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An *axr1* suppressor mutation in Arabidopsis that partially restores auxin signaling also reverses defects in jasmonate response

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Abstract: The Arabidopsis mutant *axr1* is defective in both auxin and jasmonic acid responses. A screen for *axr1-24* suppressors yielded *sar1-5*, an allele of previously described mutants that partially correct the auxin response defects in *axr1*. The new allele partially suppresses defects in jasmonate response in *axr1*. The JA concentration required for 50% inhibition of seedling root growth was 5–10 µM for *axr1-24 sar1-5.* This was at least 10-fold less than that required for *axr-24*, but 5- to 10-fold higher than for WT. In the *axr1-24* background *sar1-5* also partially restored resistance to the soil fungus *Pythium irregulare*, a trait known to require jasmonate signaling. The *axr1-24* mutant was equally resistant to JA and JA-Ile, indicating that the defect is not in formation of the JA conjugate that is required for signaling. In contrast to *axr1*, the *tir1-1* mutant that affects the same signaling pathway showed no resistance to jasmonates. Taken together, this evidence argues that the defects in jasmonate response seen in *axr1* are not a secondary result of impaired auxin signaling, but that AXR1 functions directly in jasmonate response. Both AXR1 and SAR1 potentially help to coordinate the diverse activities of these 2 important plant signaling pathways.

Key words: Auxin, jasmonic acid

1. Introduction

Plant hormones are increasingly recognized to function in complex and coordinated ways to regulate growth and development and to facilitate responses to the environment (Kombrink 2012; Wilkinson et al. 2012). Recent discoveries using hormone signaling mutants and their enhancers or suppressors have confirmed that there is considerable interaction or "cross-talk" between response pathways (Gazzarrini and McCourt 2003; Teale et al. 2008; Kazan and Manners 2012).

Among the mechanisms for coordinating hormone signaling appears to be the sharing of some protein components of the signaling pathways. We previously identified a link between auxin and jasmonate signaling through an Arabidopsis mutant showing insensitivity to methyl jasmonate (JA-Me) (Tiryaki and Staswick 2002). This turned out to be an allele of *axr1*, the well-characterized Arabidopsis mutation that confers resistance to auxins and affects a number of auxin-dependent processes. The *axr1* alleles have shorter hypocotyls, reduced apical dominance, decreased root branching, fewer root hairs, and reduced male fertility, and they are defective in regulation of several auxin-responsive genes (Lincoln et al. 1990; Timpte et al. 1995; Cernac et al. 1997). Root growth in the *axr1-24* allele that we isolated displayed a level of auxin resistance similar to that of the *axr1-3* allele and was also less inhibited by jasmonate (Tiryaki and Staswick 2002). Gene induction by JA-Me was also delayed in *axr1-24* and resistance to the opportunistic soil fungus *Pythium irregulare*, a jasmonate-dependent response, was reduced in both *axr1-24* and *axr1-3*. Together, these results indicated that *AXR1* plays an important role in responses involving jasmonate signaling.

AXR1 encodes an E1-like protein that is related to the first enzyme in the ubiquitin conjugation pathway (Leyser et al. 1993). Activity of AXR1 ultimately leads to RUB modification of the SCF^{TIR1} ligase, which then ubiquitinates target proteins for degradation via the COP9 signalsome (del Pozo et al. 2002; Kelley and Estelle 2012). Targeted proteins include the Aux/IAA transcription factors, whose elimination activates genes involved in auxin response (Gray et al. 2001; Tiwari et al. 2001; Zenser et al. 2001). TIR1 was recently shown to be a receptor and upon auxin binding leads directly to Aux/IAA-SCF^{TIR1} interaction (Dharmasiri et al. 2005; Kepinski and Leyser 2005). The auxin acts as a 'molecular glue' between TIR1 and its substrate, binding both proteins and facilitating hydrophobic packing between TIR1 and

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its substrate, presumably until ubiquitinated protein is released (Mockaitis and Estelle 2008). TIR1 is therefore an auxin receptor, and unlike most of the F-box substrate interactions, this F-box protein can be directly modified by auxin (Kepinski 2007, 2009; Vanneste and Friml 2009). A crucial insight into how auxin regulates the interaction between TIR1 and its Aux/IAA substrates was recently determined using the crystal structure of TIR1 that binds to ASK1 of the SCF^{TIR1} complex (Tan et al. 2007). However, *tir1* mutants exhibit only weak auxin response defects, suggesting the presence of additional auxin receptors.

Although direct evidence for the biochemical role of AXR1 in jasmonate signaling is still lacking, a reasonable assumption is that it is analogous to its function in auxin response. Jasmonate signaling involves a similar pathway that includes COI1, an F-box protein that is homologous to TIR1 and required for jasmonate signaling (Turner et al. 2002; Xie et al. 1998). Recent findings indicate that SCF^{COI1} targets members of the jasmonate ZIM domain (JAZ) protein family for ubiquitin-mediated degradation (Chini et al. 2007; Thines et al. 2007).

Suppressors of gene mutations are useful tools for identifying new signaling components that may have functions that were missed by direct hormone screening (McCourt 1999; Browse 2009). A screen for new mutations in the *axr1-3* background that led to a reduced resistance to 2,4-D identified a second site suppressor loci called *SAR* (*suppressor of auxin resistance*). The recessive mutant *sar1-1* partially suppresses most effects of the *axr1* phenotype including plant morphology, root growth, root hair formation, and auxin-induced gene expression (Cernac et al. 1997). Genetic analysis indicated that *SAR1* acts in the same or overlapping pathway with 2 other genes for auxin response, *TIR1*, and *AXR4* (Gray and Estelle 2000). Molecular characterization of *sar1* and *sar3* genes revealed that they encode proteins with similarity to vertebrate nucleoporins, subunits of the nuclear pore complex (Parry et al. 2006). Both *sar1* and *sar3* mutations affect the localization of the transcriptional repressor *AXR3/INDOLE ACETIC ACID17*, providing a likely explanation for suppression of the phenotype conferred by *axr1*. Furthermore, *sar1 sar3* double mutant plants accumulate polyadenylated RNA within the nucleus, indicating that *SAR1* and *SAR3* are required for mRNA export (Parry et al. 2006).

We initiated a screen for suppressors of *axr1-24* before we had determined that this mutation was allelic to the previously characterized *axr1* mutants. Rather than screening for restored sensitivity to auxin, we isolated suppressors in the absence of exogenous auxin on the basis of a plant phenotype that was closer to the wild type. In theory, this has the potential to identify genes that might not be discovered when screening under higher concentrations of hormones than a plant normally experiences (McCourt 1999). We describe here the characterization of one suppressor of *axr1*. Although allelic to *sar1*, this locus had not been previously examined for its effect on jasmonate response in the *axr1* background.

2. Materials and methods

2.1. Plant materials and growth conditions

The *axr1-24* mutant was previously described (Tiryaki and Staswick 2002). Auxin response mutants *tir1-1* and *axr2* were obtained from the Arabidopsis Biological Resource Center. *axr1-12 sar1-1* and *axr1-12 sar3-1* were kindly provided by M Estelle. Seeds were sown in Redi Earth (W.R. Grace, Cambridge, MA, USA) in either 8 × 8 × 8-cm plastic pots or plastic trays. Plants were grown at 21 °C under continuous fluorescent illumination (~100 µE m^{-2} s^{-1}). Seedlings for root inhibition assays were grown on agar media containing the inhibitors indicated using surface-sterilized seeds as previously described (Staswick et al. 2002). Plates containing seeds were incubated at 4 °C for 4 days, then placed vertically in an incubator at 23 °C and grown under 12-h fluorescent light/12-h dark cycles for the times indicated. Root lengths for inhibitor treatments are expressed as percent inhibition relative to the untreated control for each genotype. Confidence intervals (95%) were calculated using the delta method.

2.2. Mutagenesis, mutant screening, and genetic analysis

Approximately 36,000 homozygous *axr1-24* seeds were mutagenized in 50 mL of 0.3% (v/v) ethyl-methanesulfonate (EMS) (Sigma) for 24 h at room temperature. Seeds were washed 15 times with water over a 6-h period to remove residual EMS. The mutagenized seeds (M_1) were sown into trays and grown in a clean growth chamber to avoid contamination by stray seeds, under the same growth condition described before. M_1 plants were separated into 24 parental groups of approximately 1500 individuals each and M_2 seeds resulting from self-fertilization, and were harvested as independent mutant pools. To screen for suppressors of *axr1-24*, about 1000 seeds from each M_2 pool were sown in trays as described above and placed in a greenhouse at 25 °C. Candidate suppressors were identified as wild type-looking plants with larger leaves than *axr1-24*. M_3 seeds were harvested from these and tested on MS basal salt agar medium containing 50 µM JA-Me as described before.

To determine if the putative 16-1 suppressor mutation was intragenic or extragenic, the homozygous line was crossed to the wild type (L*er*). F_2 seeds were tested on 50 µM JA-Me plates for the segregation ratio of jasmonate resistant to sensitive roots. To further clarify the nature of the mutation, the double homozygous mutant (*axr1-24 sar1-5*) was backcrossed to *axr1-24* homozygotes and F_1 plants were analyzed.

2.3. Fungal inoculation

Pythium irregulare was grown and inoculated into soil containing 5-week-old individual seedlings of the wild type (Columbia) and mutants (*axr1-24* and *16-1*) with the same inoculation technique as described previously (Tiryaki and Staswick 2002). Each pot contained 9 seedlings and 9 pots were used for each genotype. After inoculation, plants were returned to the growth chamber and monitored daily for symptoms of loss of turgor and tissue collapse.

2.4. Quantitation of jasmonates

Jasmonates were quantified essentially as described previously (Staswick and Tiryaki 2004), with modifications as indicated. Aerial tissue from 3-week-old seedlings grown in soil was harvested and extracted in 80% methanol. (D_6)(+/-)-JA (generously provided by O Miersch and C Wasternack), dihydroJA-ACC, and ($^{13}C_6$)-JA-Ile were added as internal standards. The JA-Ile standard was synthesized as described earlier using ($^{13}C_6$)-L-isoleucine obtained from Cambridge Isotope Laboratories (Andover, MA, USA). GC/MS analysis was done on a Finnigan Trace GC with DSQ mass spectrometer using negative chemical ionization with methane reagent gas.

3. Results

3.1. *axr1-24* is not affected in jasmonate accumulation

We recently determined that the Arabidopsis *jar1* mutant is insensitive to JA because it fails to conjugate JA to Ile, a requirement for jasmonate-mediated inhibition of root growth (Staswick and Tiryaki 2004). To test whether the *axr1-24* phenotype in jasmonate response was related to a defect in JA-Ile accumulation, we examined seedling root growth in the presence of this conjugate. The mutant showed strong resistance to JA-Ile compared with the wild type. The concentration necessary for 50% inhibition was around 100-fold higher for *axr1-24* than for WT (Figure 1).

To further examine whether the *axr1* phenotype is related to altered JA biosynthesis or metabolism, we quantified the levels of JA, JA-Ile, JA-Leu, and the JA conjugate with the ethylene precursor 1-aminocyclopropane carboxylic acid (JA-ACC). There was no significant difference between the wild type and *axr1-24* for any of these jasmonates (Table). These results indicate that the defects in jasmonate response in *axr1-24* are not due to altered jasmonate levels, supporting the idea that *axr1* acts downstream of jasmonate metabolism.

We also examined whether TIR1, the F-box protein involved in auxin response, has a role in jasmonate signaling as well. Although root growth of *tir1* appeared to be marginally more resistant than the wild type in this experiment (Figure 1), this result was not seen in 3 other experiments using JA as the inhibitor. This suggests that for root inhibition by jasmonate, TIR1 does not substitute appreciably for COI1 in the SCF complex. *axr2*, another auxin-resistant mutant that affects auxin transport (Timpte et al. 1995; Bennett et al. 1996), was also fully sensitive to JA-Ile (Figure 1) and JA (data not shown). These results demonstrate that resistance does not necessarily confer jasmonate insensitivity.

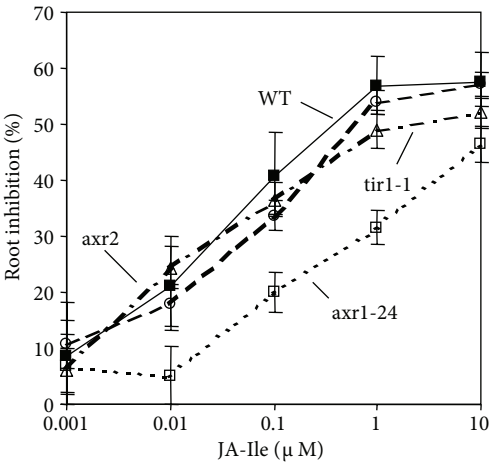

Figure 1. Inhibition of seedling root growth by JA-Ile. Seedlings were grown in agar media for 6 days at the indicated JA-Ile concentrations. Inhibition is expressed as a % of each genotype grown in the absence of inhibitor. Error bars indicate 95% confidence intervals for each ratio of the means (n = 25 primary roots per data point).

3.2. Isolation of *axr1-24* suppressors

Putative suppressors of *axr1-24* were identified among approximately 240,000 M_2 seeds representing 24 parental groups of about 1500 mutagenized *axr1-24* M_1 plants each. The screen was based on restoration of the visible wild phenotype, including increased plant stature, enhanced male fertility, and leaf size and shape that was more similar to the wild type. Seven M_2 plants showing partial reversion to the wild type were identified and progeny derived by selfing each of these plants retained the suppressed *axr1-*

Table. Quantitation of jasmonates from 3-week-old wild-type and *axr1-24* seedlings.

Genotype	WT	*axr1-24*
	(pmol g^{-1} FW)	
JA	36 ± 9	45 ± 8
JA-Ile	48 ± 10	59 ± 17
JA-Leu	11 ± 6	11 ± 6
JA-ACC	57 ± 2	66 ± 37

The values represent the mean ± SE for 3 extractions of independent tissue samples.

24 phenotype. To assess whether the putative suppressors restored sensitivity to JA-Me, M_3 seed from each was grown in the presence of 50 µM JA-Me. None were inhibited as strongly as the wild type, but several appeared less resistant to JA-Me than *axr1-24* (Figure 2).

3.3. Genetic characterization of an *axr1-24* suppressor

One putative suppressor of jasmonate response defects in *axr1-24*, line16-1, was chosen for further analysis. It showed no evidence of segregation for the suppressed phenotype in the M_2 or M_3 generations, suggesting homozygosity. Compared with *axr1-24*, line16-1 had increased plant height and increased leaf and seed pod size, although these were intermediate between that of *axr1-24* and the wild type (data not shown). Wild-type Landsberg *erecta* (L*er*) was crossed to line16-1 and the F_2 segregation ratio of root growth that was sensitive versus resistant to 50 µM JA-Me was recorded. If the suppressor was a recessive mutation and unlinked to *axr1*, then 3 of 16 F_2 progeny would be expected to have the resistant (*axr1*) phenotype. Analysis of 265 F_2 seedling roots resulted in 222 sensitive and 43 resistant. This fits a segregation ratio of 13:3 (chi-square = 1.1, P = 0.29) and suggested that the phenotype was due to a recessive extragenic suppressor mutation, not a reversion of *axr1-24* to the wild type. To verify this result, line16-1 was backcrossed to *axr1-24*. Visual analysis of 19 F_1 plants showed that all had the *axr1-24* phenotype. The F_2 generation from this cross segregated 40 sensitive to 152 resistant, which fits a 3:1 segregation ratio (chi-square = 1.9, P = 0.18). This confirmed that the suppressor phenotype was due to a monogenic recessive mutation at a locus distinct from *axr1-24*.

The observed phenotype of 16-1 was similar to the previously characterized *axr1* suppressor *sar1-1* (Cernac et al. 1997). To assess whether these were the same genes, the locus was mapped. From the cross of the wild type (L*er*) by 16-1 (ecotype Col-0), we isolated an F_3 family that was homozygous for *axr1-24* and segregating for the suppressor locus. Bulk segregate analysis of 172 JA-Me–sensitive F_3 individuals indicated linkage to chromosome 1 marker *nga280*, but not to other markers tested. This agreed with the previously determined position of *axr1* suppressor *sar1-1* on chromosome 1 (Cernac et al. 1997). To determine whether the suppressor was allelic to *sar1-1*, homozygous 16-1 plants were crossed to *axr1-12 sar1-1*. The F_1 cross showed no evidence of complementation, supporting the idea that the *axr1-24* suppressor we isolated is allelic to *sar1-1*. In contrast, the cross with *axr1-12 sar3-1* showed complementation, producing only plants with the *axr1-24* phenotype in F_1. Together, these data establish that we isolated a new *sar1* allele, hereafter called *sar1-5*.

3.4. The defect in JA response is partially corrected by *sar1-5*

In order to obtain more detailed insight into the effect of *sar1* on jasmonate signaling, the dose response of *axr1-24 sar1-5* root growth in the presence of JA was tested. Fifty percent inhibition occurred at around 10^{-5} M, which was roughly 10-fold lower than the concentration required for *axr1-24* (Figure 3). The previously described suppressor *sar1-1* in the strongly auxin-resistant *axr1-12* background was also evaluated for comparison. It had a similar level of inhibition as seen in *axr1-24 sar1-5* (Figure 3). *axr1-24 sar1-5* was also examined for its ability to restore sensitivity to IAA in root elongation. IAA inhibited root growth in *axr1-24 sar1-5* to a level intermediate between that of the wild type and *axr1-24*, although only in the range of 10^{-7} to 10^{-6} M (Figure 4).

3.5. Fungal infection in axr1 is delayed by *sar1-5*

We previously showed that *axr1* was susceptible to *P. irregulare*, demonstrating that *AXR1* is important for disease resistance (Tiryaki and Staswick 2002). To determine whether or not *sar1-5* suppresses the

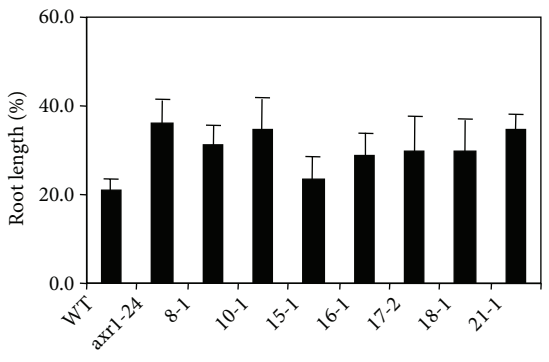

Figure 2. Root length of putative *axr1* suppressors. Seedlings were grown 4 days in the presence of 50 µM JA-Me. Root length is expressed as % of untreated for each genotype and error bars indicate 95% confidence intervals for each ratio of the means (n = 20 primary roots).

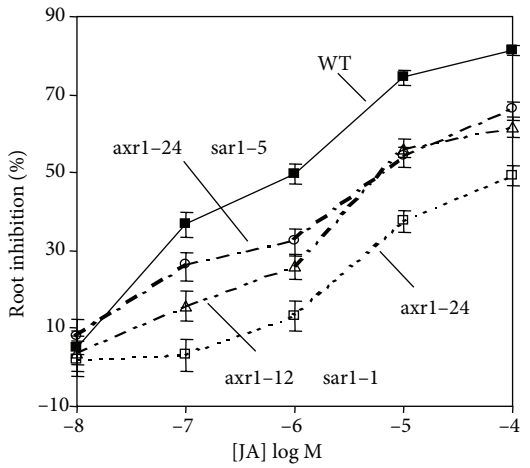

Figure 3. Effect of *sar1* on root inhibition by JA. Conditions were the same as for Figure 1 (n = 20 primary roots per data point).

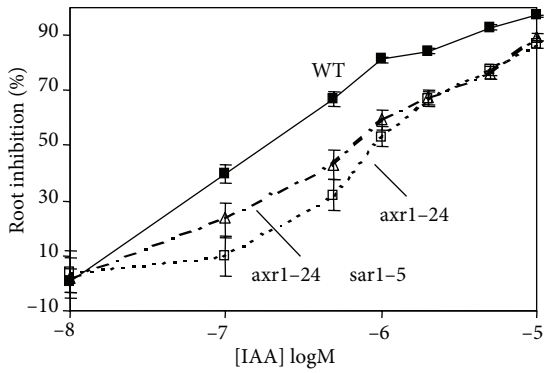

Figure 4. Suppression of *axr1* resistance to IAA by *sar1*. Conditions were the same as for Figure 1 (n = 20 primary roots per data point).

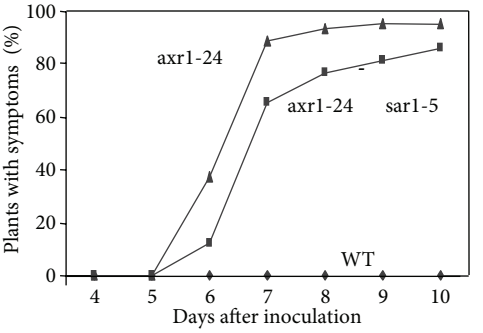

Figure 5. Sensitivity of *axr1-24 sar1-5* to fungal infection. Plants were monitored for disease symptoms at each of the days indicated after inoculation with *P. irregulare*. 81 plants were analyzed for each genotype.

susceptibility of *axr1* to *P. irregulare*, 5-week-old seedlings were inoculated at the root zone with an isolate of *P. irregulare*. The wild type was resistant, whereas the fungus caused wilting and tissue collapse in *axr1-24* (Figure 5), as observed earlier (Staswick et al. 1998). Although *axr1-24 sar1-5* was not resistant like the wild type, development of symptoms was delayed compared with *axr1-24*. About 90% of *axr1-24* plants displayed symptoms 7 days after inoculation, while only about 70% of *axr1-24 sar1-5* plants were symptomatic at the same time. Even after 10 days, less than 90% of *axr1-24 sar1-5* showed symptoms. This result is consistent with the partial restoration of jasmonate response seen in sensitivity to root inhibition.

4. Discussion

Earlier characterization of *axr1* suggested the possibility of a direct connection between the auxin and jasmonate signaling pathways through the sharing of the E1 function of AXR1 (Tiryaki and Staswick 2002). We have further explored the relationship between these pathways by isolating and characterizing a suppressor of *axr1* called *sar1-5*. *SAR1* was previously evaluated only in the context of auxin response, so it was possible that its function was specific to auxin activity.

Our results demonstrate that this is not the case. The *sar1-5* allele in the *axr1-24* background increased sensitivity of seedling root growth to jasmonates and increased resistance to infection by *Pythium irregulare*. Previous analyses have shown that these 2 assays are effective indicators of jasmonate signaling in Arabidopsis (Feys et al. 1994; Staswick et al. 1998; Vijayan et al. 1998). The requirement for jasmonate signaling in male fertility also might indicate that the partial infertility of *axr1* is due to a defect in jasmonate rather than auxin signaling (Tiryaki and Staswick 2002). If so, the increased fertility in *axr1 sar1* mutants (Cernac et al. 1997) would also be consistent with a jasmonate-signaling role for *SAR1*. Therefore, we conclude that along with *AXR1*, *SAR1* plays an important role in jasmonate signaling.

It was possible that jasmonate insensitivity in *axr1* was indirectly caused by impaired auxin response, rather than directly the result of a defect in jasmonate signaling. However, our results strengthen the case that *AXR1* is indeed functional in jasmonate signaling. Two other auxin insensitive mutants, *tir1* and *axr2*, showed no evidence of resistance to jasmonate. Because TIR1 acts in the same auxin response pathway as AXR1 (Gray and Estelle 2000), this argues that AXR1 is directly involved in jasmonate signaling. The fact that jasmonate-insensitive *coi1* mutants have a strong phenotype that is similar to that of JA null mutants also indicates that TIR1 does not appreciably complement COI1 for jasmonate response (Berger 2002).

We recently demonstrated that a defect in JA metabolism impairs jasmonate signaling in Arabidopsis. Specifically, conversion of JA to the amide conjugate with isoleucine is required for root inhibition (Staswick and Tiryaki 2004). In view of the role of AXR1 in protein degradation, it was therefore reasonable to test whether the jasmonate-associated defects in *axr1* could result from elimination of proteins required to synthesize or regulate the level of jasmonate signal, rather than downstream signaling components. However, *axr1* showed a similar level of resistance to both JA and JA-Ile (compare Figures 1 and 3), suggesting that accumulation of this conjugate does not limit jasmonate response. There was also no significant difference in the level of JA or JA conjugates between the wild type and *axr1-24*. This evidence suggests it is unlikely that AXR1 acts by regulating the level of the jasmonates.

Although *sar1-5* only partially suppresses the effects of *axr1-24* in jasmonate response, this is consistent with the results for other *sar1* alleles in auxin response. Of the 4 alleles previously identified, *sar1-1* and *sar1-2* were the strongest (Cernac et al. 1997). *sar1-1* suppresses most aspects of the *axr1* phenotype, including effects on seedling and leaf morphology, cell length, sensitivity to

auxin, and auxin-inducible gene expression (Cernac et al. 1997). However, for most of these, plants are not fully restored to the wild type either in *axr1-12* or in the milder locus *axr1-3*. As previously documented for *sar1-1* with the synthetic auxin 2,4-D (Cernac et al. 1997), we found that *sar1-5* also partially restores sensitivity to the natural auxin IAA in *axr1-24*.

In addition to *AXR1* and *SAR1*, recent evidence indicates that other genes are also involved in both auxin and jasmonate response. Suppression of *AtRBX1*, encoding an essential component of the SCF-type E3 ubiquitin ligase, reduced response to both auxin and jasmonic acid (Schwechheimer et al. 2002). On the other hand, the *eta3* mutant that strongly enhances the negative effects of *tir1* in auxin response also confers modest insensitivity to JA-Me (Gray et al. 2003). The fact that jasmonate signaling is less affected in *axr1* compared with other mutants specifically impaired in jasmonate function (e.g., *jar1* and *coi1*) suggests that there may be redundancy for *AXR1* function in jasmonate response (Tiryaki and Staswick 2002). One possible candidate is *AXL1*, which partially complements *axr1* in auxin response (del Pozo et al. 2002), but its role in jasmonate function has not been reported. Thus, there may be several additional linkages in auxin and jasmonate signaling pathways.

In summary, we have documented that *SAR1* functions in jasmonate as well as auxin signaling. While a major function of auxin is in the control of growth and development, jasmonates have a major role in protective responses to both biotic and abiotic assaults. SAR1 as well as AXR1 function in both jasmonate and auxin response as further common signaling intermediates used to accomplish diverse tasks in plants. *AXR1* mutants also show some resistance to other plant hormones including ABA, epi-brassinolide, and the ethylene precursor ACC (Tiryaki and Staswick 2002). It will be interesting to determine whether *SAR1* functions in these response pathways and how these interactions coordinate these diverse plant signals.

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