Running title: Auxin mutants compromise SAR

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Arabidopsis auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds.

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Abstract

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 signalling 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 signalling components underpinning signal perception and establishment of systemic immunity are rudimentary. Previously we showed 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 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 establishment and maintenance of systemic immunity.
Introduction

Plants growing under natural conditions are continuously subjected to a wide variety of abiotic and biotic stresses. Many of the defence 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 defence against plant pathogens were salicylic acid (SA), jasmonates (JA) and ethylene (ET) (Glazebrook, 2005) with roles more recently attributed to abscisic acid (ABA), gibberellins and auxin (Navarro et al., 2006; de Torres-Zabala et al., 2007; Wang et al., 2007; Navarro et al., 2008; Grant and Jones, 2009; Kazan and Manners, 2009). JA/ET and SA mediated defence pathways antagonise each other with JA/ET largely defining defence against necrotrophic pathogens and insects while SA is central in defence against biotrophic pathogens (Glazebrook, 2005). Despite this well-established antagonism between SA and JA/ET signalling 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 has also been shown to alter the interaction between these hormones (Thaler et al., 2002; Spoel et al., 2007). The contribution of auxin to defence appears to fit within the biotroph - necrotroph, SA - JA/ET antagonism model with several recent reports linking auxin with susceptibility to the hemi-biotroph 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 signalling networks. Conversely, mutations in key auxin signalling 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 defence mechanisms. Navarro et al., (2006) showed that this detection of non-self led to suppression of the F-box auxin receptor, TIR1, and its AFB paralogs through post-transcriptional silencing by miRNAs. The importance of these SCFTIR1/AFB receptor / ubiquitination complexes was confirmed by the increased growth of virulent Pseudomonas syringae pv tomato (Pst) in transgenic lines over-expressing 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 thaliana* lacking the disease resistance gene *RPS2*, was found to increase levels of the auxin indole acetic acid (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 (NAA) promoted the development of disease following infection with virulent *Pst*. Full genome microarray experiments of plants treated with the SA analogue benzothiadiazole S-methylester (BTH) revealed inhibition of auxin signalling pathways (Wang et al., 2007). This inhibition was predicted to result from the stabilisation of auxin repressor proteins in a mechanism apparently independent from the post-transcriptional 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 defence marker *PR1*. SA induced expression of *PR1* was diminished with NAA co-treatment. Inoculation of *Arabidopsis* with *Plectosphaerella cucumerina* induces expression of both *PR1* and the JA/ET dependent marker, defensin *PDF1*; susceptible auxin signalling mutants were not significantly affected in this response but pre-treatment of plants with the auxin transport inhibitor tri-iodobenzoic acid (TIBA) which yielded similar susceptibility did act to reduce *PDF1* expression while enhancing *PR1* expression (Llorente et al., 2008). Interestingly, infection of the stabilised repressor of auxin signalling *axr2-1* produced a significantly greater induction of the JA biosynthesis gene *LOX3* than in wildtype 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 susceptible 3 (PBS3) acts on 4-substituted benzoates, perhaps by
priming or inducing SA biosynthesis (Okrent et al., 2009). In rice, GH3.8 decreases free IAA levels when over-expressed, most probably by increasing the amount of IAA conjugated to aspartate (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 signalling pathway activation, though the suppression of SA or JA responses may be crucial. The importance of auxin in plant defence responses is further emphasised by the number of plant pathogens that synthesise auxin in order to facilitate virulence (reviewed in (Robert-Seilianiantz 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 necrotising 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 localised MAMPs triggered immunity (Mishina and Zeier, 2007).

Recently, SAR has been reported following challenge with virulent *Pseudomonas 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 (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 are, however, associated with JA signalling (Truman et al., 2007). The importance of this transient JA associated response has lead to speculation that SAR may depend on a synergistic interaction of JA and SA signalling networks. In addition to dual defence networks being involved, there is now compelling, but sometimes contradictory evidence that multiple signals, including both methyl salicylate (MeSA) and a lipid derived molecule, that may or may not be azelaic acid, and terpenoids, are exported through the phloem from immunised leaves together with non-essential signals such as increased JA.
levels (Maldonado et al., 2002; Chaturvedi et al., 2007; Park et al., 2007; Truman et al., 2007; Vlot et al., 2008; 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 genes up-regulated in systemic leaves following avirulent challenge which are also regulated by auxin. We therefore tested a selection of auxin signalling mutants for their capacity to establish SAR. The auxin influx transporter AUX1 and AXR4 which controls the localisation of AUX1 (Bennett et al., 1996; Kepinski and Leyser, 2005; Dharmasiri et al., 2006) were both required for SAR to moderately or strongly virulent *Pseudomonas 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 which 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 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 lead to the identification of 394 significantly differentially expressed genes (Truman et al., 2007) compared to either the DC3000hrp mutant which cannot deliver bacterial effector proteins into the plant cell or virulent DC3000. There were no significant differences in gene expression between DC3000hrp or DC3000 indicating that only the presence of avrRpm1 was responsible for reconfiguring gene expression and 4hpi Type III effectors did not impact on transcription in systemic tissue. The majority of these genes (369) were up-regulated and they contained a significant over-representation 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 catalyse the conversion of tryptophan to indole-3-acetaldoxime, a key branching point which may be converted into IAA, the antimicrobial phytoalexin camalexin or indolic glucosinolate defence 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 tryptophan and indolic glucosinolate (IG) biosynthesis is also strongly induced (Celenza et al., 2005). Over-expression 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 probesets 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 on disease resistance responses (Davies et al., 1999; Zhang et al., 2007; Ding et al., 2008). Recently tryptophan conjugates of IAA have been shown to be potent inhibitors of auxin signalling, 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 dataset 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(\textit{avrRpm1}) relative to a wildtype DC3000 challenge as rapidly as 4hpi in genes known to be involved in the biosynthesis of tryptophan, auxin and other derivatives of tryptophan (Figure 1).

Most striking, the induction of \textit{CYP79B2} and \textit{CYP79B3} is accompanied by increases in other transcripts that would act to commit indole-3-acetaldoxime to IG biosynthesis - \textit{CYP83B1}, \textit{UGT74B1} and the sulfotransferase encoded by \textit{SOT16} (Bak et al., 2001; Grubb et al., 2004; Piotrowski et al., 2004). However, IAA may also be synthesised 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 towards camalexin biosynthesis, because \textit{CYP71A13} and \textit{PAD3} which catalyse the initial and final steps in this pathway, respectively, are both up-regulated (Schuhegger et al., 2006; Nafisi et al., 2007). No \textit{GH3} family genes known to conjugate IAA were seen to increase significantly. The \textit{GH3} family member \textit{JAR1}, whose product conjugates isoleucine to JA (Staswick et al., 2002), and \textit{PBS3} (Okrent et al., 2009), which may be involved in conjugating SA precursors, both exhibited marginally increased expression.

\textit{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 increase in auxin during the early phase of establishment of SAR (Supplemental Table. 1 online). A number of plants with altered auxin homeostasis due to mutation or ectopic over-expression of components in auxin biosynthesis exhibit gross morphological abnormalities and are unsuitable for undertaking SAR experiments. To address a role of auxin signalling in SAR we tested \textit{Arabidopsis} auxin signalling or auxin biosynthesis mutants that exhibit no gross morphological abnormalities. The immunising effect resulting from \textit{RPM1} mediated recognition of DC3000(\textit{avrRpm1}) was tested the auxin F-box receptor mutant \textit{tir1} (Kepinski and Leyser, 2005). In three independent
experiments no SAR effect was observed in *tir1* plants challenged with virulent *Pseudomonas syringae pv maculicola* (*Psm*) in secondary leaves following an initial inoculation with DC3000(*avrRpm1*) (Figure 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 in the *cyp79b2/cyp79b3* double mutant in two independent experiments (Fig. 2B). This *cyp79b* double mutant is blocked in the synthesis of indole-3-acetaldoxime and has reduced IAA and is the only route to 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* mutant resembled *tir1* in its compromised SAR.

**Auxin transport mechanisms are critical to JA SA signalling dynamics**

The transcriptional responses of *axr4*, *aux1*, *tir1* and *cyp79b2/cyp79b3* were examined using qRT-PCR. A subset of genes were selected from available microarray data, together with classical markers of SAR and SA-associated defence 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 (Figure 3A). By contrast inoculation with DC3000*hrp* mutant which is unable to deliver bacterial effectors, or virulent DC3000 (data not shown) does not induce *At5g56980* systemically. It therefore seems likely that auxin signalling plays no role in the generation or perception of the mobile signal(s) responsible for this phase of the elaboration of SAR, though redundancy in auxin signalling and biosynthesis mean we cannot exclude this possibility. Many of the genes up-regulated systemically by DC3000(*avrRpm1*) 4hpi 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* (Figure 3B-D), including *JAZ10*, encoding one member of a family of negative regulators of JA signalling (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, ~60 fold, in systemic leaves at 4 hpi; this relative induction declines exponentially over the next six hours (Figure 3B – note log2 scale, Supplemental Table 2 online). The early expression of *JAZ10* fits neatly into a model of the development of SAR in which attenuation of JA signalling would see JA responsive genes return to basal levels prior to the induction of...
SA-mediated responses known to be essential to establishment of SAR. *JAZ10*, shares this expression pattern with indole-3-acetaldoxime biosynthesis component *CYP79B3*, indole glucosinolate biosynthesis component *CYP83B1*, camalexin biosynthesis component *PAD3* and 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, 10 hpi) and three genotypes (Col 0, *aux1*, *axr4*) reveal strongly co-regulated expression of these genes with correlation coefficients between genes ranging from 0.70 - 0.96 (Supplemental Table 3 online). All, with the exception of *ILL5*, are up-regulated in *aux1* in response to DC3000(*avrRpm1*) though not as strongly as wild-type (Figure 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 wild-type steadily increases over time (Figure 3D). Similar trends were observed in *axr4*, with the exception of *PAD3* (Supplemental Figure 1 online), 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 co-expressed with *JAZ10*, transcripts associated with SA-mediated defence responses and the later stages of SAR development are not induced by DC3000(*avrRpm1*) 4 hpi. *PR1* is a classical marker for SAR, while *FMO1* has recently been identified as an important regulator of both basal defences 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 were substantially lower in *aux1* compared with wild-type (Figure 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 Col0 and *aux1* plants 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(\textit{avrRpm1}) challenge in Col0 (Fig. 5A), levels of free SA and conjugated SA were significantly reduced (~3 fold less, \( p < 0.01 \)) in \textit{aux1} responding to DC3000(\textit{avrRpm1}), compared with wild-type (Fig. 5A & B). SA levels in \textit{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 promote increase susceptibility to \textit{Pst} (Koornneef and Pieterse, 2008), accumulated to significantly higher levels in \textit{aux1} responding to DC3000(\textit{avrRpm1}) compared with Col0 (Fig. 5C & 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; Yasuda et al., 2008; de Torres Zabala et al., 2009). 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 \textit{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).

\textit{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 \textit{aux1} challenged leaves the suggested changes in indolic compounds (Fig. 6 and data not shown). LC-MS was used to measure the relative abundance of the main aliphatic and indolic glucosinolates. Despite the transcriptional up-regulation of almost the entire IG biosynthesis pathway from 4hpi onwards no major glucosinolate changes in systemic responding leaves of wild type plants were evident following a DC3000(\textit{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) (Figure 6A) and six aliphatic glucosinolates were seen in \textit{aux1} (Supplemental Figure 2). Notably, 1-methoxy-3-indolylmethyl glucosinolate showed an opposite behaviour and was significantly more abundant in \textit{aux1} responding to DC3000(\textit{avrRpm1}) compared with only subtle increases in Col0 (Fig.6B).
Camalexin levels increase in 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 Figure 2G). 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 the camalexin biosynthetic branch being affected. This interpretation was supported by monitoring tryptophan levels. Tryptophan increases in unchallenged leaves in response to a DC3000(avrRpm1) immunizing challenge in both Col0 and aux1 however no significant differences in tryptophan are evident are observed between the DC3000 and DC3000(avrRpm1) challenges (Fig. 6D). Therefore, it seems unlikely that changes to primary metabolism or the biosynthesis of tryptophan are responsible for the alterations to indolic metabolites observed in aux1. In addition, we profiled three flavonoids (Fig. 6E and Supplemental Fig. 2) 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 behaviour of an unknown ion whose mass and daughter spectrum are consistent with an indolic glucoside (Fig. 6F). m/z 322, an ion detected with negative mode ionisation 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 spectrum 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 signalling networks and reconfigure metabolism in favour of defence or disease. One strategy that is currently emerging is the recruitment of phytohormones to sequentially engage a range of defence responses in defined temporal windows. Rather than regulation by a single hormone, the balance between multiple hormones antagonistically or synergistically impacts on defence responses dependent upon the host genotype and pathogen lifestyle (Thaler et al., 2002; Mur et al., 2006; Robert-Seilanianzt et al., 2007; Navarro et al., 2008; Grant and Jones, 2009). Recently it has been shown that jasmonates play an important role in establishment of SAR activated by an incompatible challenge (Chaturvedi et al., 2007; Truman et al., 2007) prior to foliar accumulation of SA in distal responding tissues (Vlot et al., 2008). This study now provides evidence that auxin perception and transport mutants are also involved in 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 1 online). 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 establishment of SAR we cannot, based upon the modified profiles shown in (Fig. 6 and Supplemental Fig. 2), 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 (i) the substrate specificity of the AUX1 transporter is incompletely characterised and (ii) 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 $cyp79b2b3$ double mutant indole glucosinolate and camalexin biosynthesis is abolished and IAA levels 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
signalling mutants on SAR may therefore 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 and Supplemental Fig. 2), it may be that this disruption in biosynthesis feedbacks into the control of auxin signalling by impacting on the transport and localisation of auxin. Another possibility is that AUX1 and AXR4 are engaged in the transport of another indole derived metabolite that plays an important signalling role in SAR.

Alternatively, the importance of auxin signalling 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 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 signalling 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 is not attenuated but enhanced. This may reflect a mechanism of promoting SA antagonism of the early JA signalling responses involved in 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., 2008). These data underline the complexity both of the establishment of systemic immunity and the interplay of phytohormones involved in defence. Both JA and ET signalling are essential for the induced systemic resistance (ISR) brought about through the recognition of non-pathogenic rhizobacteria (van Loon et al., 1998). The aux1 mutant confers ethylene 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 signalling to modulate SA cross-talk.

Two components of a proteasomal degradation signalling complex SGT1B and TIR1 have now been identified as essential components in the signal transduction required for SAR. We originally hypothesised that the impact of sgt1b on SAR resulted from its JA signalling role
(Truman et al., 2007) but it may prove that its contribution to auxin signalling (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 defence responses, whereby auxin homeostasis directly affected the expression of expansins. Over-expression of these cell wall loosening enzymes was 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. 3) consistent with a model of SAR restricting leaf expansion thus potentially, allowing cellular resources to be deployed towards 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 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 on 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 MAMPs perception (Ward et al., submitted). 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 (Figure 6F). This further indicates that AUX1 is required for normal regulation of several classes of aromatic defence compounds.

Based upon these findings we speculate the following model for activation of SAR (Fig. 7B). Following perception of an immunising signal in systemically responding leaves, SAR is triggered initially through an increase in jasmonates which leads to induction of components of the indole biosynthetic pathway (Fig. 1). Concomitantly JA signalling is negatively regulated through a feedback loop mediated by co-induction of JAZ transcripts. This transition provides
competency to produce defense signalling components (e.g. indole glucosinolates and camalexin, Fig. 6 & Supplemental Fig. 2) 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 Figure 3) and interaction with the TIR receptor to activate auxin regulated transcription. Establishment of SAR requires a further transition from auxin to SA mediated signalling. This phase may be mediated through feedback repression of auxin signalling through the activities of auxin-inducible GH3 genes encoding auxin-conjugating enzymes (Zhang et al., 2007; Ding et al., 2008). Activation of SA defences 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 colonisation of immunised tissue. Indeed, it has recently been shown that elevated expression of transcripts encoding components of the tryptophan biosynthetic and metabolic pathways together with altered camalexin, indolic glucosinolates and other indolic compounds in Arabidopsis mlo mutants contribute positively towards 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. Plant Physiol. in press). Collectively these data highlight roles for indolic compounds in elaborating broad spectrum defense responses. It is unlikely that focussing upon how a single metabolite contributes to resistance will be meaningful as broad spectrum defence conferred by SAR reflects the deployment of multiple defence 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 phytohormones interact to mediate biotic interactions (Bari and Jones, 2009; Grant and Jones, 2009). The temporal organisation of these interactions appears to be essential for positive outcomes; spatial organisation and distribution of signalling components may also be vital. A mechanistic understanding of the temporal/spatial regulation of these interacting hormones is an immediate challenge.
Methods

Microarray Data Mining

Affymetrix whole genome GeneChip data previously described in Truman et al., 2007 and available at http://affymetrix.arabidopsis.info/narrays (identifier NASCARRAYS-403) was interrogated using gene lists obtained from biosynthetic pathway data available at The Arabidopsis Information Resource (www.arabidopsis.org). The microarray scan data was processed using the Robust Multi-array 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 normalised data.

Maintenance of Plants and Bacteria

All bacterial strains were grown, cultured, and maintained as described in de Torres et al., (2003). A. thaliana plants were germinated and grown as described (de Torres et al., 2003) using the following growth parameters; 110 µEinstein’s in short-days (10h light) at 65% relative humidity. Day temperature was 23°C, dark temperature 21°C. Plants were used between 4-5 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 OD_{600} 0.2 (≈2 × 10^8 cfu ml^{-1}) in 10 mM MgCl₂. Inoculations were undertaken in the morning, at least two hours after dawn, and completed within 2 h. For measuring SAR bacterial growth, initial challenges were either mock (10 mM MgCl₂) inoculation or challenge with DC3000(\textit{avrRpm1}) at 1 × 10^8 cfu ml^{-1}. After 2 days, secondary leaves were infiltrated with either \textit{P. syringae pv. maculicola} M4 at 5 \times 10^5 cfu ml^{-1} or DC3000 at 5 \times 10^4 cfu ml^{-1}. Bacterial growth titres were determined either 3 or 4 days 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 Corporation, California, USA) following the manufacturer's instructions. Quantitative PCR was performed on the cDNA using the QuantiTect SYBR Green PCR kit (Qiagen, West Sussex, UK) on a Rotor-Gene 6000 (Corbett Research, Cambridge, UK). *Actin 2* was used as an internal standard to normalise cDNA content in the samples. Relative expression levels were calculated using the ΔΔCt 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 Supplementary Table 4 online.

**Hormone and Metabolite Measurements**

Quantitative hormone measurements using isotopically labelled standards were conducted by LC/MS/MS using the protocol described in Forcat et al., 2008. Other metabolites were quantified from the same extracts with the same gradient by using appropriate Single Reaction Monitoring of ion pairs.

Glucosinolates were measured in negative mode but using a 2μl injection volume. Identification was based on MS^n and UV and the compounds quantified using transitions based on parent/sulphite ions ([M-H]-/ [SO3H]-).

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

**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. cRNA was derived from mRNA isolated from distal systemically responding Col0 leaves 4 h after challenge with either *Pst* DC3000 or DC3000(*avrRpm1*) as described in Truman et al. 2007. The genes plotted represent all the significant changes observed in the biosynthesis of tryptophan, 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.

**Figure 2: Auxin signalling, transport and biosynthesis mutants fail to develop effective systemic immunity.**

Multiplication of virulent *P. syringae* 3 or 4 days after inoculation in SAR-induced plants. Leaves were either mock (10 mM MgCl₂) inoculated or challenged with DC3000(*avrRpm1*) at 1 × 10⁸ 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 measured 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 measured 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 4 or 5 replicates.

**Figure 3: The kinetics of systemic transcriptional responses to avirulent inoculation is disrupted in the *aux1* mutant.**

The accumulation of transcripts encoding key signalling components were analysed in systemic responding tissue by qRT-PCR. (A) RT-PCR shows the rapid accumulation of *At5g56980*, a marker for 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 defences (hrp). The error bar shows the standard deviation of three
replicates. A strong response was also observed in the aux1, axr4, tir1 and cyp79b2/cyp79b3 mutants. (B-D) JAZ10, CYP79B3, CYP83B1, PAD3, ILL5 and ILL6 share a common expression pattern. (B) Up-regulation in response to DC3000avrRpm1 compared to DC3000 declines linearly over time in wildtype A. thaliana. Induction of these genes is not abolished in aux1 (C) but exhibit an inverted response relative to wildtype (D). qRT-PCR analyses were repeated in two or three independent experiment with similar results.

Figure 4: Gene expression associated with salicylic acid accumulation and the establishment of SAR is 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 defences and SAR were 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 wildtype expression levels.

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 wildtype and aux1 plants 21h after inoculation with DC3000 hrpA (hrp), DC3000 (DC) and DC3000(avrRpm1) (avr). Isotopically labelled 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 of dry weight ± the standard error. Significant differences (p-value ≤0.05) between corresponding treatments in aux1 and wildtype are marked (a). Similar hormone profiles were determined in two experiments.

Figure 6: SAR associated changes in the distribution of indolic compounds are dependent on AUX1.

LC/MS/MS analysis of metabolites in systemic tissue 21h after infection with DC3000hrpA (hrp), DC3000 (DC) and DC3000avrRpm1 (avr). (A, B) Small increases in the abundance of the IG 1-methoxy-3-indolyl glucosinolate in the wildtype response are strongly enhanced in
aux1 plants challenged with DC3000avrRpm1 while several aliphatic glucosinolates and 4-methoxy-3-indolyl-methyl glucosinolate are significantly less abundant in aux1. (C, D, Supplemental Figure 2) SAR induced accumulation of the phytoalexin camalexin is abolished and basal levels massively reduced with aux1 whereas tryptophan the key precursor to these indolic compounds responds to avirulent challenge and accumulates to wildtype levels in aux1. (E) Several flavonoids including kaempferol-glucoside-rhamnoside are significantly less abundant in aux1. (F) An ion detected with negative mode ionisation previously identified as PAMP responsive in elicited leaves is also SAR responsive in an AUX1 dependent fashion. The mass and daughter spectrum of this ion are consistent with an indolic glucoside. Error bars show the SE of three replicates. Significant differences (Students t test; p-value ≤0.05) between DC3000 hrpA- and DC3000(avrRpm1) within a genotype are marked (a) while significant differences between corresponding treatments in aux1 and wildtype are marked (b).

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 on both the magnitude and timing of jasmonate and salicylate defense response. Mutants in auxin transport impair the perception or transduction of the SAR signalling network to undergo the transition from the early JA defensive phase to the later SA phase.

B) Model describing temporal phases of hormone signalling underpinning establishment of systemic immunity. Perception of the immunizing signal in systemically responding leaves activates JA signalling networks mediated through the COI1 jasmonate receptor which leads to induction of components of the indole biosynthetic pathway. Concomitantly JA signalling is negatively regulated through a feedback loop mediated by co-induction of JAZ transcripts. This transition provides competency to produce defense signalling components such as indole glucosinolates 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 signalling. This phase may be mediated through feedback repression of auxin signalling through the activities
of auxin-inducible $GH3$ genes encoding auxin-conjugating enzymes. Activation of SA defences through NPR1 dependent signalling pathways primes the plant to respond more rapidly to future pathogen challenges completes the hormonal transitions programmed by gene-for-gene activated SAR.
Supplemental data:

Supplemental Figure 1: *axr4* suppresses the induction of systemic responses to avirulent challenge and alters the kinetics of their expression.

Supplemental Figure 2: *aux1* impacts on the abundance of a wide range of secondary metabolites.

Supplemental Figure 3: Expansin genes are suppressed systemically in response to avirulent challenge.

Supplemental Table 1: Hormone profiling in systemic responding leaves following specific immunizing challenges.

Supplemental Table 2: The kinetics of systemic transcriptional responses to avirulent inoculation are disrupted in the *aux1* mutant.

Supplemental Table 3: The systemic expression of genes controlling the homeostasis of indolic compound correlate with the negative regulator of JA responses *JAZ10*.

Supplemental Table 4: Primers used for qRT-PCR reactions as described in Methods.
Impact of the *aux1* mutant on systemic response to *avrRpm1*

Log2 ratio *aux1* *avr* vs Col0 *avr*

- **8h**
  - FMO1: -3
  - PR1: 3

- **48h**
  - FMO1: 1
  - PR1: -1
A

Jasmonate mediated responses

Salicylate mediated responses

Strength of response

time

wildtype

aux1

B

Establishment of SAR

4 hpi

21 hpi

Pathogen challenge (avr recognition)

JAZ

COI1

JA

Aux/IAA

TIR1

IAA

flavonoids

Camalexin

Indole glucosinolates

GH3

NPR1

Defense

JA signalling

auxin signalling

SA signalling