., 2014), delivery of HopX1 by DC3000 cor did not induce chlorosis in jaz5/10 leaves (Fig. S5e).

JAZ5 and JAZ10 are regulated in a complex manner that is partly dependent on COR and requires additional bacterial signals

We next compared JAZ5 and JAZ10 dynamics during infection by qRT-PCR. Initial JAZ5 and JAZ10 transcript accumulation at
6 hpi was similar in DC3000 and DC3000Δcfa6ΔcmaΔ, indicative of a COR-independent MAMP response (Fig. 7a,b). Subsequent COR accumulation contributed to c. 50% of JAZ5 and c. 80% of JAZ10 induction at 12 and 18 hpi, respectively (Fig. 7a,b; compare DC3000 vs DC3000Δcfa6ΔcmaΔ). Interestingly, COI1 was required for nearly all JAZ10, but only c. 50% of JAZ5, transcript accumulation (Fig. 7b), suggesting that COR induction of JAZ10 is primarily achieved through COI1.

Both JAZ5 and JAZ10 are required to protect against COR phytophobicity. Congruently, JAZ5 and, particularly, JAZ10 expression is dependent on both COR and additional stimuli, most probably bacterial-derived signals, as COR alone cannot sustain the expression of these two JAZs (Fig. 6). Differential splicing of JAZ10 can lead to splice variants lacking the Jas domain PY motif, resulting in enhanced resistance to ubiquitin-mediated proteasomal degradation (Chung et al., 2010). Following DC3000 challenge, we detected JAZ10.1 and JAZ10.3, but not JAZ10.4 (which accumulates during the wound response; Chung & Howe, 2009). The sustained JAZ10 increase during infection (Fig. 6b) is largely attributable to JAZ10.3 from 8 hpi (Fig. 7c). JAZ10.3 is predicted to bind to COI1, but is not efficiently ubiquitinated, therefore attenuating JA signalling (Chung et al., 2010). Both sustained increases in JAZs and specific accumulation of the ubiquitination-resistant JAZ10.3 isofrom are complementary mechanisms capable of modulating JA signalling.

Transcriptional changes in jaz5/10 leaves infected with DC3000

JAZs are known to bind a number of transcription factors. Removal of JAZ5 and JAZ10 would be predicted to derepress transcription factors, either constitutively or following pathogen challenge. We compared expression profiles between Col-0 and jaz5/10 unchallenged and DC3000-challenged leaves sampled at 6, 8, 12 and 16 hpi using mRNA-seq. High-stringency gFOLD analysis (Feng et al., 2012) identified 550 genes with a differential fold change of three or greater between Col-0 and jaz5/10 across all time points (Table S3a). At this stringency, MYC2 and its paralogues MYC3, MYC4 and AIB1, implicated in susceptibility to P. syringae (Fernandez-Calvo et al., 2011) and insect resistance (Schweizer et al., 2013), were not differentially expressed (qRT-PCR validation; Fig. S6). Neither were the JASMONATE-ASSOCIATED MYC2-LIKE1 (JAM1s), recently shown to be negative regulators of jasmonate signalling (Sasaki-Sekimoto et al., 2013). By contrast, the expression of other basic helix-loop-helix-encoding transcription factors, common targets of JAZs, were strongly modified, with seven members being induced in the jaz5/10 background and 11 being suppressed (Table S4).

Cluster 1, comprising 97 genes suppressed in the jaz5/10 mutant compared with Col-0 (Fig. S7a), was highly enriched in genes associated with defence responses (Fig. S7b), implicating a positive role for JAZ5/10 in immune defence homeostasis. Apart from the suppression of classical markers of defence, such as PR5, BGL2 and a chitinase (CHL), and an over-representation of redox components, previously characterized defence signalling components implicated in the regulation of SA-mediated defence were significantly over-represented. ALD1, encoding AGD2-LIKE DEFENSE RESPONSE PROTEIN 1, is required for the accumulation of SA during P. syringae infection, interacting with PAD4 and ICS1 and contributing to the regulation of the
abundance and responsiveness of MAMP receptor levels (Cecchini et al., 2015). Suppression of NIMIN1 and NIMIN2, whose proteins co-operate with NPR1 to monitor and appropriately activate defences, such as PRI induction (Hermann et al., 2013), would compromise SA-inducible defences, as would the suppression of AtNUDX6, encoding a positive regulator of NPR1-dependent SA signalling (Ishikawa et al., 2010).

In parallel, SA-independent ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) immunity signalling could be compromised by the suppression of transcripts for the defence regulator flavin-dependent monooxygenase 1 (FMO1; Bartsch et al., 2006). FMO is linked to the suppression of cytochrome P450 transcripts CYP710A1 (Nafisi et al., 2007), CYP71A13 and PAD3 (Griibel & Zeier, 2010), encoding components of secondary metabolite pathways leading to camalexin and indole-3-acetonitrile production, thus implicating weakened broad-spectrum-inducible defences in jaz5/10.

Of the four WRKY transcription factors suppressed in Cluster 1 (WRKY5, 58, 51, 62 and 75), WRKY38 and WRKY62 are proposed to be negative regulators of basal defence, interacting with histone deacetylase HDA19 to fine-tune plant basal defence responses (Kim et al., 2008). WRKY62 has been shown to be involved in SA-mediated suppression of JA signalling downstream of NPR1 (Mao et al., 2007), whereas AtWRKY75, in co-operation with AtWRKY28, transcriptionally regulates SA- and JA/ET-dependent defence signalling pathways (Chen et al., 2013).

The impact on the modulation of SA and JA signalling in Cluster 1 is reflected in Cluster 2, comprising 69 genes suppressed in jaz5/10 leaves at 6 hpi with DC3000 (Fig. S7c) and having an over-representation of genes associated with the ontological molecular function ‘hydrolase activity’ (Fig. S7d). Most striking is the suppression of JA biosynthetic and signalling components, including lipoxygenase 2 (LOX2), allene oxide synthase (AOS), allene oxide cyclase (AOC2) and the JA marker Vegetative Storage Protein 2 (VSP2). Particularly notable was the suppression of CYP94B3 encoding a JA-Ile hydroxylase that catalyses the formation of 12-OH-JA-Ile from JA-Ile (Kitaoka et al., 2011). These transcriptional changes are consistent with the unexpected lower JA levels seen in jaz5/10 (Fig. 4).

Discussion

This study provides the first demonstration of functional co-operativity between JAZs. The two phylogenetically distinct JAZs, JAZ5 and JAZ10 (Oh et al., 2013), collectively function to mitigate COR virulence functions and contribute to innate immunity.

These findings evolved from unexpected JA dynamics measured in DC3000- and DC3000cor-challenged leaves of wild-type and coil1. Contrary to the early expression of marker genes, such as PDF1.2, which infer JA biosynthesis (Glazebrook, 2005), JA accumulated very late in the infection process, well after bacterial multiplication and thus incompatible with a direct role in the suppression of SA defences (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Kazan & Lyons, 2014). Moreover, inoculation with DC3000cor further dramatically enhanced this late JA accumulation. These results contrast with the prediction that transcriptional activation of JA signalling and biosynthetic pathways by COR would increase JA levels (Cui et al., 2005; Laurie-Berry et al., 2006). Furthermore, coil1 leaves challenged with DC3000cor accumulated markedly more JA than did Col-0, further indicating a significant COI1-independent contribution to JA accumulation in DC3000cor-infected leaves. Thus, a complex multilayered defence network or feedback mechanism in response to DC3000 and its phytotoxin COR act to constrain JA accumulation until late in the infection process.

High-resolution microarray analysis of DC3000- and DC3000hrp-infected leaves confirmed the early induction of JA biosynthetic transcripts and a significant, but complex, induction of JAZs in both basal defence and disease progression. A strong, sustained increase in JAZs at 6–7 hpi occurred before, or coincident with, the first detectable trace of COR and paralleled the induction of JA biosynthetic genes. In the absence of early elevated JA levels, we hypothesized that this sustained accumulation of JAZs may restrict jasmonate biosynthesis and signalling. Reduced JAZ accumulation reported in leaves challenged with DC3000cor may explain the hyperaccumulation of JA (Demianski et al., 2012); however, JAZ5/10 dynamics were also MAMP responsive. Counter-intuitively, despite inducing more JA, DC3000cor grows less, suggesting that the early SA increases are epistatic to elevated JA and that JAZs may ameliorate the signalling potential of JA. Although COR alone strongly induced JAZ5 and JAZ10, this induction was transient and required additional co-stimulatory signals provided by the pathogen. This is resonant of ‘two-signal’ models which are central to vertebrate immunity and, more recently, have been evoked as mechanisms underpinning aspects of human (Fontana & Vance, 2011) and plant (Lindeberg et al., 2012) innate immunity. Moreover, the JAZ10.3 splice variant accumulated from 8 hpi, indicative of differential splicing during infection and highlighting a further potential mechanism to restrict pathogen modulation of JA signalling (Yan et al., 2007; Chung et al., 2010). We propose that MAMP triggered immunity primes immunosurveillance and selected JAZ induction and/or COR provide a second discriminatory signal, leading to sustained activation of JAZs and differential splicing of JAZ10.

JAZ proteins modulate growth and defence responses through the interplay between JAZ and DELLA proteins (Wild et al., 2012; Yang et al., 2012) and repress an expanding range of transcription factors involved in various developmental responses (Fernandez-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011). However, surprisingly little is known about the extent to which individual JAZs collaborate to regulate specific biological processes. Using reverse genetic approaches, we identified unexpected JAZ co-operativity. Single loss-of-function mutations in all tested jaz alleles did not significantly modify DC3000 infection phenotypes. However, the jaz5/10 double mutant, but no other combinations, including those with jaz5 or jaz10, resulted in remarkably strong chlorosis. jaz5/10 lines were moderately more susceptible to DC3000, albeit weakly so compared with the hypersusceptible pad4 or sid2. In root growth assays,
jaz5/10 mutants showed similar sensitivity to MeJA and COR as other jaz double mutants, but chlorosis was conditional on the production of COR. Co-infiltration of DC3000 or with COR, but not MeJA or JA-Ile, could induce strong chlorosis in jaz5/10. In addition, HopX delivery could not induce chlorosis in jaz5/10 leaves.

Although MYC2, MYC3 and MYC4 are required for full susceptibility to DC3000 (Nickstadt et al., 2004; Laurie-Berry et al., 2006; Fernandez-Calvo et al., 2011), no sustained induction was evident in challenged or unchallenged jaz5/jaz10 leaves. DC3000-induced chlorosis was not enhanced in myc2/jaz5/jaz10 leaves and was minimal in MYC2 overexpression lines. Thus, other transcription inducers or repressors (Wild et al., 2012; Yang et al., 2012), tethered by JAZ5/10, probably contribute to COR-induced chlorosis. Indeed, mRNA-seq profiling revealed that jaz5/10 plants had significantly fewer transcripts associated with defence responses, particularly SA-mediated processes, than Col-0. This was reflected in strongly suppressed JA biosynthesis and signalling transcripts in jaz5/10 mutants within 6 h of DC3000 infection, helping to explain why the anticipated increase in JA was not measured in these plants.

JA responses are fine-tuned through a number of JAZ interactions, including homo- and heterodimerization, concentration- and ligand-dependent differential binding to COI1, and direct or indirect interaction with transcriptional repressors, such as TOPLESS (Pauwels et al., 2010). For example, JAZ8 is susceptible to in vivo 26S proteasome degradation by COR, but more resistant to JA-mediated degradation than is JAZ10 (Shyu et al., 2012). Moreover, there are COI1-independent jasmonate responses, suggesting that other F-Box proteins are involved in jasmonate signalling (Geng et al., 2012). Although biotic challenge leads to transcriptional changes in JAZs, no direct role for JAZs in plant–pathogen interactions has yet been clearly established. One-quarter of transgenic lines overexpressing JAZ1 lacking the Jas domain phenocopied coil mutants show JA insensitivity and increased resistance to DC3000 infection (Thines et al., 2007). A similar proportion of plants overexpressing JAZ1N305A and JAZ1L305A, which disrupts the JA-Ile/COR-dependent COI1–JAZ interaction, also conferred JA insensitivity, some of which additionally showed increased resistance to DC3000 infection (Shyu et al., 2012). Thus, the overexpression of dominant negatives can interfere with DC3000 proliferation; however, no evidence for altered susceptibility to jaz1 has been shown. Similarly, jaz10 antisense lines showed moderately enhanced symptom development, but not increased susceptibility to DC3000 (Demianski et al., 2012). We found that jaz5/10 chlorosis was specific to exogenous COR, but not JA-Ile or MeJA. JAZ5 can be directly or indirectly involved in transcriptional repression through either its two EAR domains (Kagale et al., 2010) or via its interaction with the negative regulators TOPLESS (Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012) and NINJA (Pauwels et al., 2010). Furthermore, JAZ5 lacks the highly conserved LPIARR motif found in many JAZ proteins that interact strongly with COI1 in a JA-Ile-dependent manner (Shyu et al., 2012). JAZ5 could be eliminated via a COI1-independent mechanism, or require molecules other than JA-Ile for its degradation. JAZ10 could also function as a negative regulator of JA responses. jaz10 loss-of-function mutants show JA hypersensitivity (Yan et al., 2007). Thus, both JAZ5 and JAZ10 could act as negative regulators of a broad range of JA responses, with COR being particularly effective at activating these responses.

COR-producing P. syringae induces strong chlorosis on tomato, but not Arabidopsis, leaves. COR-induced cell death in tomato is via the modulation of the photosynthetic machinery and/or reactive oxygen signalling (Ishiga et al., 2009). DC3000 infection suppressed chloroplast peroxiredoxin (Prx) and NADPH-dependent thioredoxin reductase C (NTRC) in tomato and Arabidopsis, but COR only suppressed Prx and NTRC in tomato (Ishiga et al., 2012). Neither chloroplast Prx nor NTRC were differentially regulated in our mRNA-seq experiment. Tomato SlJAZ2, SlJAZ6 and SlJAZ7 proteins interact with SlCOI1 in the presence of COR. However, silencing of these genes in tomato and Nicotiana benthamiana led to no significant differences in COR-induced chlorosis or enhanced bacterial multiplication (Ishiga et al., 2013), suggesting that additional pathogen-associated factors are required for COR virulence in Arabidopsis, as inferred by the JAZ5/10 dynamics observed in this study.

In summary, our data reveal unexpected JA dynamics during DC3000 infection of Arabidopsis and co-operativity between JAZ5 and JAZ10 which restricts COR phytotoxicity. This co-operativity is remarkable as it protects against a pathogen virulence factor that has evolved as a plant hormone mimic. The future challenge is to understand the complex regulatory network of JAZ proteins, the biological relevance of JAZ–JAZ homo- and heterodimers and the identity of the interacting transcription factors/repressors involved in this particular biological process.

Acknowledgements

We gratefully acknowledge support from the British Biotechnology and Science Research Council (Grants BB/C514115/1 and BB/F005903/1) and a Chinese Government Studentship to B.Z. The members of the Plant Responses to Environmental STress consortium are thanked for microarray design, labelling and data extraction. We thank Selena Gimenez-Ibanez for the HopX derivatives, Paul O’Neil for assistance with clustering and Orkun Soyer for helpful discussions. Pathogen work was carried out under Plant Health License PHL 204B/6739.

Author contributions

References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Jasmonic acid (JA) accumulates late in leaves infected with virulent DC3000.

Fig. S2 The majority of DC3000-induced jasmonic acid (JA) is derived from de novo JA biosynthesis.

Fig. S3 Reverse transcription-polymerase chain reaction (RT-PCR) validation of jaz5, jaz10 and jaz5/10 knock-out lines.

Fig. S4 Jasmonate sensitivity to seedling root growth of different double jaz mutant combinations compared with the jasmonate insensitive coi1-16.

Fig. S5 The jaz5/10 chlorotic phenotype is not dependent on MYC2.

Fig. S6 Expression profiles of four JAZ-targeted MYC genes in wild-type and jaz5/10 mutant backgrounds.

Fig. S7 Gene clusters discriminating jaz5/10 and Col-0 plants in non-induced leaves or early after leaf infection with DC3000.

Table S1 Summary of pathogen infection phenotypes of JAZ T-DNA insertion lines, including response of double and triple mutant combinations used in this study.

Table S2 JAZ and jasmonate biosynthetic gene expression derived from CATMA arrays, reporting a compatible interaction, a basal defence response or the impact of DC3000 type III effectors.

Methods S1 Primers used for reverse transcription-polymerase chain reaction (RT-PCR) and their respective amplicon size.

Table S3 Differential gene expression between Col-0 and jaz5/10 mutants as determined by gFOLD, and selected gene clusters derived from gFOLD analysis discriminating Col-0 from jaz5/10 mutants.

Table S4 Basic helix-loop-helix transcription factor genes differentially regulated between wild-type Col-0 challenge and the jaz5/10 mutant following infection.

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.

About New Phytologist

- **New Phytologist** is an electronic (online-only) journal owned by the New Phytologist Trust, a not-for-profit organization dedicated to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews.

- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication ‘as ready’ via Early View – our average time to decision is <27 days. There are no page or colour charges and a PDF version will be provided for each article.

- The journal is available online at Wiley Online Library. Visit [www.newphytologist.com](http://www.newphytologist.com) to search the articles and register for table of contents email alerts.

- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient, our USA Office (np-usaoffice@lancaster.ac.uk)

- For submission instructions, subscription and all the latest information visit [www.newphytologist.com](http://www.newphytologist.com)