

# Mutations in an Auxin Receptor Homolog AFB5 and in SGT1b Confer Resistance to Synthetic Picolinate Auxins and Not to 2,4-Dichlorophenoxyacetic Acid or Indole-3-Acetic Acid in Arabidopsis<sup>[W]</sup>

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Although a wide range of structurally diverse small molecules can act as auxins, it is unclear whether all of these compounds act via the same mechanisms that have been characterized for 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA). To address this question, we used a novel member of the picolinate class of synthetic auxins that is structurally distinct from 2,4-D to screen for *Arabidopsis* (*Arabidopsis thaliana*) mutants that show chemically selective auxin resistance. We identified seven alleles at two distinct genetic loci that conferred significant resistance to picolinate auxins such as picloram, yet had minimal cross-resistance to 2,4-D or IAA. Double mutants had the same level and selectivity of resistance as single mutants. The sites of the mutations were identified by positional mapping as At4g11260 and At5g49980. At5g49980 is previously uncharacterized and encodes auxin signaling F-box protein 5, one of five homologs of TIR1 in the *Arabidopsis* genome. TIR1 is the recognition component of the Skp1-cullin-F-box complex associated with the ubiquitin-proteasome pathway involved in auxin signaling and has recently been shown to be a receptor for IAA and 2,4-D. At4g11260 encodes the tetratricopeptide protein SGT1b that has also been associated with Skp1-cullin-F-box-mediated ubiquitination in auxin signaling and other pathways. Complementation of mutant lines with their corresponding wild-type genes restored picolinate auxin sensitivity. These results show that chemical specificity in auxin signaling can be conferred by upstream components of the auxin response pathway. They also demonstrate the utility of genetic screens using structurally diverse chemistries to uncover novel pathway components.

The small molecule plant hormone auxin (indole-3-acetic acid [IAA]) regulates and influences many aspects of the growth and development of plants. Treatment of plants with exogenous IAA can result in a variety of physiological and morphological effects, including loss of apical dominance, effects on cell elongation and division, and adventitious root formation (Davies, 1995). A wide range of chemically diverse synthetic small molecules can also produce effects similar to treatment with the natural hormone (Woodward and Bartel, 2005). Some of these compounds have found use as herbicides, the most well known being 2,4-dichlorophenoxyacetic acid (2,4-D; Sterling and Hall, 1997). 2,4-D has proven to be a useful chemical probe of auxin action because it is a potent and stable xenobiotic compound that is not subject to the many

endogenous homeostatic and metabolic mechanisms that can affect IAA (Ljung et al., 2002). Screens for *Arabidopsis* (*Arabidopsis thaliana*) mutants that are resistant to the action of 2,4-D have defined several genetic loci, including *AXR1*, *AXR2*, *AXR3*, *AXR5*, and *AXR6* (Leyser et al., 1993, 1996; Nagpal et al., 2000; Hellmann et al., 2003; Yang et al., 2004), and mutations in these loci are cross-resistant to IAA, demonstrating the functional similarity of the two auxins. An additional locus, *TIR1*, has been identified from a screen using an auxin transport inhibitor and mutations at this locus were also found to confer resistance to both 2,4-D and IAA (Ruegger et al., 1998).

Investigation of these and other mutants has led to the understanding that auxin signal transduction involves the ubiquitin-proteasome pathway (Leyser, 2002; Dharmasiri and Estelle, 2004). IAA and 2,4-D stimulate the ubiquitination and degradation of the Aux/IAA family of transcriptional regulators that includes *AXR2* (IAA7), *AXR3* (IAA17), and *AXR5* (IAA1). These proteins interact with DNA-binding auxin response factor transcription factors to regulate auxin-responsive genes (Guilfoyle and Hagen, 2001; Liscum and Reed, 2002). Their degradation results in derepression of auxin-regulated genes that in turn leads to the physiological and morphological events associated with auxin action. Specific ubiquitination of the proteins

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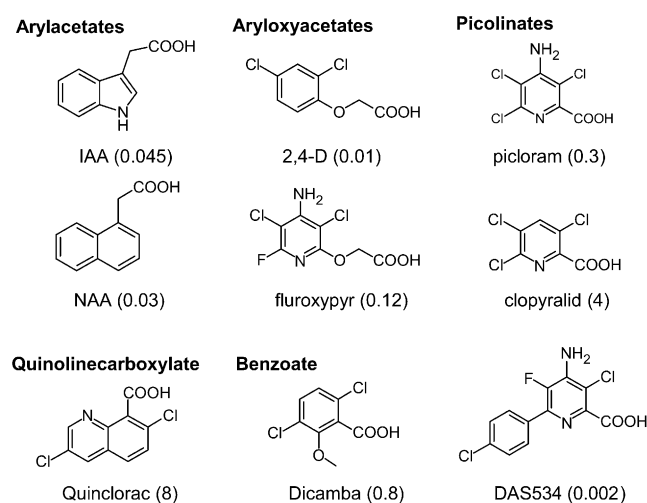
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targeted for degradation is accomplished by a Skp1-cullin-F-box protein (SCF) ubiquitin E3 ligase complex (Gray et al., 2001; Risseuw et al., 2003). The SCF components implicated in the Arabidopsis auxin response have been identified as ASK1, AXR6, CUL1, and TIR1, respectively (Gray et al., 1999). The cullin portion of the complex requires an additional subunit, the RING-finger protein RBX1 (Gray et al., 2002), and modification by the ubiquitin-related protein, RUB1, to be fully functional (del Pozo and Estelle, 1999). A variety of proteins responsible for the conjugation and deconjugation of RUB1 have been identified as affecting auxin response and these include AXR1 (Leyser et al., 1993; del Pozo et al., 2002). The functional SCF complex recruits an E2 ubiquitin ligase via the cullin subunit to ultimately catalyze the ubiquitination reaction of the target protein.

Proteins targeted for ubiquitination are recruited to the SCF complex via interaction with the F-box protein and this subunit is thought to confer a significant level of specificity to the action of the SCF complex (Leyser, 2002; Dharmasiri and Estelle, 2004). The first F-box protein implicated in 2,4-D and IAA-mediated SCF function was TIR1, a member of the Leu-rich repeat (LRR) subclass of the large F-box protein family in Arabidopsis (Ruegger et al., 1998; Gagne et al., 2002). The SCF<sup>TIR1</sup> complex has been shown to interact with target Aux/IAA proteins in response to 2,4-D and IAA treatment in both in vivo and in vitro systems (Gray et al., 2001; Dharmasiri et al., 2003; Kepinski and Leyser, 2004), thus directly confirming the important biochemical role of this complex in the action of these auxinic compounds. Most significantly, TIR1 has recently been shown to act directly as an auxin receptor because it can bind and functionally respond to IAA and 2,4-D (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Three close homologs of TIR1, called auxin signaling F-box protein 1 (AFB1), AFB2, and AFB3, have also been shown to participate in the auxin response (Dharmasiri et al., 2005b). Mutants that are deficient in all four of these proteins are auxin insensitive and exhibit severe developmental and morphological phenotypes. Thus, these four homologs are thought to collectively modulate the plant response to IAA and 2,4-D (Dharmasiri et al., 2005b).

These discoveries have clearly demonstrated the utility of 2,4-D as a chemical surrogate for IAA to dissect the auxin response. 2,4-D has been in commercial use as an herbicide for more than 50 years and is still a staple of many effective herbicide treatments. Further herbicide research has led to the discovery and development of at least 19 commercial herbicides (<http://www.plantprotection.org/HRAC/moa2001.htm>) and considerably more experimental compounds that act via the auxin mode of action. These comprise at least four structurally distinct classes that include aryloxyacetates, such as 2,4-D, picolinates, benzoates, and quinoline carboxylates, examples of which are shown in Figure 1. These herbicidal auxins exhibit a broad range of potencies and differential selectivities to



**Figure 1.** Chemical structures of IAA, several commercial auxinic herbicides, and the experimental herbicide DAS534. The concentrations of herbicide ( $\mu\text{M}$ ) required to produce a growth reduction of Arabidopsis roots by 50% relative to untreated controls ( $\text{GR}_{50}$ ) in our assay system are shown in parentheses.

various plant species (Sterling and Hall, 1997). Despite their structural differences, many studies have shown that these synthetic auxins induce similar morphological, physiological, and molecular events (Chang and Foy, 1983; Boerjan et al., 1992; Grossmann et al., 1996; Dargeviciute et al., 1998; Pufky et al., 2003). However, it is presently unclear whether these structurally different auxinic compounds act via the same mechanisms and signal transduction components that have been characterized for 2,4-D and IAA.

The diversity of synthetic auxins provides a useful suite of compounds to apply a chemical genetic approach to dissecting auxin responses (Blackwell and Zhao, 2003; Zhao et al., 2003; Armstrong et al., 2004). We have screened for Arabidopsis mutations that confer differential resistance to 2,4-D and picolinate auxins using a novel picolinate auxin as an initial probe. We reasoned that if IAA, 2,4-D, and picolinate auxinic compounds all promote common downstream events, as suggested by the similar physiological and morphological responses to the compounds (Chang and Foy, 1983; Boerjan et al., 1992; Grossmann et al., 1996; Dargeviciute et al., 1998; Pufky et al., 2003), then identification of mutants that are selectively resistant to picolinate chemistries could preferentially identify mutations involved in upstream events close to or at the site of chemical interaction. Using this strategy, we have succeeded in isolating and characterizing several mutant alleles at two genetic loci that confer selective resistance to the picolinate class of herbicidal auxins. Our results indicate that picolinate auxins appear to act via the SCF ubiquitination mechanism (like 2,4-D and IAA), but some different upstream components are involved in their action. Significant chemical selectivity therefore resides within these components. These studies also provide chemical genetic tools to

differentiate and dissect events in the auxin pathway and to distinguish the action of at least two classes of synthetic auxins.

## RESULTS

### Structure and Potency of a Novel Picolinate Auxin

One of the symptoms of treatment of Arabidopsis seedlings with natural or synthetic auxins is inhibition of root growth (Estelle and Somerville, 1987). This provides a convenient and quantitative measurement of auxin potency. In our assay system, seedlings were germinated and grown on auxin-containing medium and so were continuously exposed to auxin. The concentrations producing 50% reduction in Arabidopsis root growth ( $GR_{50}$ ) for a variety of synthetic auxins and for the natural auxin IAA were determined using this method. We found that a novel picolinate auxin, 4-amino-3-chloro-6-(4-chlorophenyl)-5-fluoro-pyridine-2-carboxylic acid (DAS534), had a  $GR_{50}$  of 2 nM, which was 5-fold lower than that of 2,4-D and 22-fold lower than that of IAA (Fig. 1). DAS534 was also more potent on Arabidopsis than several hundred synthetic auxin analogs from the Dow AgroSciences chemical files (data not shown). DAS534 contains a 6-phenyl ring substitution on the picoline ring (Fig. 1) and is a member of a series of 6-aryl-4-aminopicolinate auxinic herbicides (Balko et al., 2004). Because of the significant differences in both structure and potency of DAS534 relative to 2,4-D and IAA, we undertook a chemical genetic screen to assess whether we could recover mutants that were selectively resistant to DAS534 and not resistant to 2,4-D and IAA.

### Identification and Genetic Characterization of Mutants Resistant to DAS534

DAS534-resistant plants were isolated by screening ethyl methanesulfonate (EMS)-mutagenized  $M_2$  Columbia-0 (Col-0) seedlings growing on agarose media containing 10 nM DAS534. This concentration was sufficient to produce marked auxinic effects on the seedlings, including significant inhibition of root growth. From a total of 780,100 EMS  $M_2$  seedlings, 125 putative resistant mutants were identified by visual inspection of root length. Seeds were recovered from 33 of these plants and the phenotype confirmed in secondary screens. We then selected lines that showed low or no cross-resistance to 0.045  $\mu$ M 2,4-D. Seven lines resistant to DAS534 had negligible resistance to 2,4-D at this concentration and were further evaluated. One additional line (R090) showing robust resistance to both DAS534 and 2,4-D was also retained for comparison.

To determine the inheritance pattern of the mutations and the number of loci represented, the lines were crossed with each other, with wild-type Col-0 plants, and with the known auxin-resistance mutant *axr1-3*. Progeny of the crosses were analyzed for resis-

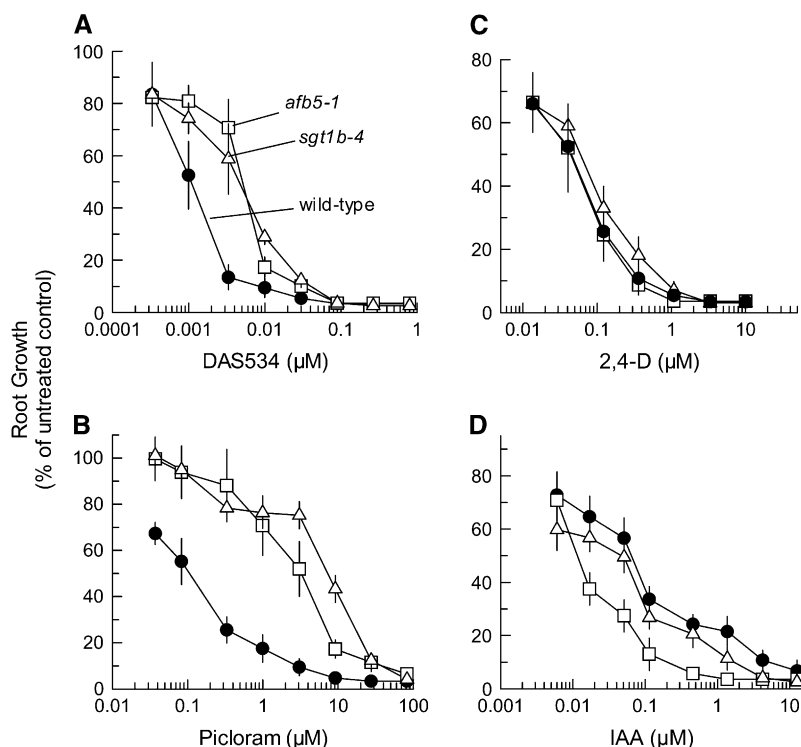
tance to DAS534. All of the mutations were recessively inherited. The mutant R090 showing no auxin selectivity appeared to be allelic with *axr1-3* and was not examined further. The other seven selectively resistant mutant lines fell into two complementation groups. Based on our subsequent identification of the genes in which the mutations occurred, the mutations in one complementation group were named *afb5-1*, *afb5-2*, *afb5-3*, and *afb5-4*. The mutants in the other complementation group were named *sgt1b-4*, *sgt1b-5*, and *sgt1b-6*. All of these mutant lines were phenotypically normal when grown on agarose medium or in soil and had normal fertility. Mutant seedlings exhibited normal gravitropism and had normal morphology under etiolating conditions. The *sgt1b* mutants typically exhibited slightly longer roots (approximately 15%) than wild-type plants when grown on agarose medium lacking herbicide.

### Chemical Selectivity of Resistance

The dose responses of root growth of the mutant lines treated with a variety of auxins were measured to characterize the level and chemical spectrum of resistance in detail. Both *afb5* and *sgt1b* mutants had 6- to 8-fold resistance to DAS534 (Fig. 2A). No significant differences in resistance between lines within each complementation group were noted (data not shown). The mutants were very cross-resistant to the picolinate auxin herbicide picloram (Fig. 2B), showing 26- to 60-fold increases in  $GR_{50}$  over that of wild type. The *sgt1b* mutants were slightly more resistant than the *afb5* mutants. In contrast to the response to DAS534 and picloram, the lines from both complementation groups showed negligible resistance to 2,4-D (Fig. 2C) and a slight increase in sensitivity to IAA (Fig. 2D).

Wild-type, *afb5*, and *sgt1b* seedlings growing on 5 nM DAS534 are shown in Figure 3. At this low concentration of DAS534, wild-type plants have elongated hypocotyls and are unable to fully expand their cotyledons (Fig. 3, A and F), whereas hypocotyl elongation and cotyledon expansion of *afb5-4* and *sgt1b-4* are unaffected by DAS534 (Fig. 3, B, D, G, and I). Four additional herbicidal compounds were also tested on *afb5-4*. *afb5-4* had 50-fold resistance to the picolinate auxinic herbicide clopyralid. However, it showed no resistance to the benzoate auxin dicamba or to a close analog of the aryloxyacetate auxin, fluroxypyr, or to 1-naphthylacetic acid. It also exhibited no difference in response to the auxin transport inhibitor, naphthylphthalamic acid, compared to wild type (data not shown).

A foliar spray application of picloram at 200 g/ha on wild-type Col-0 Arabidopsis plants growing in the greenhouse produced profound morphological effects typical of auxinic herbicides and severely inhibited plant growth, whereas the application had minimal effect on *afb5-1* (Fig. 4). This is in contrast to the effect of 2,4-D, which induces auxinic symptoms and severe growth reduction to a similar extent on both wild-type and mutant plants at 50 g/ha. Thus, the chemical



**Figure 2.** Differential effects of auxins on root growth of mutants from complementation groups 1 and 2. Root lengths of Arabidopsis seedlings were measured 8 d after seeding on plates containing a range of concentrations of the test compounds. Data are plotted as a percentage of the root length of untreated plants in the same experiment. Root lengths ( $\pm$ SD) for untreated controls were 14.9 ( $\pm$ 2.3), 13.9 ( $\pm$ 1.3), and 19.4 ( $\pm$ 1.7) mm for wild-type, *afb5*, and *sgt1b* plants, respectively. The symbols used for wild-type plants, *afb5-1*, and *sgt1b-4* are denoted in A. Treatments are DAS534 (A), picloram (B), 2,4-D (C), and IAA (D). Error bars represent the SD from five individual root measurements.

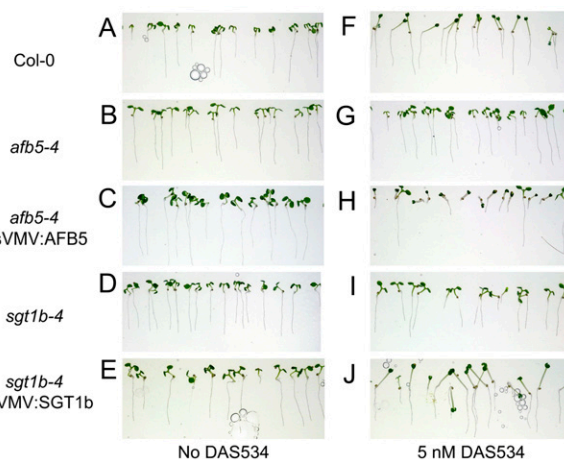
selectivity of resistance seen in seedling root assays is maintained in adult plants with foliar exposure to the auxin herbicides.

### Mapping and Identification of Picolinate Auxin-Resistance Mutations

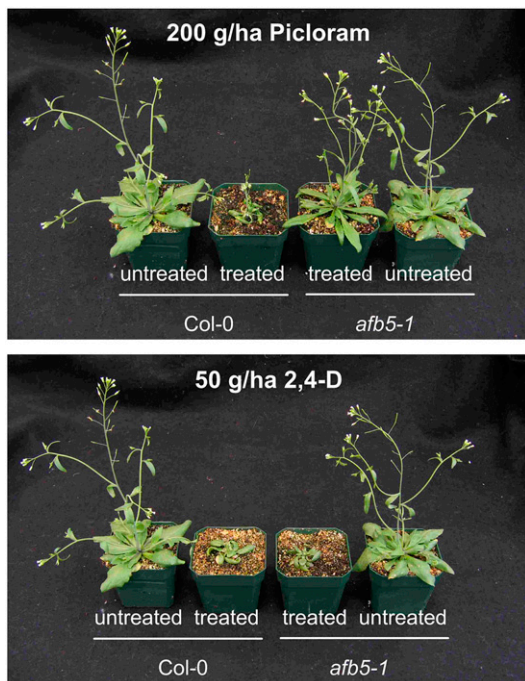
The *afb5* and *sgt1b* mutants displayed clear chemical selectivity in their resistance profiles toward the picolinate class of auxin herbicides. To identify the genes involved in this chemical selectivity, one mutation from each complementation group (*afb5-1* and *sgt1b-4*) was genetically mapped. The mutation in *afb5-1* was mapped to a 200-kb interval at around 105 cM on chromosome 5 (see Supplemental Fig. S1). This interval contains 47 genes, including At5g49980, annotated by The Arabidopsis Information Resource (TAIR) as a homolog of TIR1. TIR1 is an F-box protein involved in 2,4-D and IAA-mediated SCF function and has recently been shown to be a receptor for auxin (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). *tir1* mutants are resistant to 2,4-D and IAA (Ruegger et al., 1998). Because of this possible linkage to auxin function, At5g49980 was PCR amplified from genomic DNA from the *afb5* mutants and Col-0 plants and compared by DNA sequence analysis. All four *afb5* lines contained G-to-A mutations in At5g49980 relative to the Col-0 sequence, resulting in changes in the encoded polypeptide. The mutations in the deduced amino acid sequences from *afb5-2* and *afb5-4* introduce stop codons at amino acid residues 220 and 134, respectively. The mutations in *afb5-1* and *afb5-3* introduce amino acid changes in the

encoded polypeptide sequence, producing an R-to-K mutation at position 609 in *afb5-1* and a C-to-Y mutation at position 451 in *afb5-3* (Fig. 5; see multiple sequence alignment in Supplemental Fig. S2).

There are six members of the F-box protein subclass that includes TIR1 in the Arabidopsis genome (Gagne



**Figure 3.** DAS534-resistant mutants and complementation with *AFB5* and *SGT1b*. A to E, Untreated seedlings. F to J, Seedlings grown in the presence of 5 nM DAS534. The primary visible effects at this sublethal concentration of DAS534 are loss of cotyledon decurvature and increased hypocotyl elongation (F). *afb5-4* and *sgt1b-4* are resistant to this effect (G and I), whereas mutants transformed with the corresponding wild-type gene regain the response (H and J). The *afb5-4* CsVMV:*AFB5* line (H) appears to have an increased response relative to Col-0 (F).



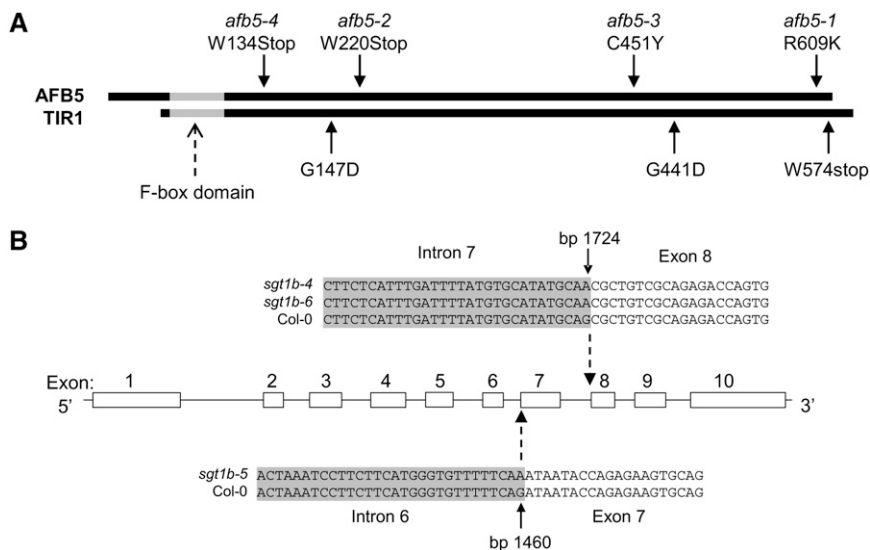
**Figure 4.** Selective resistance of *afb5-1* to foliar application of picloram. Seventeen-day-old Col-0 and *afb5-1* Arabidopsis rosettes growing in the greenhouse were sprayed with picloram at 200 g/ha (top) or 2,4-D at 50 g/ha (bottom). Photographs were taken 12 d after treatment.

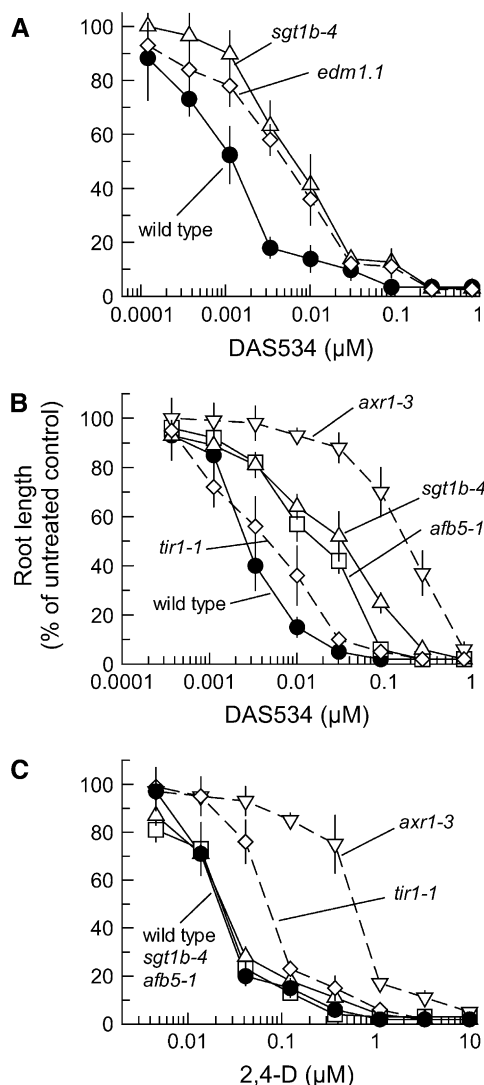
et al., 2002). The three closest homologs of TIR1 have been named AFB1, AFB2, and AFB3, and they have been shown collectively to regulate the response to IAA and 2,4-D (Dharmasiri et al., 2005b). For consistency, we have denoted the two remaining homologs (At4g24390 and At5g49980) as AFB4 and AFB5, respectively. To confirm that the picolinate auxin-resistance phenotype was caused by the identified lesions in AFB5, the auxin-resistance phenotype was comple-

mented by transformation of the *afb5-4* mutant line with a construct containing a wild-type copy of AFB5 driven by the cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1998). Ten independent transformants were identified that restored auxin sensitivity to the mutant (Fig. 3H). Some of these transformants appeared to be hypersensitive to DAS534 because their roots were significantly shorter than those of wild-type plants when grown on herbicide-containing media. These data confirm that the mutation in AFB5 is responsible for the resistance phenotype of this complementation group. Because the mutants are resistant to picolinate auxins, and not to 2,4-D, and all contain lesions in the Leu-rich F-box protein AFB5, this protein appears to be specifically involved in mediating the effects of picolinate auxins.

The mutation in *sgt1b-4* was mapped to a 100-kb interval on chromosome 4 at around 36 cM (see Supplemental Fig. S1). This interval contains 17 genes, including At4g11260, which encodes the tetratricopeptide repeat-containing protein SGT1b. A mutation in SGT1b has been shown to enhance the level of resistance to 2,4-D in the *tir1* mutant background (Gray et al., 2003). The deletion mutant line *edm1* lacks seven genes (At4g11220–At4g11280), including SGT1b (Tor et al., 2002). We found that this deletion mutant had a similar level of resistance to DAS534 as *sgt1b-4* (Fig. 6A). It was also very cross-resistant to picloram (20-fold; data not shown) similar to the *sgt1b* mutants. To determine whether the DAS534-resistant plants in this complementation group contained mutations in SGT1b, At4g11260 was PCR amplified from genomic DNA from all three mutants. Sequence analysis showed that all three mutants contained lesions producing alterations of the predicted 3' intron splice sites of At4g11260. Mutants *sgt1b-4* and *sgt1b-6* contained G-to-A transitions that disrupt the predicted 3' splice site of intron 7, whereas mutant *sgt1b-5* contained a G-to-A transition that disrupts the 3' splice site of intron 6 (Fig. 5B).

**Figure 5.** Mutation sites in AFB proteins and SGT1b that confer resistance to picolinate auxins. A, Relative positions of the mutations conferring auxin resistance in the polypeptide sequences of AFB5 (this work) and TIR1 (Ruegger et al., 1998; Alonso et al., 2003) were determined by alignment using Clustal within the Vector NTI suite (see Supplemental Fig. S2). The F-box domain is denoted in gray. Note that AFB5 contains a 46-residue N-terminal extension relative to TIR1. A similar length N-terminal extension is also found in AFB4, but not in the four AFBs associated with the IAA and 2,4-D response (Dharmasiri et al., 2005b). B, Exons of SGT1b are shown as boxed regions. Sites of mutations occur at the 3' splicing site of intron 6 in *sgt1b-5* and intron 7 in *sgt1b-4* and *sgt1b-6* of the gene. The nucleotide sequences of the 3' intron-exon boundaries are shown. The intron portion is shaded gray and the mutation sites are arrowed.





**Figure 6.** Comparison of the effect of DAS534 and 2,4-D on previously characterized mutants. Measurements were performed as described in Figure 2. Root lengths ( $\pm$ SD) for untreated controls were 21.5 ( $\pm$ 1.8), 21.2 ( $\pm$ 3.2), 24.1 ( $\pm$ 3.1), 20.0 ( $\pm$ 2.1), 25.0 ( $\pm$ 2.1), and 31.5 ( $\pm$ 3.1) mm for Col-0, *afb5-1*, *sgt1b-4*, *edm1*, *tir1-1*, and *axr1-3* plants, respectively. A, Effect of DAS534 on *edm1* and *sgt1b-4*. The deletion mutant line *edm1* lacks seven genes (At4g11220–At4g11280), including *SGT1b* (Tor et al., 2002). Effect of DAS534 (B) and 2,4-D (C) on *axr1-3* and *tir1-1* compared with *afb5-1* and *sgt1b-4*. Symbol labeling is shown in B and is the same for C.

To confirm that the mutations in At4g11260 were responsible for the DAS534-resistant phenotype, the mutant *sgt1b-4* was transformed with a construct containing the wild-type At4g11260 gene driven by the constitutive CsVMV promoter (Verdaguer et al., 1998).  $T_2$  seeds were tested for DAS534 sensitivity. Using the same process as was employed for identification of *AFB5* transformants, 10 independent transformants were identified that were sensitive to DAS534, confirming that the lesions in At4g11260 are responsible for the resistance to DAS534. An example of an *sgt1b-4*

line containing the transgene that restores sensitivity to DAS534 is shown in Figure 3J.

### Comparison with Other Auxin-Resistant Mutants

The mutations we identified in *AFB5* and *SGT1b* conferring picolinate-selective resistance implicate the SCF ubiquitin ligase complex in the molecular mode of action of these herbicides. Several other mutants associated with this complex (e.g. *tir1*, *axr1*) or its ubiquitination targets (e.g. *axr2*) in the auxin response have been characterized and possess varying phenotypes and levels of resistance to 2,4-D or IAA (Lincoln et al., 1990; Ruegger et al., 1998; Nagpal et al., 2000). However, their chemical resistance profile to picolinate auxins has not been previously established so their relative resistance to 2,4-D, DAS534, and picloram was evaluated.

There was no significant chemical selectivity in *axr1-3* because it had a high level of resistance to DAS534 as well as to 2,4-D (Fig. 6, B and C). It was also resistant to picloram (Table I). The relative degree of resistance of *axr1-3* to the picolinate auxins was somewhat higher than that of *afb5* and *sgt1b* mutants, indicating that *axr1* has a greater overall effect on auxinic responses. This is consistent with the general role of AXR1 in the activation of SCF complexes by rubinylation (Schwechheimer et al., 2002). *tir1-1* mutants have significantly lower levels of resistance to 2,4-D than *axr1* (Ruegger et al., 1998; Fig. 6C), consistent with the findings that additional SCF complex components (AFB1–AFB3) are involved in response to this synthetic auxin (Dharmasiri et al., 2005b). We found that the modest level of resistance of *tir1-1* to DAS534 was similar to that of 2,4-D (Fig. 6, B and C; Table I). Thus, the marked chemical selectivity we observed with the *afb5* and *sgt1b* mutants was not apparent with this mutant. The *tir1-1* mutant was slightly more resistant to picloram than DAS534, but this resistance was considerably less than the strong resistance of *afb5* or *sgt1b* mutants to this compound (Table I). The resistance levels of *tir1-1* and *axr1-3* to 2,4-D in our study were similar to those previously described (Estelle and Somerville, 1987; Ruegger et al., 1998), indicating that the results we

**Table I.** Resistance of *afb5* and *sgt1b* and three other auxin-resistant mutants to various herbicidal auxins

Root lengths were measured as described in Figure 2. The fold increase in resistance was calculated by dividing the mutant root GR<sub>50</sub> by the wild-type GR<sub>50</sub>. ND, Not determined.

Mutants	Fold Increase in Resistance over Wild Type			
	DAS534	Picloram	2,4-D	IAA
<i>afb5</i>	5.6	26	1.5	0.4
<i>sgt1b</i>	8.4	60	1.8	0.7
<i>axr1-3</i>	80	160	28	ND
<i>tir1-1</i>	2.0	7.7	2.8	ND
<i>axr2-1</i>	6.7	5.0	7.5	ND

obtained in our assay system are comparable to previous work. AXR2 is one of the short-lived Aux/IAA transcriptional regulators targeted for SCF-mediated ubiquitination and mutations in this locus have been found to confer a dominant 2,4-D resistance phenotype (Wilson et al., 1990; Nagpal et al., 2000). The level of resistance of *axr2-1* to DAS534, picloram, and 2,4-D was similar (Table I), so there was also no pronounced chemical specificity in the synthetic auxin resistance of this mutant.

#### Resistance Phenotype of *afb5 sgt1b* Double Mutants

Mutations in either *AFB5* or *SGT1b* cause similar levels of resistance to DAS534. Plants containing homozygous mutations in both *AFB5* and *SGT1b* were generated to determine whether the level of resistance to DAS534 was similar or increased over that of the single-mutant lines. Three double-mutant lines were compared with the single-mutant parental lines for resistance to DAS534, picloram, and 2,4-D. The single and double mutants exhibited similar levels of resistance (5- to 10-fold; data not shown); thus, the resistance mechanisms in *afb5* and *sgt1b* are not additive. The double mutants also exhibited no obvious deleterious phenotype in growth or fertility, similar to the single mutants. This result suggests that *AFB5* and *SGT1b* are involved in the same response pathway to DAS534.

## DISCUSSION

### Chemically Selective Auxin Resistance

We have used a novel picolinate auxin to uncover mutants in two loci that exhibit chemically selective auxin resistance. Both loci encode components associated with the SCF ubiquitination pathway, one of which is a homolog of the IAA and 2,4-D receptor protein. This pathway has been shown to be the signal transduction mechanism for plant responses to 2,4-D and IAA (Leyser, 2002; Dharmasiri and Estelle, 2004). Although other auxin-resistant mutants such as *rib1*, *aux1*, and *eir1* have been observed to have some chemical specificity, their differential resistance is due to the specificity of pumps and cellular uptake mechanisms (Yamamoto and Yamamoto, 1998; Marchant et al., 1999; Poupart and Waddell, 2000) rather than in components of auxin signal transduction. None of the three examples of mutants within the auxin signal transduction pathway that we tested, *tir1*, *axr1*, and *axr2*, displayed the marked chemical selectivity in responses that we observed with *afb5* and *sgt1b*. Thus, the mutants we have identified appear to be unique in showing differential resistance in the signal transduction pathway. This indicates that there are significant differences in chemical perception of synthetic auxins within upstream components of the pathway. This may contribute to subtle variations in the effects of different synthetic auxins and suggests that they do

not necessarily have equivalent downstream effects. For example, some evidence for differential gene expression in etiolated Arabidopsis seedlings treated with 2,4-D, IAA, and picloram has been found (Pufky et al., 2003).

### AFB Redundancy and Interaction with Auxins

Several studies have indicated that the F-box protein TIR1 acts as the recognition component of the auxin-responsive SCF complex and interacts with certain Aux/IAA proteins to target them for ubiquitination and proteasomal degradation (Gray et al., 2001; Dharmasiri et al., 2003; Kepinski and Leyser, 2004). The five homologs of TIR1 in the Arabidopsis genome share 45% to 87% amino acid identity (see phylogenetic tree in Supplemental Fig. S2). The three proteins that are most closely related to TIR1 (61%–72% amino acid identity) have been named AFB1, AFB2, and AFB3 and appear to have similar and overlapping roles to TIR1 (Dharmasiri et al., 2005b). Combinations of mutations in these four genes show increasingly severe 2,4-D resistance phenotypes such that the quadruple mutant is insensitive to 2,4-D (Dharmasiri et al., 2005b). No characterization of the roles of the more distantly related AFB4 and AFB5 (45% and 46% amino acid identity to TIR1, respectively) has been described, to our knowledge. Our data show that mutations in *AFB5* confer selective resistance to picolinate auxins, especially picloram, while conferring very low or no cross-resistance to 2,4-D and IAA. This suggests that a significant proportion of plant responses to picolinate auxins is mediated via an SCF<sup>AFB5</sup> ubiquitin ligase. This is exemplified by the observation that adult *afb5* plants have normal sensitivity to treatment with herbicidal levels of 2,4-D, but are quite resistant to picloram (Fig. 4). The only remaining AFB without phenotypic characterization is AFB4, a close homolog of AFB5 (80% amino acid sequence identity). Both AFB4 and AFB5 contain an N-terminal extension of 46 residues relative to TIR1 and other AFBs (Fig. 4; alignment in Supplemental Fig. S2). The similarity of AFB4 and AFB5 may indicate that AFB4 could also interact with picolinate auxins.

Recent studies have indicated that IAA and 2,4-D can directly bind to the TIR1 F-box protein of the SCF complex to increase its affinity for the target Aux/IAA proteins (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). AFB1, AFB2, and AFB3 are also implicated in the response to IAA and 2,4-D (Dharmasiri et al., 2005a, 2005b). Our data are consistent with a similar mechanism for picolinate auxins that involves AFB5. The SCF<sup>AFB5</sup> ubiquitin ligase complex may be readily modulated by picolinate auxins and not by 2,4-D. Because the *tir1* mutant we tested showed slight resistance to the picolinate auxins, as well as to 2,4-D (Fig. 5, B and C), SCF<sup>TIR1</sup> may mediate some portion of the response to all of these compounds. This could account for the 7-fold resistance of *tir1-1* to picloram (Table I). The high nonselective level of resistance of

*axr1* to all of the auxins we tested indicates that multiple SCFs are involved in both 2,4-D and picolinate auxin responses, consistent with AXR1 activating a wide range of SCF complexes via rubinylation (Schwechheimer et al., 2002). However, the relative contribution of each SCF complex to the response may vary depending on the auxin chemistry applied.

The *afb5* mutant alleles exhibited a high level of resistance (approximately 30-fold) to picloram, whereas they had relatively less resistance (approximately 6-fold) to the more potent auxin DAS534. One rationale for the increased auxinic potency of DAS534 may be that it interacts strongly with all AFBs, whereas picloram may interact primarily via AFB5. Similarly, IAA and 2,4-D appear to act primarily via TIR1 and the homologous AFB1, AFB2, and AFB3 (Dharmasiri et al., 2005a). Plant responses to IAA may not be significantly mediated by SCF<sup>AFB5</sup> because none of the *afb5* alleles gave resistance to IAA and the mutants had no obvious endogenous auxin-related phenotypes as seedling or adult plants as do many IAA/2,4-D-resistant mutants (Woodward and Bartel, 2005). Indeed, the slight hypersensitivity of *afb5* alleles to IAA may indicate that some indirect negative interaction could occur. Differences in response could arise from a lower affinity of AFB5 for IAA and 2,4-D (relative to TIR1 and AFB1, AFB2, or AFB3), thus leading to a significantly higher threshold for response. Additional biochemical assays of SCF<sup>AFB</sup> function using the appropriate components (Dharmasiri et al., 2003) will be required to determine the relative affinities of the AFBs for various auxins. The functional diversity and variations in response of AFBs to various auxins could also be affected by differences in the time and location of expression of the AFB proteins. Detailed analysis of the relative expression of the AFB gene family may help elucidate this. An alternative and intriguing explanation of the lack of cross-resistance of *afb5* plants to IAA is that AFB5 interacts with another undetermined endogenous small molecule. Herbicidal picolinate auxins may therefore mimic the activity of this compound rather than IAA.

The LRR F-box proteins are composed of an N-terminal F-box that interacts with other components of the SCF complex and a C-terminal LRR domain that interacts with proteins targeted for ubiquitination (Gray et al., 1999; Gagne et al., 2002). The four resistance mutations in AFB5 are all in the LRR domain of the protein, two of which introduce stop codons into the N-terminal portion of the domain and two of which introduce amino acid changes into the C-terminal region. We did not detect any phenotypic difference between the four alleles, indicating that a highly conservative amino acid substitution at the extreme C terminus of the protein (R609K) has the same effect as truncations at the N terminus (W134stop, W220stop). Both sites of amino acid substitutions, Arg-609 and Cys-451, are completely conserved in all six TIR1-related AFBs, suggesting that they are essential for auxin-related F-box protein functionality (Supple-

mental Fig. S2). The three characterized *tir1* alleles are also point mutations in the C-terminal LRR domain in amino acids that are conserved in the six TIR1-related AFBs (Fig. 5A), rather than in the N-terminal F-box domain (Ruegger et al., 1998; Alonso et al., 2003). As TIR1 has been shown to directly bind auxins (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005), the residues conferring auxin resistance in TIR1 and AFB5 may be close to the site of chemical interaction.

### Role of SGT1b in Auxin Action

The second locus that we identified conferring picolinate-specific auxin resistance encodes SGT1b, a tetratricopeptide-repeat protein that is highly conserved throughout eukaryotes (Lingelbach and Kaplan, 2004). In yeast (*Saccharomyces cerevisiae*), it is an essential gene that physically associates with a variety of multiprotein complexes, including SCF ubiquitin ligase. It has been shown to interact in substoichiometric amounts with Skp1p (the cullin scaffold protein) of the SCF complex, but is not required for in vitro ubiquitin ligase enzymatic activity (Kitagawa et al., 1999). Genetic evidence has indicated that it may be involved in promoting association and turnover of SCF and other complexes with the appropriate target (Lingelbach and Kaplan, 2004). In plants, SGT1b has recently been shown to be a component of R gene-mediated disease resistance, and mutant plants lacking SGT1b have increased susceptibility to certain plant pathogens (Muskett and Parker, 2003). In addition, a mutation that increases the rather weak auxin resistance of *tir1-1* (*eta3*) was recently discovered to be an allele of *SGT1b* (Gray et al., 2003). The *eta3* mutants showed decreased degradation of Aux/IAA proteins in response to IAA, suggesting that SGT1b is required for some elements of auxin-mediated SCF function.

Our data indicate that the effect of mutations in *SGT1b* has a significantly greater effect on resistance to picolinate auxins than on resistance to 2,4-D or IAA. The level of resistance of the three *sgt1b* alleles (and the deletion mutant *edm1*) to DAS534 and picloram was approximately 8-fold and 60-fold, respectively, whereas there was negligible resistance to 2,4-D (approximately 1.7-fold). Gray et al. (2003) observed a modest level of resistance to 2,4-D (approximately 2-fold) in a *sgt1b* mutant that increased to 7-fold resistance in the *sgt1b tir1-1* double mutant. In contrast, we observed no difference in picolinate-resistance of *sgt1b afb5* double mutants relative to that of the single mutants. This may indicate that SCF<sup>AFB5</sup> function has a greater dependency on SGT1b than SCF<sup>TIR1</sup> such that loss of function of either SGT1b or AFB5 is equivalent in terms of picolinate resistance, whereas both SGT1b and TIR1 functions need to be attenuated to manifest significant resistance to 2,4-D. SGT1b may alter the affinity of the various SCF complexes for their auxin effector (Gray et al., 2003) It will be of interest to evaluate whether differences in synergy by mutations in SGT1b also occur in combination with mutations in



other AFBs. It is also intriguing to speculate whether there is any direct chemical interaction of picolinate auxins with SGT1b, possibly in complex with AFB5 and its targets.

### Relevance to Herbicidal Auxin Resistance and Selectivity in the Field

There are now many well-characterized auxin-resistant *Arabidopsis* mutants (Woodward and Bartel, 2005). However, there are relatively few cases of field resistance developing to herbicidal auxins, especially in comparison to other herbicidal modes of action in widespread use. This lack of resistance development in the field may be due to the redundancy in auxin receptors (AFBs) and other components of the auxin signal response and the fitness penalties that may occur because of mutations in the pathway. However, an isolated occurrence of field resistance to picolinate auxins has been documented in yellow starthistle (*Centaurea solstitialis*) and appears to have arisen from heavy localized use of picloram. This resistant biotype carries a recessive mutation (Sabba et al., 2003) that confers selective resistance to picloram and clopyralid, but not to 2,4-D, and it also has normal growth and morphology (Fuerst et al., 1996). Thus, the picloram-resistant field biotype appears to have some of the characteristics associated with the mutations that we have identified in *Arabidopsis*. In contrast, another well-characterized auxin-resistant biotype of wild mustard (*Brassica kaber* syn. *Sinapis arvensis*) contains a dominant mutation that confers nonselective resistance to 2,4-D and picloram (Jugulam et al., 2005).

Herbicidal auxins can have varying potencies and selectivities toward various plant species that can be dependent on foliar uptake, translocation, and metabolism of the compound, as well as plant morphology. However, in several cases, this does not completely account for their weed spectrum or potency (Hall and Vanden Born, 1988; Sterling and Hall, 1997; Grossmann, 2000). This suggests that some degree of selectivity may occur at or close to the target site. There is evidence for differential cellular uptake of synthetic auxins by auxin permeases (Delbarre et al., 1996; Imhoff et al., 2000; Hoessel et al., 2005) that could account for some differences in herbicidal efficacy between different auxins. Our present data suggest that some degree of synthetic auxin selectivity and potency may also lie within components of the SCF ubiquitin ligase machinery, including the F-box protein receptors.

### Utility of Auxin Diversity in Chemical Genetics

Our work demonstrates the utility of using a differential screen with structurally diverse chemistries to uncover and characterize novel phenotypes and mutations. For example, in this case, the mutations we describe would not emerge from resistance screens using 2,4-D or IAA. The structural diversity within

synthetic auxins may have additional utility in carefully designed chemical genetic studies to differentiate and dissect auxin-mediated biological processes. Conversely, better understanding of the molecular interactions of these compounds with the upstream signaling components may lead to new and improved herbicidal molecules.

## MATERIALS AND METHODS

### Materials

Seeds of *tir1-1*, *axr1-3*, and *axr2-1* were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Seeds of *edm1* were provided by Dr. Eric Holub (Horticulture Research International). DAS534 was synthesized as described in Supplemental Methods S1. Clopyralid was obtained from the Dow AgroSciences compound files. All other chemical reagents were obtained from Sigma Chemical Company.

### Mutant Screens

Sterilized EMS-mutagenized M<sub>2</sub> Col-0 seeds (Lehle Seeds) were sterilized by washing four times in 15% commercial bleach, 0.5% SDS solution, then rinsed with sterile water. Seeds were stratified at 4°C for 3 to 4 d. Petri plates (15-cm diameter) were prepared containing 50 mL of modified Murashige and Skoog basal medium (Sigma) with 1 mM MES buffer, 0.8% Suc, 0.42% agarose (SeaKem), and 10 nM DAS534. Sterilized seeds were distributed on top of the medium and 30 mL of medium were then poured on top and allowed to solidify. The plates were moved to a growth chamber at 22°C, illuminated at 75  $\mu\text{E m}^{-2} \text{s}^{-1}$  on a 16-h-light/8-h-dark cycle for 10 d. Resistant seedlings were identified as plants with long roots and fully expanded cotyledons. Herbicide-resistant seedlings were transferred to petri plates containing Murashige and Skoog medium lacking DAS534 and allowed to recover in the growth chamber for 7 to 10 d. After recovery, seedlings were transferred to soil (HP Promix) and allowed to grow to maturity in a greenhouse at 22°C with supplemented light on a 16-h-light/8-h-dark cycle. Selection of lines for detailed study was based on the strength of resistance, elimination of potential siblings, and health and fertility of adult plants.

### Root Growth Assays

*Arabidopsis* (*Arabidopsis thaliana*) seedling auxin response assays were performed using growth medium supplemented with 0.8% Suc and 0.4% agarose. Compounds were added to the medium as concentrated solutions dissolved in dimethyl sulfoxide. Controls had the appropriate amount of dimethyl sulfoxide added without inhibitor. Plates were incubated for 8 d at 23°C under continuous fluorescent lighting (50 to 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). Root measurements were made by carefully extracting individual plants from the medium and measuring the length of the tap root. Five roots were measured at each concentration of compound.

### Greenhouse Tests

*Arabidopsis* seedlings were grown for 2 weeks in a growth chamber (23°C; continuous light at 120–150  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), then taken to the greenhouse for 3 d after which plants were sprayed with picloram or 2,4-D using a track sprayer to deliver the appropriate rate. Plants were then grown in the greenhouse for 12 d (22°C under supplemented light with a 14-h-light/10-h-dark cycle) prior to photography and evaluation.

### Genetic Analyses and Mapping

F<sub>1</sub> seeds from crosses between DAS534-resistant and wild-type lines were tested for resistance to DAS534 by plating the seeds on medium containing 10 nM DAS534. The roots of DAS534-resistant seedlings were as long as seedlings plated on medium lacking the herbicide, whereas DAS534-sensitive roots were approximately 50% shorter.

To generate mapping populations, homozygous *afb5-1* and *sgt1b-4* M<sub>3</sub> plants (in the Col-0 background) were crossed with wild-type Landsberg

*erecta* (*Ler*). F<sub>2</sub> seeds were germinated on medium containing 10 nM DAS534. Plants resistant to the herbicide were identified and allowed to recover on medium without herbicide for 7 d, then transplanted to soil. A single leaf was removed at the rosette stage for genomic DNA isolation. Mapping was performed using single-nucleotide polymorphism (SNP) markers from Cho et al. (1999) or identified by comparing sequences from the *Ler* Random Sequence Database (The Institute for Genomic Research [TIGR]) with the complete sequence of *Arabidopsis* Col-0 (TAIR). Verified SNPs were used as molecular markers to map the mutation using a fluorescence polarization SNP-mapping technique (Hsu et al., 2001). All primers were custom synthesized by Operon. All other reagents were supplied in the AcycloPrime-FP SNP detection kit from Perkin-Elmer. Fluorescence polarization was read in a LJJL BioSystems Analyst 96-384 meter (Molecular Devices). Information on the markers used in the mapping experiments is listed in Supplemental Table S1. The genes At5g49980 and At4g11260 were PCR amplified from *afb5-1* by Pfu Turbo DNA Hotstart DNA polymerase (Stratagene) and sequenced using Applied BioSystems BigDye terminator, version 3.1, cycle-sequencing kit and run on an ABI 3100.

### Complementation of Mutant Phenotypes

The gene At5g49980 was PCR amplified from genomic DNA of Col-0 using the primers 5'-CACCAAAAATGACACAAGATCGCTCAGAAATGTC-3' and 5'-TCCTACACTTACCCATTTTCATTCCTCCG-3' and the gene At4g11260 similarly amplified using the primers 5'-CACCAAAAATGGCCAAGGAAT-TAGCAGAGAAA-3' and 5'-TCGAGCAGAAACCAAAAAAGAAAAACA-3'. The PCR products were cloned into pENTR 1A (Invitrogen) and independently recombined into the binary plant transformation vector pNT4274 using Gateway (Invitrogen) with the strong constitutive CsVMV (Verdaguer et al., 1998) and the *nos* terminator. The transformation vector also contained a *nptII* gene as a selectable marker. Vectors were transformed into *Agrobacterium* strain GV3101 containing the helper plasmid pMP90RK and used to transform either *afb5-4* (for the strain containing the At5g49980 vector) or *sgt1b-4* (for the strain containing the At4g11260 vector) via the floral-dip procedure (Clough and Bent, 1998). Kanamycin-resistant T<sub>1</sub> plants were transplanted to soil and allowed to self-fertilize. T<sub>2</sub> seed was tested for susceptibility to DAS534 as described above.

### Identification of Double Mutants

Genomic DNA was isolated from 95 F<sub>2</sub> plants generated from a cross of *afb5-1* and *sgt1b-4*. The At5g49980 (*AFB5*) and At4g11260 (*SGT1b*) genes were amplified by PCR and evaluated by DNA sequence analysis for mutations. Eight plants containing mutations in both genes were identified and progeny from three of them were compared with the single-mutant parental lines for resistance to DAS534, picloram, and 2,4-D.

### Supplemental Data

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

**Supplemental Figure S1.** Map-based cloning of DAS534-resistance mutations.

**Supplemental Figure S2.** Sequence comparisons of AFBs.

**Supplemental Table S1.** Synthesis of DAS534.

**Supplemental Methods S1.** Description of SNP markers used in mapping.

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