Arabidopsis Auxin Mutants Are Compromised in Systemic Acquired Resistance and Exhibit Aberrant Accumulation of Various Indolic Compounds[1][W][OA]

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Systemic acquired resistance is a widespread phenomenon in the plant kingdom that confers heightened and often enduring immunity to a range of diverse pathogens. Systemic immunity develops through activation of plant disease resistance protein signaling networks following local infection with an incompatible pathogen. The accumulation of the phytohormone salicylic acid in systemically responding tissues occurs within days after a local immunizing infection and is essential for systemic resistance. However, our knowledge of the signaling components underpinning signal perception and the establishment of systemic immunity are rudimentary. Previously, we showed that an early and transient increase in jasmonic acid in distal responding tissues was central to effective establishment of systemic immunity. Based upon predicted transcriptional networks induced in naive Arabidopsis (*Arabidopsis thaliana*) leaves following avirulent *Pseudomonas syringae* challenge, we show that a variety of auxin mutants compromise the establishment of systemic immunity. Linking together transcriptional and targeted metabolite studies, our data provide compelling evidence for a role of indole-derived compounds, but not auxin itself, in the establishment and maintenance of systemic immunity.

Plants growing under natural conditions are continuously subjected to a wide variety of abiotic and biotic stresses. Many of the defense mechanisms employed to mitigate the effects of these stresses have been found to involve signal transduction pathways controlled by phytohormones. The first hormones to be marked as central players in defense against plant pathogens were salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Glazebrook, 2005), with roles more recently attributed to abscisic acid (ABA), gibberellins, and auxin (Navarro et al., 2006, 2008a; de Torres-Zabala et al., 2007; Wang et al., 2007; Grant and Jones, 2009; Kazan and Manners, 2009). JA/ET- and SA-mediated defense pathways antagonize each other, with JA/ET largely defining defense against necrotrophic pathogens and insects, while SA is central in defense against biotrophic pathogens (Glazebrook, 2005). Despite this well-established antagonism between SA and JA/ET signaling pathways, examples of synergism also exist depending on the relative phytohormone concentrations (Mur et al., 2006). Temporal separation of the two responses and their direct effects in distal tissues have also been shown to alter the interaction between these hormones (Thaler et al., 2002; Spoel et al., 2007). The contribution of auxin to defense appears to fit within the biotroph-necrotroph, SA-JA/ET antagonism model, with several recent reports linking auxin with susceptibility to the hemibiotroph *Pseudomonas syringae* (Navarro et al., 2006; Chen et al., 2007; Wang et al., 2007; Zhang et al., 2007) and negative cross talk between SA and auxin signaling networks. Conversely, mutations in key auxin signaling components or chemical inhibition of auxin transport lead to an increased susceptibility to the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* (Llorente et al., 2008).

Perception of evolutionarily conserved microbe-associated molecular markers (MAMPs) by the host plant initiates basal defense mechanisms. Navarro et al. (2006) showed that this detection of nonself led to suppression of the F-box auxin receptor, TRANSPORT INHIBITOR RESPONSE1 (TIR1), and its AUXIN SIGNALING F-BOX (AFB) paralogs through posttranscriptional silencing by microRNAs. The importance of these $SCF^{TIR1/AFB}$ receptor/ubiquitination complexes was confirmed by the increased growth of virulent *P. syringae* pv *tomato* (*Pst*) in transgenic lines overexpressing *AFB1*. Virulent pathogens are capable of surmounting MAMP-triggered immunity through the deployment of a constellation of protein effectors and phytotoxins. One such protein effector, AvrRpt2, which promotes virulence in Arabidopsis (*Arabidopsis thaliana*) lacking the disease resistance gene *RPS2*, was found to increase levels of the auxin indole acetic acid

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(IAA). Transgenic plants expressing AvrRpt2 in an *rps2* null background exhibited a variety of phenotypes associated with altered auxin physiology (Chen et al., 2007). Both Chen et al. (2007) and Wang et al. (2007) reported that treatment of plants with the synthetic auxin 1-naphthalacetic acid promoted the development of disease following infection with virulent *Pst*. Full genome microarray experiments of plants treated with the SA analog benzothiadiazole *S*-methylester revealed inhibition of auxin signaling pathways (Wang et al., 2007). This inhibition was predicted to result from the stabilization of auxin repressor proteins in a mechanism apparently independent from the posttranscriptional pathway induced by MAMPs. The antagonism of auxin transcriptional responses to SA was found to be reciprocal, at least in the case of the SA-associated defense marker *PR1*. SA-induced expression of *PR1* was diminished with 1-naphthalacetic acid cotreatment. Inoculation of Arabidopsis with *P. cucumerina* induces the expression of both *PR1* and the JA/ET-dependent marker, defensin *PDF1*; susceptible auxin signaling mutants were not significantly affected in this response, but pretreatment of plants with the auxin transport inhibitor triiodobenzoic acid, which yielded similar susceptibility, did act to reduce *PDF1* expression while enhancing *PR1* expression (Llorente et al., 2008). Interestingly, infection of the stabilized repressor of auxin signaling, *axr2-1*, produced a significantly greater induction of the JA biosynthesis gene *LOX3* than in wild-type plants, indicating that auxin may act differentially in affecting separate branches of pathogen-responsive, JA-mediated responses.

Additional evidence for the relationship between SA and auxin has come from studying members of the *GH3* family of early auxin-responsive genes. *GH3* genes encode acyl-adenylate/thioester-forming enzymes, capable of conjugating amino acid residues to a number of substrates. In Arabidopsis, GH3.5 has been shown to adenylate both SA and IAA (Zhang et al., 2007). An activation-tagged mutant of *GH3.5* resulted in both elevated levels of SA and increased *PR1* expression as well as higher levels of free IAA following infection. A model has been proposed whereby GH3.5 plays dual roles, positively modulating auxin responses and increasing susceptibility in a compatible interaction, while during an incompatible interaction, GH3.5 positively modulates SA responses, increasing resistance. By contrast, GH3.12/AvrPphB Susceptible3 (PBS3) acts on 4-substituted benzoates, perhaps by priming or inducing SA biosynthesis (Okrent et al., 2009). In rice (*Oryza sativa*), GH3.8 decreases free IAA levels when overexpressed, most probably by increasing the amount of IAA conjugated to Asp (Ding et al., 2008). Increased IAA conjugation was associated with increased resistance to *Xanthomonas oryzae* pv *oryzae* and was accompanied by significantly decreased free SA levels. In addition, transcripts of genes associated with both SA-mediated disease resistance and JA biosynthesis decreased. These data indicate that the resistance conferred by GH3.8 is independent of either salicylate or jasmonate signaling pathway activation, although the suppression of SA or JA responses may be crucial. The importance of auxin in plant defense responses is further emphasized by the number of plant pathogens that synthesize auxin in order to facilitate virulence (for review, see Robert-Seilaniantz et al., 2007).

Systemic acquired resistance (SAR) is a broad-spectrum, lasting resistance found in naive leaves distal to an infection event (Durrant and Dong, 2004; Grant and Lamb, 2006). Originally associated with response to a local necrotizing pathogen, most SAR studies focus on incompatible interactions induced by classic gene-for-gene interactions (Durrant and Dong, 2004). However, induced resistance has also been reported for compatible fungal (Caruso and Kuc, 1977; Cohen and Kuc, 1981) and bacterial (Attaran et al., 2009) infections. A similar resistance has also been described for the systemic response to localized MAMP-triggered immunity (Mishina and Zeier, 2007).

Recently, SAR has been reported following challenge with virulent *P. syringae* (Attaran et al., 2009), while previous studies have reported systemic induced susceptibility (Cui et al., 2005) in Arabidopsis and systemic induced tolerance in tomato (*Solanum lycopersicum*; Block et al., 2005). These conflicting data may arise from the time of challenge (Griebel and Zeier, 2008), differing growth conditions, or the age of challenged plants.

This study focuses upon the molecular basis of the establishment of SAR resulting from incompatible interactions, which is ultimately associated with the accumulation of SA in the systemic tissue. In Arabidopsis challenged with avirulent *Pst*, the first detectable systemic gene expression responses to infection, however, are associated with JA signaling (Truman et al., 2007). The importance of this transient JA-associated response has led to speculation that SAR may depend on a synergistic interaction of JA and SA signaling networks. In addition to dual defense networks being involved, there is now compelling, but sometimes contradictory, evidence that multiple signals, including both methyl salicylate and a lipid-derived molecule that may or may not be azelaic acid, and terpenoids are exported through the phloem from immunized leaves together with nonessential signals such as increased JA levels (Maldonado et al., 2002; Park et al., 2007; Truman et al., 2007; Chaturvedi et al., 2008; Vlot et al., 2008b; Attaran et al., 2009; Jung et al., 2009; Shah, 2009). Understanding the nature and interaction of these signals represents a major challenge in the field (Shah, 2009).

Reanalysis of our early systemic transcriptional data (Truman et al., 2007) identified a subset of genes upregulated in systemic leaves following avirulent challenge that are also regulated by auxin. Therefore, we tested a selection of auxin signaling mutants for their capacity to establish SAR. The auxin influx transporter AUX1 and AXR4, which controls the localization of

AUX1 (Bennett et al., 1996; Kepinski and Leyser, 2005; Dharmasiri et al., 2006), were both required for SAR to moderately or strongly virulent *P. syringae*, along with the auxin receptor TIR1 (Bennett et al., 1996; Kepinski and Leyser, 2005; Dharmasiri et al., 2006). Here, we present our findings that disruption of auxin transport results in significant changes in the abundance of several indolic compounds that may be central to the development of SAR. Furthermore, the dynamics of JA-SA cross talk in *aux1* and *axr4* mutants appears to be shifted, indicating that a complex interplay of phytohormones is at work during the establishment of SAR.

RESULTS

Auxin and Indole Glucosinolate Biosynthetic Pathways Are Up-Regulated during the Systemic Response to Avirulent Infection

Previous global profiling of systemic transcriptional responses in Arabidopsis following an *RPM1*-mediated incompatible interaction with *Pst* DC3000 carrying the avirulence gene *avrRpm1* led to the identification of 394 significantly differentially expressed genes (Truman et al., 2007) compared with either the DC3000*hrp* mutant, which cannot deliver bacterial effector proteins into the plant cell, or virulent DC3000. There were no significant differences in gene expression between DC3000*hrp* and DC3000, indicating that only the presence of *avrRpm1* was responsible for reconfiguring gene expression and that type III effectors at 4 h post inoculation (hpi) did not impact transcription in systemic tissue. The majority of these genes (369) were up-regulated, and they contained a significant overrepresentation of genes encoding components of aromatic amino acid biosynthesis pathways and pathways incorporating aromatic amino acids into secondary metabolites. Of particular interest is the induction of two genes encoding the cytochrome P450s CYP79B2 and CYP79B3; both these enzymes catalyze the conversion of Trp to indole-3-acetaldoxime, a key branching point that may be converted into IAA, the antimicrobial phytoalexin camalexin, or indole glucosinolate (IG) defense compounds (Mikkelsen et al., 2000; Zhao et al., 2002; Glawischnig et al., 2004; Bednarek et al., 2009; Clay et al., 2009). In addition to these enzymes, the MYB transcription factor *ATR1*, which has been shown to positively regulate components of both Trp and IG biosynthesis, is also strongly induced (Celenza et al., 2005). Overexpression of each of these three genes has been shown to result in increased IAA and IG levels. The synthesis of camalexin is also enhanced if elicited by chemical treatment, infection (Zhao et al., 2002; Celenza et al., 2005; Malitsky et al., 2008), or via expression of a constitutively active form of MKK9 (Xu et al., 2008). In addition to these elements, critical to controlling auxin homeostasis, two probe sets targeted to three genes predicted to encode auxin conjugate hydrolases were strongly induced. *ILL5*, *ILL6*, and *IAR3* encode proteins that most likely function in releasing conjugated auxin into the pool of free IAA. This function contrasts to the GH3 family of proteins, some of whose members have been shown to conjugate IAA and impact disease resistance responses (Davies et al., 1999; Zhang et al., 2007; Ding et al., 2008). Recently, Trp conjugates of IAA have been shown to be potent inhibitors of auxin signaling; thus, some auxin conjugate hydrolases will have the dual effect of releasing active IAA and removing a negative regulator (Staswick, 2009). Since these highlighted expression profiles could confer a change in auxin homeostasis during the earliest stages of the development of SAR, we interrogated our data set with a list of genes predicted or known to be involved in the biosynthesis of indolic compounds. We identified significant changes in response to DC3000(*avrRpm1*) relative to a wild-type DC3000 challenge as rapidly as 4 hpi in genes known to be involved in the biosynthesis of Trp, auxin, and other derivatives of Trp (Fig. 1).

Most striking, the induction of *CYP79B2* and *CYP79B3* is accompanied by increases in other transcripts that would act to commit indole-3-acetaldoxime to IG biosynthesis: *CYP83B1*, *UGT74B1*, and the sulfotransferase encoded by *SOT16* (Bak et al., 2001; Grubb et al., 2004; Piotrowski et al., 2004). However, IAA may also be synthesized through degradation of IGs to indole-3-acetonitrile and conversion to IAA by nitrilases such as NIT3 (Grubb et al., 2004), whose transcripts are moderately increased during SAR. Indole-3-acetaldoxime precursor may also be directed toward camalexin biosynthesis, because *CYP71A13* and *PAD3*, which catalyze the initial and final steps in this pathway, respectively, are both up-regulated (Schuhegger et al., 2006; Nafisi et al., 2007). No *GH3* family genes known to conjugate IAA were seen to increase significantly. The *GH3* family member *JAR1*, whose product conjugates Ile to JA (Staswick et al., 2002), and *PBS3* (Okrent et al., 2009), which may be involved in conjugating SA precursors, both exhibited marginally increased expression.

Auxin Biosynthesis and Transport Mutants Are Compromised in SAR

The impact of transcripts associated with the biosynthesis of indolic compounds, including auxin, was unexpected, because we cannot detect increases in auxin during the early phase of the establishment of SAR (Supplemental Table S1). A number of plants with altered auxin homeostasis due to mutation or ectopic overexpression of components in auxin biosynthesis exhibit gross morphological abnormalities and are unsuitable for undertaking SAR experiments. To address a role of auxin signaling in SAR, we tested Arabidopsis auxin signaling or auxin biosynthesis mutants that exhibit no gross morphological abnormalities. The immunizing effect resulting from *RPM1*-

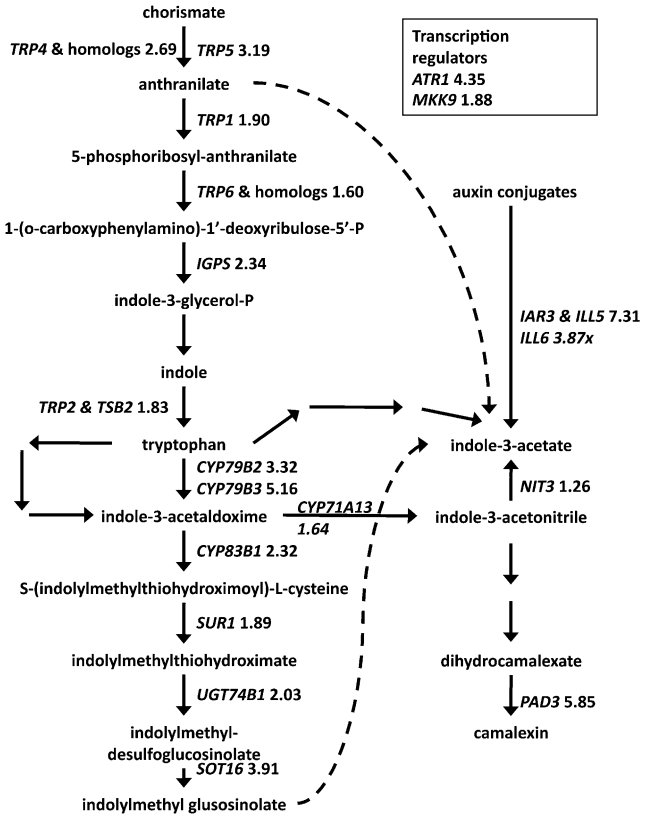

Figure 1. Indole biosynthetic components are induced systemically following avirulent bacterial challenge. Affymetrix ATH1 arrays were used to profile systemic responses to bacterial infection in three independent experiments. Copy RNA was derived from mRNA isolated from distal systemically responding Col-0 leaves at 4 h after challenge with either *Pst* DC3000 or DC3000(*avrRpm1*) as described by Truman et al. (2007). The genes plotted represent all the significant changes observed in the biosynthesis of Trp, auxin, and other indolic compounds together with regulatory components known to control these pathways. Fold change values represent ratios of expression changes in DC3000(*avrRpm1*)-responding leaves relative to DC3000 challenge.

mediated recognition of DC3000(*avrRpm1*) was tested in the auxin F-box receptor mutant *tir1* (Kepinski and Leyser, 2005). In three independent experiments, no SAR effect was observed in *tir1* plants challenged with virulent *P. syringae* pv *maculicola* in secondary leaves following an initial inoculation with DC3000(*avrRpm1*) (Fig. 2A) or more virulent DC3000 (data not shown). Strikingly, SAR response was also completely abolished in the auxin polar transport mutants *aux1* (*aux1-22*) in five independent experiments, *axr4* (*axr4-2*) in three independent experiments, and the *cyp79b2/cyp79b3* double mutant in two independent experiments (Fig. 2B). This *cyp79b* double mutant is blocked in the synthesis of indole-3-acetaldoxime, has reduced IAA, and is the only route to the production of IG and camalexin (Zhao et al., 2002). *axr4* acts in the same pathway as *aux1*, actively disrupting trafficking of AUX1 to the plasma membranes of epidermal cells (Dharmasiri et al., 2006). Like *cyp79b2/cyp79b3*, both *aux1* and *axr4* mutants resembled *tir1* in its compromised SAR.

Auxin Transport Mechanisms Are Critical to JA and SA Signaling Dynamics

The transcriptional responses of *axr4*, *aux1*, *tir1*, and *cyp79b2/cyp79b3* were examined using quantitative reverse transcription (qRT)-PCR. A subset of genes was selected from available microarray data, together with classical markers of SAR and SA-associated defense responses that are expected to define the establishment of SAR. At 4 hpi, all four mutants showed a significant increase in the systemic expression of *At5g56980*, originally used as a marker of early systemic responses (Truman et al., 2007) following avirulent challenge (Fig. 3A). By contrast, inoculation with the DC3000*hrp* mutant, which is unable to deliver bacterial effectors, or virulent DC3000 (data not shown) does not induce *At5g56980* systemically. Therefore, it seems likely that auxin signaling plays no role in the generation or perception of the mobile signal(s) responsible for this

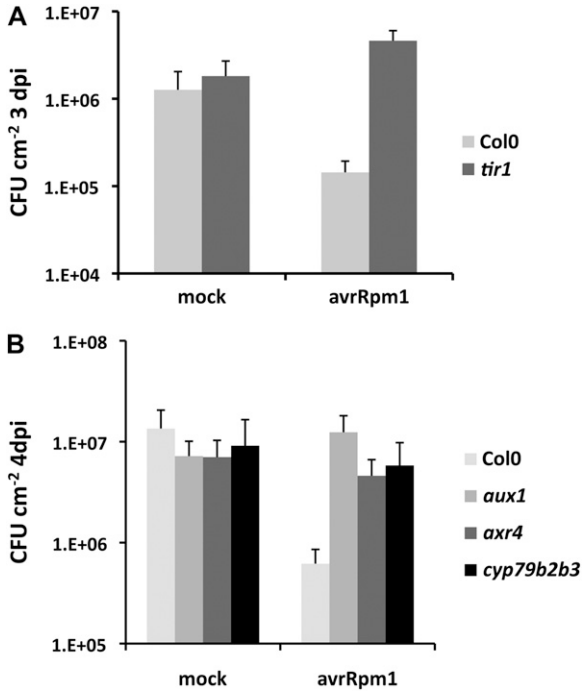

Figure 2. Auxin signaling, transport, and biosynthesis mutants fail to develop effective systemic immunity. Multiplication of virulent *P. syringae* at 3 or 4 d after inoculation in SAR-induced plants. Leaves were either mock (10 mM $MgCl_2$) inoculated or challenged with DC3000(*avrRpm1*) at 1×10^8 cfu mL^{-1}. Two days later, secondary leaves were challenged with *P. syringae* pv *maculicola*. A, SAR is abolished in the auxin receptor mutant *tir1*. Bacterial growth was measured at 4 dpi. B, Attenuation or abolition of SAR in the auxin transport mutants *aux1* and *axr4* and the indole-3-acetaldoxime biosynthesis double mutant *cyp79b2/cyp79b3*. Bacterial growth was measured at 3 dpi. These experiments were repeated two or three times with similar results, using either DC3000 or *P. syringae* pv *maculicola*. Error bars indicate the SE of four or five replicates.

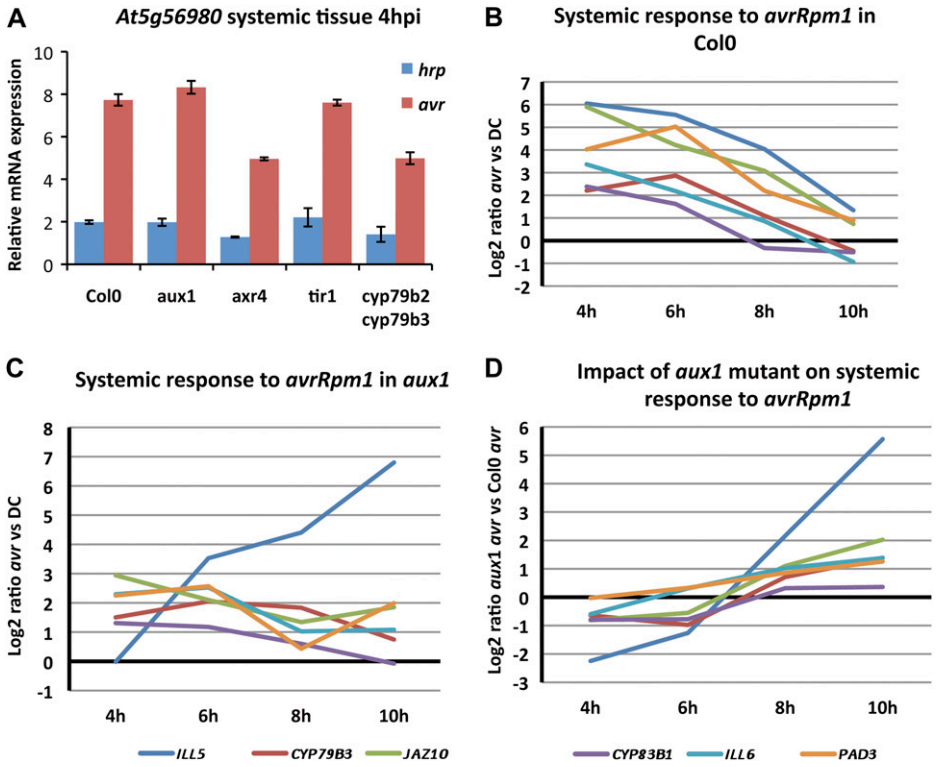

Figure 3. The kinetics of systemic transcriptional responses to avirulent inoculation is disrupted in the *aux1* mutant. The accumulation of transcripts encoding key signaling components was analyzed in systemically responding tissue by qRT-PCR. A, RT-PCR shows the rapid accumulation of *At5g56980*, a marker for the perception of a systemic signal(s) in systemic tissue after avirulent (*avr*) challenge compared with responses to the disarmed *hrpA* mutant of *Pst* DC3000 capable of eliciting basal defenses (*hrp*). The error bar shows the SD of three replicates. A strong response was also observed in the *aux1*, *axr4*, *tir1*, and *cyp79b2*/*cyp79b3* mutants. B to D, *JAZ10*, *CYP79B3*, *CYP83B1*, *PAD3*, *ILL5*, and *ILL6* share a common expression pattern. B, Up-regulation in response to DC3000(*avrRpm1*) compared with DC3000 (DC) declines linearly over time in wild-type Arabidopsis. C and D, Induction of these genes is not abolished in *aux1* (C) but exhibits an inverted response relative to the wild type (D). qRT-PCR analyses were repeated in two or three independent experiment with similar results.

phase of the elaboration of SAR, although redundancy in auxin signaling and biosynthesis mean we cannot exclude this possibility. Many of the genes up-regulated systemically by DC3000(*avrRpm1*) at 4 hpi are induced only transiently, correlating with the transient increase in JA levels in systemic leaves. The transcriptional dynamics of at least a subset of these genes is inverted in *aux1* (Fig. 3, B–D), including *JAZ10*, encoding one member of a family of negative regulators of JA signaling (Chini et al., 2007; Thines et al., 2007; Chung and Howe, 2009). The initial induction of *JAZ10* by DC3000(*avrRpm1*) in relation to DC3000 is very strong, approximately 60-fold, in systemic leaves at 4 hpi; this relative induction declines exponentially over the next 6 h (Fig. 3B, note the $\log_2$ scale; Supplemental Table S2). The early expression of *JAZ10* fits neatly into a model of the development of SAR in which the attenuation of JA signaling would see JA-responsive genes return to basal levels prior to the induction of SA-mediated responses known to be essential to the establishment of SAR. *JAZ10* shares this expression pattern with the indole-3-acetaldoxime biosynthesis component *CYP79B3*, the IG biosynthesis component *CYP83B1*, the camalexin biosynthesis component *PAD3*, and the putative auxin conjugate hydrolases *ILL5* and *ILL6*. RT-PCR measurements of the systemic responses to DC3000 and DC3000(*avrRpm1*) across four time points (4, 6, 8, and 10 hpi) and three genotypes (Columbia [Col-0], *aux1*, and *axr4*) reveal strongly coregulated expression of these genes, with correlation coefficients between genes ranging from 0.70 to 0.96 (Supplemental Table S3). All, with the exception of *ILL5*, are up-regulated in *aux1* in response to DC3000(*avrRpm1*), although not as strongly as the wild type (Fig. 3C). In *aux1*, there is a modest decrease in the level of induction of these genes over time, again with the exception of *ILL5*, which dramatically increases. However, their expression in SAR-responsive tissue relative to the wild type steadily increases over time (Fig. 3D). Similar trends were observed in *axr4*, with the exception of *PAD3* (Supplemental Fig. S1), consistent with their function in a shared pathway. Based upon these data, *aux1* was chosen for further detailed analysis of compromised SAR responses.

In contrast to the genes coexpressed with *JAZ10*, transcripts associated with SA-mediated defense responses and the later stages of SAR development are not induced by DC3000(*avrRpm1*) at 4 hpi. *PR1* is a classical marker for SAR, while *FMO1* has recently been identified as an important regulator of both basal defenses and SAR (Uknes et al., 1992; Bartsch et al., 2006; Mishina and Zeier, 2006). Both these genes were expressed at higher levels in *aux1-22* at 8 hpi, but by 48 hpi they were substantially lower in *aux1* compared with the wild type (Fig. 4). The differential impact of *aux1* on SA-responsive genes mirrors its impact on JA-responsive genes. It is tempting to speculate that a delayed, reduced, but extended accumulation of JA explains this disruption.

The *aux1* Mutant Distorts the Hormone Profile of SAR Leaves

Plant hormone balance plays an important role in plant-pathogen interactions (Grant and Jones, 2009). Liquid chromatography coupled with mass spectroscopy (LC-MS) was employed to measure SA, JA, and ABA in the systemic leaves of Col-0 and *aux1* plants at 21 hpi (Forcat et al., 2008). This time point was chosen, based upon preliminary qRT-PCR experiments, as being representative of a stage in the development of SAR when the transient accumulation of JA has passed and pathways leading to elevated SA were being engaged. While free SA levels were only moderately increased following an avirulent DC3000(*avrRpm1*) challenge in Col-0 (Fig. 5A), levels of free SA and conjugated SA were significantly reduced (approximately 3-fold less; $P < 0.01$) in *aux1* responding to DC3000(*avrRpm1*) compared with the wild type (Fig. 5, A and B). SA levels in *aux1* were slightly, but not significantly, higher with avirulent inoculation than with virulent bacteria but lower than with the disarmed mutant. Both JA and ABA, phytohormones known to promote increase susceptibility to *Pst* (Koornneef and Pieterse, 2008), accumulated to significantly higher levels in *aux1* responding to DC3000 (*avrRpm1*) compared with Col-0 (Fig. 5, C and D). The antagonism between JA and SA is well established (Koornneef et al., 2008; Koornneef and Pieterse, 2008); more recently, it has been shown that ABA plays an important role in bacterial virulence and that ABA suppresses chemically induced SAR (de Torres-Zabala et al., 2007, 2009; Yasuda et al., 2008). Auxin has been shown to act antagonistically to ABA in the regulation of stomatal opening, and both hormones also interact in the regulation of root development (Rock and Sun, 2005). Enhanced ABA levels in *aux1* may act to suppress both SA biosynthesis and responses to SA, as appears to occur during local DC3000 infection (de Torres Zabala et al., 2009).

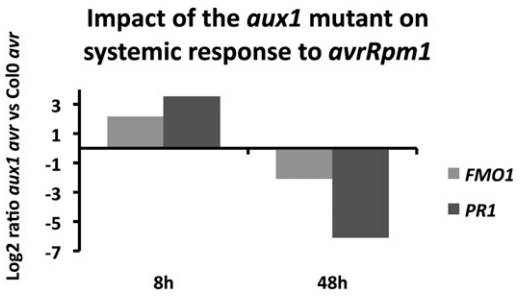

Figure 4. Gene expression associated with SA accumulation and the establishment of SAR are perturbed in *aux1* plants. The expression of two key markers of SAR, *PR1*, a classical marker for SAR, and *FMO1*, an important regulator of both basal defenses and SAR, was compared in *aux1* and wild-type plants. qRT-PCR measurements representative of two separate experiments reflect the impact of *aux1* on SA-associated gene expression at different stages in the development of SAR relative to wild-type expression levels.

aux1 Changes the Ratios of Indolic Compounds during SAR

The same tissue extracts used for phytohormone measurements were profiled for a range of indolic compounds predicted to be affected by SAR based on changes in the global profiling of transcriptional responses, and indicative ion signatures seen in NMR and flow infusion electrospray mass spectrometry of *aux1*-challenged leaves suggested changes in indolic compounds (Fig. 6; data not shown). LC-MS was used to measure the relative abundance of the main aliphatic glucosinolates and IGs. Despite the transcriptional up-regulation of almost the entire IG biosynthesis pathway from 4 hpi onward, no major glucosinolate changes in systemically responding leaves of wild-type plants were evident following a DC3000(*avrRpm1*) immunizing challenge. Strikingly, a reduction in the levels of 4-methoxy-3-indolyl-methyl glucosinolate, the specific glucosinolate identified as a precursor of callose biosynthesis (Clay et al., 2009; Fig. 6A), and six aliphatic glucosinolates was seen in *aux1* (Supplemental Fig. S2). Notably, 1-methoxy-3-indolylmethyl glucosinolate showed an opposite behavior and was significantly more abundant in *aux1* responding to DC3000(*avrRpm1*) compared with only subtle increases in Col-0 (Fig. 6B).

Camalexin levels increase in the wild type following avirulent inoculation (Fig. 6C). In *aux1*, however, camalexin levels are severely reduced under all treatments. Although no role has been demonstrated for camalexin in conferring resistance to bacteria, it is predicted to be important in the broad-spectrum resistance associated with SAR. Consistent with these data, the pattern of camalexin accumulation in the wild type and mutant is matched by the pattern of its precursor, dihydrocamalexic acid (Supplemental Fig. S2G). Both *PAD3* and *CYP79B3* are induced in *aux1* and display the altered dynamics illustrated in Figure 3. These results are consistent with the prediction that entry into this branch of aromatic secondary metabolism is disrupted in *aux1*, rather than progress along

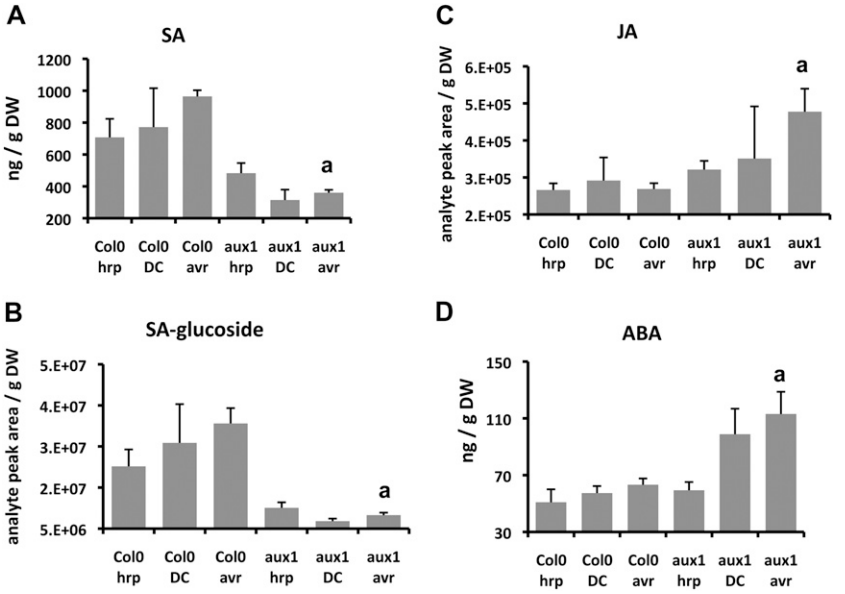

Figure 5. AUX1 affects the balance of key hormone levels in systemic leaves responding to infection. LC-MS/MS was used to measure changes in selected hormones simultaneously in the systemic leaves of wild-type and *aux1* plants at 21 hpi with DC3000*hrpA*$^-$ (hrp), DC3000 (DC), and DC3000(*avrRpm1*) (avr). Isotopically labeled standards were used to quantify SA (A) and ABA (D), while internal standards were not used for SA glucoside (B) and JA (C). Changes in hormone levels are expressed as the average quantity or analyte peak area of three replicates per gram dry weight (DW) ± SE. Significant differences ($P \leq 0.05$) between corresponding treatments in *aux1* and the wild type are marked (a). Similar hormone profiles were determined in two experiments.

the camalexin biosynthetic branch being affected. This interpretation was supported by monitoring Trp levels. Trp increases in unchallenged leaves in response to a DC3000(*avrRpm1*) immunizing challenge in both Col-0 and *aux1*; however, no significant differences in Trp are evident between the DC3000 and DC3000(*avrRpm1*) challenges (Fig. 6D). Therefore, it seems unlikely that changes to primary metabolism or the biosynthesis of Trp are responsible for the alterations to indolic metabolites observed in *aux1*. In addition, we profiled three flavonoids (Fig. 6E; Supplemental Fig. S2) in systemic tissues. The change exhibited by kaempferol-glucoside-rhamnoside (Fig. 6E) typifies the common pattern of accumulation for all three compounds, with moderate increases in response to avirulent challenge in the wild type but significant reductions in *aux1* regardless of treatment. The extent of impact of *aux1* on indolic profiles can be seen by the behavior of an unknown ion whose mass and daughter spectrum are consistent with an indolic glucoside (Fig. 6F). Ion mass-to-charge ratio (*m/z*) 322 was detected with negative mode ionization and previously identified as MAMP responsive in elicited leaves; *m/z* 322 is also SAR responsive in an AUX1-dependent fashion (Fig. 6F). The mass and daughter spectra of this ion are consistent with an indolic glucoside.

DISCUSSION

Plant-pathogen interactions are rapid and dynamic, with both host and pathogen constantly wrestling to modify signaling networks and reconfigure metabolism in favor of defense or disease. One strategy that is currently emerging is the recruitment of phytohormones to sequentially engage a range of defense responses in defined temporal windows. Rather than regulation by a single hormone, the balance between multiple hormones antagonistically or synergistically impacts defense responses dependent upon the host genotype and pathogen lifestyle (Thaler et al., 2002; Mur et al., 2006; Robert-Seilaniantz et al., 2007; Navarro et al., 2008b; Grant and Jones, 2009). Recently, it has been shown that jasmonates play an important role in the establishment of SAR activated by an incompatible challenge (Truman et al., 2007; Chaturvedi et al., 2008) prior to foliar accumulation of SA in distal responding tissues (Vlot et al., 2008a). This study now provides evidence that auxin perception and transport mutants are also involved in the establishment and maintenance of SAR.

Despite the up-regulation of several genes involved in auxin biosynthesis and homeostasis during SAR, no significant accumulation of IAA could be detected at either 8 or 24 hpi (Supplemental Table S1). We may have missed a transient IAA peak, as foliar IAA homeostasis is strongly regulated (Ljung et al., 2005), but sustained alterations to IAA and IAA conjugates are not associated with SAR. While our data strongly suggest a key role for auxin in the establishment of SAR, we cannot, based upon the modified profiles shown here (Fig. 6; Supplemental Fig. S2), rule out the possibility that unknown but related indole-derived compounds other than IAA may influence the SAR response. This is entirely plausible given that (1) the substrate specificity of the AUX1 transporter is incompletely characterized and (2) the recent demonstration that AUX1 can transport the structurally unrelated 2,4-dichlorophenoxyacetic acid (Carrier et al., 2008). Additionally, no significant changes in the expression patterns of GUS driven by the synthetic auxin promoter *DR5* were observed in leaves systemic to avirulent challenge (data not shown).

In the *cyp79b2/cyp79b3* double mutant, IG and camalexin biosynthesis is abolished and IAA levels are

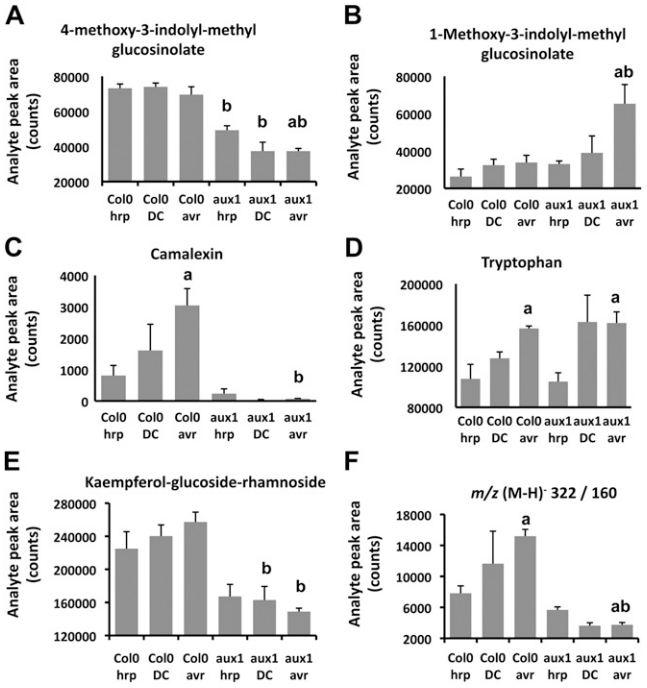

Figure 6. SAR-associated changes in the distribution of indolic compounds are dependent on AUX1. LC-MS/MS analysis of metabolites in systemic tissue at 21 hpi with DC3000*hrpA* (hrp), DC3000 (DC), and DC3000*avrRpm1* (avr). A and B, Small increases in the abundance of the IG 1-methoxy-3-indolyl glucosinolate in the wild-type response are strongly enhanced in *aux1* plants challenged with DC3000(*avrRpm1*), while several aliphatic glucosinolates and 4-methoxy-3-indolyl-methyl glucosinolate are significantly less abundant in *aux1*. C and D, SAR-induced accumulation of the phytoalexin camalexin is abolished and basal levels are massively reduced with *aux1*, whereas Trp, the key precursor to these indolic compounds, responds to avirulent challenge and accumulates to wild-type levels in *aux1* (Supplemental Fig. S2). E, Several flavonoids, including kaempferol-glucoside-rhamnoside, are significantly less abundant in *aux1*. F, An ion detected with negative mode ionization previously identified as PAMP responsive in elicited leaves is also SAR responsive in an AUX1-dependent fashion. The mass and daughter spectra of this ion are consistent with an indolic glucoside. Error bars show the SE of three replicates. Significant differences (Student's *t* test; $P \leq 0.05$) between DC3000 *hrpA*$^-$ and DC3000 (*avrRpm1*) within a genotype are marked (a), while significant differences between corresponding treatments in *aux1* and wild type are marked (b).

reduced. However, other auxin biosynthetic pathways are able to compensate, to some extent, for the loss of this route to IAA (Zhao et al., 2002). Perturbation of this branch of secondary metabolism has recently been shown to have an impact on many other pathways both proximal and distal; therefore, the effect of this mutation on SAR may be derived from its impact on any one of a plethora of metabolites (Malitsky et al., 2008). The impact of auxin signaling mutants on SAR, therefore, may not relate to the total abundance of, or direct response to, auxin. Rather, auxin may play a crucial role in balancing the homeostasis of various indolic compounds, some of which may prove critical to systemic immunity. In the case of flavonoids (Fig. 6E; Supplemental Fig. S2), it may be that this disruption in biosynthesis feeds back into the control of auxin signaling by impacting the transport and localization of auxin. Another possibility is that AUX1 and AXR4 are engaged in the transport of another indole-derived metabolite that plays an important signaling role in SAR.

Alternatively, the importance of auxin signaling in the establishment of SAR may arise from its modulation of JA/ET-SA cross talk, possibly through antagonism with ABA. Both the reduction in SA levels and the increased levels of ABA in *aux1*-responding tissue would be predicted to contribute to enhanced susceptibility to bacterial infection. Intriguingly, the suppression of SA-associated gene expression is only seen in the later stages of SAR in *aux1* (Fig. 4). A model depicting the predicted impact of the *aux1* mutant on the dynamics of JA/ET-SA signaling in SAR is presented in Figure 7A. Gene expression responses earlier in the development of SAR indicate that SA levels and the response to SA are not attenuated but enhanced. This may reflect a mechanism of promoting SA antagonism of the early JA signaling responses involved in the establishment of SAR (Truman et al., 2007). Our results strongly support the interpretation that under the infection conditions used in these experiments, the *aux1* mutant is unable to appropriately marshal the shift from one defensive position (early jasmonate-based response) to another (later SA-mediated response). Loss of DELLA proteins controlling responses to GA have also been found to disrupt JA/ET-SA cross talk, leading to enhanced resistance to bacteria (Navarro et al., 2008b). These data underline the complexity of both the establishment of systemic immunity and the interplay of phytohormones involved in defense. Both JA and ET signaling are essential for the induced systemic resistance brought about through the recognition of nonpathogenic rhizobacteria (van Loon et al., 1998). The *aux1* mutant confers ET insensitivity for several root traits (Pickett et al., 1990; Swarup et al., 2007; Negi et al., 2008). While no distinct role for ET in SAR has yet been defined, it may be that it acts through auxin signaling to modulate SA cross talk.

Two components of a proteasomal degradation signaling complex, SGT1B and TIR1, have now been identified as essential components in the signal transduction required for SAR. We originally hypothesized that the impact of *sgt1b* on SAR resulted from its JA signaling role (Truman et al., 2007), but it may prove that its contribution to auxin signaling (Gray et al., 2003) is equally vital to the development of SAR.

A clear link has been established between auxin, the AUX1-related auxin influx carrier LAX3, and cell wall enzymes in promoting lateral root emergence (Swarup et al., 2008). Recently, Ding et al. (2008) identified one specific mechanism for auxin in the elaboration of plant defense responses, whereby auxin homeostasis directly affected the expression of expansins. Overexpression of these cell wall-loosening enzymes was

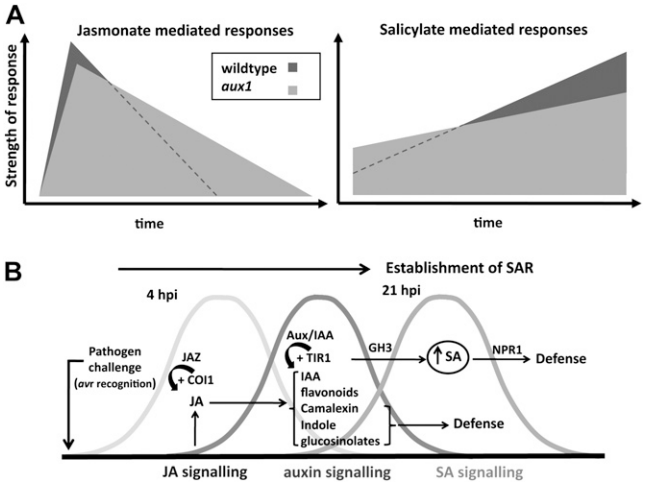

Figure 7. Prediction of the impact of auxin transport perturbation on the phytohormone-mediated defense responses and a proposed model integrating hormonal signal transitions during the establishment of SAR. A, The *aux1* mutant globally impacts both the magnitude and timing of jasmonate and salicylate defense response. Mutants in auxin transport impair the perception or transduction of the SAR signaling network to undergo the transition from the early JA defensive phase to the later SA phase. B, Model describing temporal phases of hormone signaling underpinning the establishment of systemic immunity. Perception of the immunizing signal in systemically responding leaves activates JA signaling networks mediated through the COI1 jasmonate receptor, which leads to the induction of components of the indole biosynthetic pathway. Concomitantly, JA signaling is negatively regulated through a feedback loop mediated by coinduction of *JAZ* transcripts. This transition provides competency to produce defense signaling components such as IGs and camalexin and a biologically active indole derivative, most likely auxin, that requires the functional auxin importer AUX1. AUX1 facilitates IAA import (possibly fine-tuned by flavonoids) and interaction with the TIR receptor to activate auxin-regulated transcription. Establishment of SAR requires a further transition from auxin- to SA-mediated signaling. This phase may be mediated through feedback repression of auxin signaling through the activities of auxin-inducible *GH3* genes encoding auxin-conjugating enzymes. Activation of SA defenses through NPR1-dependent signaling pathways primes the plant to respond more rapidly to future pathogen challenges and completes the hormonal transitions programmed by gene-for-gene-activated SAR.

associated with increased susceptibility to bacteria. Expression of at least one expansin (*EXPA1*) is suppressed systemically in response to avirulent challenge at 8 and 10 hpi (Supplemental Fig. S3), consistent with a model of SAR restricting leaf expansion, thus potentially allowing cellular resources to be deployed toward cell wall reinforcement. Notably, expression of *EXPA1* is lower in *aux1* leaves systemic to avirulent challenge by 10 hpi. While the expression of *EXPA1* does not explain the susceptibility conferred by *aux1*, it reinforces the links between auxin homeostasis and the establishment of SAR.

It seems clear that control over the homeostasis of indolic compounds is of key importance for the normal establishment of systemic immunity and that this breaks down in *aux1* plants. Flavonoids have been implicated in the alteration of auxin transport (Brown et al., 2001), with quercetin treatments ameliorating the physiological effects of auxin efflux mutants and ectopic flavonoid accumulation inhibiting auxin transport (Besseau et al., 2007; Santelia et al., 2008). Here, we have shown that *aux1* impacts the regulation of at least three classes of aromatic secondary metabolites (Fig. 6). It is likely that auxin transport affects several pathways. Recent global metabolite profiling of basal immune responses in our laboratory has identified several metabolite peaks elicited through MAMP perception (J. Ward, S. Forcat, M. Bennett, J. Mansfield, W. Truman, and M. Grant, unpublished data). One such compound is putatively β-D-glucopyranosyl indole-3-carboxylic acid, previously shown to accumulate in response to the root pathogen *Pythium sylvaticum* (Bednarek et al., 2005), which is also induced during SAR and suppressed in *aux1* (Fig. 6F). This further indicates that *AUX1* is required for normal regulation of several classes of aromatic defense compounds.

Based upon these findings, we put forth the following model for the activation of SAR (Fig. 7B). Following perception of an immunizing signal in systemically responding leaves, SAR is triggered initially through an increase in jasmonates, which leads to the induction of components of the indole biosynthetic pathway (Fig. 1). Concomitantly, JA signaling is negatively regulated through a feedback loop mediated by coinduction of *JAZ* transcripts. This transition provides competency to produce defense signaling components (e.g. IGs and camalexin; Fig. 6; Supplemental Fig. S2) and a biologically active indole derivative, most likely auxin, that requires the functional auxin importer AUX1. AUX1 facilitates IAA import, possibly fine-tuned by flavonoids (Brown et al., 2001; Fig. 6; Supplemental Fig. S3), and interaction with the TIR receptor to activate auxin-regulated transcription. Establishment of SAR requires a further transition from auxin to SA-mediated signaling. This phase may be mediated through feedback repression of auxin signaling through the activities of auxin-inducible *GH3* genes encoding auxin-conjugating enzymes (Zhang et al., 2007; Ding et al., 2008). Activation of SA defenses to prime the plant to respond more rapidly to future pathogen challenges completes the hormonal transitions programmed by gene-for-gene-activated SAR.

Currently, it remains to be determined which, if any, of the metabolites identified so far act to directly restrict pathogen colonization of immunized tissue. Indeed, it has recently been shown that elevated expression of transcripts encoding components of the Trp biosynthetic and metabolic pathways, together with altered camalexin, IGs, and other indolic compounds in Arabidopsis *mlo* mutants, contribute positively toward antifungal defense. Consistent with these observations, the triple mutant *mlo2/cyp79B2/cyp79B3* exhibited wild-type infection responses to the adapted pathogen *Golovinomyces orontii* (Consonni et al., 2010). Collectively, these data highlight roles for indolic compounds in elaborating broad-spectrum

defense responses. It is unlikely that focusing upon how a single metabolite contributes to resistance will be meaningful, as broad-spectrum defense conferred by SAR reflects the deployment of multiple defense compounds, contributing differentially and incrementally, to enhance resistance to pathogens with diverse lifestyles. The host cannot predict the nature of the ensuing infection and must mitigate against all eventualities. This would be most effectively achieved by the deployment, or at least priming for the deployment, of a range of small molecules capable of the initial containment of invading pathogens before full-blown defense responses can be instigated. More and more information is emerging to suggest that phytohormones interact to mediate biotic interactions (Bari and Jones, 2009; Grant and Jones, 2009). The temporal organization of these interactions appears to be essential for positive outcomes; spatial organization and distribution of signaling components may also be vital. A mechanistic understanding of the temporal/spatial regulation of these interacting hormones is an immediate challenge.

MATERIALS AND METHODS

Microarray Data Mining

Affymetrix whole genome GeneChip data previously described by Truman et al. (2007) and available at http://affymetrix.arabidopsis.info/narrays (identifier NASCARRAYS-403) were interrogated using gene lists obtained from biosynthetic pathway data available at The Arabidopsis Information Resource (www.arabidopsis.org). The microarray scan data were processed using the Robust Multiarray Average method as implemented in the Bioconductor package RMA (Irizarry et al., 2003; Gentleman et al., 2004). Mean ratios and SE were derived from this RMA-normalized data.

Maintenance of Plants and Bacteria

All bacterial strains were grown, cultured, and maintained as described by de Torres et al. (2003). Arabidopsis (*Arabidopsis thaliana*) plants were germinated and grown as described (de Torres et al., 2003) using the following growth parameters: 110 μE in short days (10 h of light) at 65% relative humidity. Day temperature was 23°C, and night temperature was 21°C. Plants were used between 4 to 5 weeks for pathogen assays, RNA extraction, and metabolite analyses.

Bacterial Inoculations

For RNA and metabolite profiling, leaves were inoculated with a 1-mL needleless syringe on their abaxial surface with a bacterial suspension adjusted to an optical density at 600 nm of 0.2 (approximately 2×10^8 colony-forming units [cfu] mL^{-1}) in 10 mM $MgCl_2$. Inoculations were undertaken in the morning, at least 2 h after dawn, and completed within 2 h. For measuring SAR bacterial growth, initial challenges were either mock (10 mM $MgCl_2$) inoculation or challenge with DC3000(*avrRpm1*) at 1×10^8 cfu mL^{-1}. After 2 d, secondary leaves were infiltrated with either *Pseudomonas syringae* pv *maculicola* M4 at 5×10^5 cfu mL^{-1} or DC3000 at 5×10^4 cfu mL^{-1}. Bacterial growth titers were determined either 3 or 4 d later as described (de Torres et al., 2006).

RNA Extraction and qRT-PCR

Total RNA was isolated as described previously (de Torres et al., 2003). cDNA was generated from 1 μg of total RNA with SuperScriptIII (Invitrogen) following the manufacturer's instructions. Quantitative PCR was performed on the cDNA using the QuantiTect SYBR Green PCR kit (Qiagen) on a Rotor-Gene 6000 (Corbett Research). *Actin2* was used as an internal standard to normalize cDNA content in the samples. Relative expression levels were calculated using the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001). Expression levels were calculated either as the relative change in gene expression using an appropriate reference sample or as the log (base 2) ratio of two specific conditions. The primers used for RT-PCR and amplicon size are described in Supplemental Table S4.

Hormone and Metabolite Measurements

Quantitative hormone measurements using isotopically labeled standards were conducted by LC-MS/MS using the protocol described by Forcat et al. (2008). Other metabolites were quantified from the same extracts with the same gradient using appropriate single reaction monitoring of ion pairs.

Glucosinolates were measured in negative mode but using a 2-μL injection volume. Identification was based on MS/MS and UV light, and the compounds were quantified using transitions based on parent/sulfite ions ($[M-H]^-/[SO_3H]^-$).

Camalexin, dihydrocamalexic acid, and Trp were quantified by a separate analysis in positive ion mode (transitions 201/142, 247/143, and 205/146), and identification was by comparison with authenticated standards (camalexin and dihydrocamalexic acid were a kind gift from Erich Glawischnig). The flavonol glycosides were identified by MS/MS and UV light and were measured simultaneously (transitions 579/187, 595/287, and 741/287) with the other compounds in positive ionization mode. Transitions for the glycoside of indole carboxylic acid (322/160) were included in the hormone analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *axr4* suppresses the induction of systemic responses to avirulent challenge and alters the kinetics of their expression.

Supplemental Figure S2. *aux1* impacts the abundance of a wide range of secondary metabolites.

Supplemental Figure S3. Expansin genes are suppressed systemically in response to avirulent challenge.

Supplemental Table S1. Hormone profiling in systemically responding leaves following specific immunizing challenges.

Supplemental Table S2. The kinetics of systemic transcriptional responses to avirulent inoculation is disrupted in the *aux1* mutant.

Supplemental Table S3. The systemic expression of genes controlling the homeostasis of indolic compound correlates with the negative regulator of JA responses, *JAZ10*.

Supplemental Table S4. Primers used for qRT-PCR as described in "Materials and Methods."

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