An in-frame deletion mutation in the degron tail of auxin coreceptor IAA2 confers resistance to the herbicide 2,4-D in *Sisymbrium orientale*


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The natural auxin indole-3-acetic acid (IAA) is a key regulator of many aspects of plant growth and development. Synthetic auxin herbicides such as 2,4-D mimic the effects of IAA by inducing strong auxin-signaling responses in plants. To determine the mechanism of 2,4-D resistance in a *Sisymbrium orientale* (Indian hedge mustard) weed population, we performed a transcriptome analysis of 2,4-D-resistant (R) and -susceptible (S) genotypes that revealed an in-frame 27-nucleotide deletion removing nine amino acids in the degron tail (DT) of the auxin coreceptor Aux/IAA2 (SoIAA2). The deletion allele cosegregated with 2,4-D resistance in recombinant inbred lines. Further, this deletion was also detected in several 2,4-D-resistant field populations of this species. *Arabidopsis* transgenic lines expressing the SoIAA2 mutant allele were resistant to 2,4-D and dicamba. The IAA2-DT deletion reduced binding to TIR1 in vitro with both natural and synthetic auxins, causing reduced association and increased dissociation rates. This mechanism of synthetic auxin herbicide resistance assigns an in planta function to the DT region of this Aux/IAA co-receptor for its role in synthetic auxin binding kinetics and reveals a potential biotechnological approach to produce synthetic auxin-resistant crops using gene-editing.

TIR1/AFB | dicamba | herbicide resistance | plant hormones | target-site resistance

**Significance**

Synthetic auxin herbicides intersect basic plant developmental biology and applied weed management. We investigated resistance to 2,4-D in the Australian weed *Sisymbrium orientale* (Indian hedge mustard). We identified a mechanism involving an in-frame 27-bp deletion in the degron tail of auxin coreceptor IAA2, one member of the gene family of Aux/IAA auxin co-receptors. We show that this deletion in IAA2 is a gain-of-function mutation that confers synthetic auxin resistance. This field-evolved mechanism of resistance to synthetic auxin herbicides confirms previous biochemical studies showing the role of the Aux/IAA degron tail in regulating Aux/IAA protein degradation upon auxin perception. The deletion mutation could be generated in crops using gene-editing approaches for cross-resistance to multiple synthetic auxin herbicides.


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implications of mutations in the degron motif of Aux/IAA proteins. Currently, 41 different weed species have evolved resistance to synthetic auxin herbicides (10). Herbicide-resistance mechanisms involve mutations in the gene encoding the target-site protein(s) as well as nontarget site changes to alter herbicide movement or rate of degradation to nonphytotoxic forms (11). Synthetic auxin herbicide–resistant weeds have been reported to have rapid metabolic degradation of the herbicide (12), reduced herbicide translocation to the growing point (13, 14), and increased expression of transmembrane kinase and receptor proteins (15). Except for the IAA16 receptor proteins (15), and increased expression of transmembrane kinase and receptor proteins (15).

In 2005, a population of the weed species Sisymbrium orientale (Indian hedge mustard) was reported to be resistant to the synthetic auxin herbicides 2,4-D and 2-methyl-4-chlorophenoxyacetic acid in South Australia (17). In the resistant population, 2,4-D translocation was reduced (18), and progeny tests revealed that the resistance was inherited as a single dominant allele (19). Given the importance of synthetic auxins to agriculture and the recent introduction of engineered resistance to 2,4-D and dicamba in soybean and cotton (20, 21), we investigated the mechanism of resistance in natural populations of the weed S. orientale. We performed a transcriptome analysis on recombinant inbred lines (RILs) derived from a cross between 2,4-D-resistant (R) and -susceptible (S) genotypes and found an in-frame 27-bp deletion in the degron tail (DT) of the auxin coreceptor Aux/IAA2 (SoIAA2). This loss of nine amino acids due to DT deletion reduced binding of both natural and synthetic auxins to TIR1 and conferred cross-resistance to 2,4-D and dicamba in soybean and cotton (20, 21), we investigated the mechanism of 2,4-D resistance in natural populations of the weed S. orientale. Segregation of SoIAA2Δ27. The heritable association between SoIAA2Δ27 and the resistance phenotype (evaluated as visual injury on a scale of 0 to 100%) was confirmed by forward genetics through segregation and genotyping analysis. Susceptible progeny of a self-pollinated heterozygous individual (70% visual injury after 250 g/active ingredient [ai]/ha - 2,4-D treatment) were homozygous for SoIAA2Δ27, or heterozygous for SoIAA2Δ27 and wild-type (WT) SoIAA2, suggesting a segregating population for this locus. Population P28, although resistant, did not contain SoIAA2Δ27, suggesting a different synthetic auxin herbicide–resistance mechanism may have evolved in this population. Overall, sequence data identified a 27-base pair deletion in SoIAA2 as the candidate mutation associated with resistance to 2,4-D in several S. orientale populations from Port Broughton, Australia.

Evaluation of Potential Nontarget Site Mechanisms of Auxin Resistance. To rule out other potential herbicide-resistance mechanisms not related to the Aux/IAA candidate, experiments using [14C] 2,4-D were performed to evaluate differences in herbicide absorption, translocation, and metabolism between R and S populations. There were no differences in maximum herbicide absorption (Amax R: 91% ± SE 0.9% and S: 92% ± SE 0.8%) or time for 90% of maximum herbicide absorption (T90 R: 2.9 h ± SE 0.3 h and S: 3.5 h ± SE 0.2 h) between populations. In untreated plants, GH3.3 and IAA19 had no detectable transcripts for both genotypes, while SoIAA2 had low expression (Fig. 1C). Both S and R plants had similar increased GH3.3 expression levels after 2,4-D treatment; however, relative expression of both IAA19 and IAA2 increased in S while expression in R did not increase following 2,4-D treatment (Fig. 1C).

**Results**

**Identification of IAA2 as a Candidate Gene for Herbicide Resistance through RNA-Sequencing.** The transcriptomes of untreated S. orientale plants were sequenced, consisting of six F4 RILs derived from a cross between 2,4-D-resistant PB-R (Port Broughton–Resistant) and susceptible S (Cross A, 3 R-RILs and 3 S-RILs) and six F3 RILs from a cross between 2,4-D-resistant PB-R2 and susceptible S (Cross B, two R-RILs, and four S-RILs). Average reads per sample were 6.7 × 106 with >40 quality control score. Only one transcript, SoIAA2, had differential expression between R-RILs and S-RILs of both PB-R and PB-R2 populations based on cutoff criteria of false discovery rate (FDR) <0.05, fold change of >2, and a consistent expression pattern in all R-RIL replicates compared to all S-RIL replicates. The IAA2 protein encoded by SoIAA2 (3.3-fold lower expression in R than S, FDR <0.0001) showed a high-sequence similarity to the Arabidopsis IAA2 protein encoded by At3g23030 (SI Appendix, Fig. 1). No single-nucleotide changes in any transcripts were identified that were shared among all R-RILs and different from all S-RILs. Inspection of the read alignments to SoIAA2 identified a gap in read coverage for all R-RILs, suggesting a deletion in the R allele, whereas all S-RILs had continuous read coverage at this position (SI Appendix, Fig. 2). Transcription analysis led to the hypothesis that resistance was linked to this deletion mutation in SoIAA2.

**SoIAA2 Gene-Sequencing.** A 239-bp region of the SoIAA2 gene that includes the predicted deletion was amplified and sequenced from three R-RILs and three S-RILs. The 27-bp deletion in the R allele (gene and messenger RNA [mRNA] referred to as SoIAA2Δ27) was confirmed (Fig. 1A). The deletion results in an in-frame protein lacking nine amino acids (aa 73 through 81; protein referred to as SoIAA2Δ9) comprising most of the DT region between the degron and the PBl domain (Fig. 1A). None of the S-RILs contained a deletion in SoIAA2, showing that the deletion was correlated with resistance.

The presence of SoIAA2Δ27 in other 2,4-D resistant populations of S. orientale was investigated. Eight populations from South Australia, including parent populations PB-R and PB-R2 were screened for resistance to 2,4-D, with four populations identified as resistant. The SoIAA2 gene was sequenced to determine the presence or absence of SoIAA2Δ27 in individual plants from each population (SI Appendix, Table 1). Full-length SoIAA2 was present in individuals from four susceptible populations, P15, P31, P49, and P50. All individuals from the two parent-resistant populations, PB-R and PB-R2, contained SoIAA2Δ27. Individual plants from resistant population P17 were found to be homozygous for SoIAA2Δ27, or heterozygous for SoIAA2Δ27 and wild-type (WT) SoIAA2, suggesting a segregating population for this locus. Population P28, although resistant, did not contain SoIAA2Δ27, suggesting a different synthetic auxin herbicide–resistance mechanism may have evolved in this population. Overall, sequence data identified a 27-base pair deletion in SoIAA2 as the candidate mutation associated with resistance to 2,4-D in several S. orientale populations from Port Broughton, Australia.
radiolabeled compound for both populations at all time points (SI Appendix, Fig. 3D and Table 2). The half-life of 2,4-D in R plants (200 h ± SE 22) and S plants (118 h ± SE 42) could not account for the difference in phenotype. Taken together, these results indicate that changes in absorption, translocation, and metabolism are not the mechanism of 2,4-D resistance in this R population.

**Expression of SoIAA2Δ27 in Arabidopsis Confers 2,4-D and Dicamba Resistance.** To test whether SoIAA2Δ27 was sufficient to confer 2,4-D resistance, Arabidopsis was transformed with an empty pFGC5941 vector (Ø), SoIAA2, and SoIAA2Δ27 under the CaMV35S promoter (SI Appendix, Fig. 4). Genotypes were confirmed by PCR, and plants containing the empty vector and WT SoIAA2 did not show obvious changes in plant phenotype (Fig. 2A). Arabidopsis-transformed lines that were heterozygous for SoIAA2Δ27 had lanceolate shape leaves and a minor reduction in plant size. Plants homozygous for SoIAA2Δ27 had strong morphological abnormalities, dwarfism, a reduced number of reproductive organs, and lower seed production. Total biomass of transformed plants homozygous for SoIAA2Δ27 was substantially lower compared to Col-0 and lines transformed with SoIAA2 or a null vector (Fig. 2B).

A root-growth assay in the presence and absence of IAA and synthetic auxins was performed using the transformed Arabidopsis lines. Col-0 (WT) and plants containing the null vector (Ø2 and Ø4) and SoIAA2 (S1 and S7) were susceptible to 2,4-D (0.5 μM) and dicamba (20 μM), with root elongation of 5 to 6% relative to the ethanol control in the presence of 2,4-D and root elongation of 10 to 13% relative to the ethanol control in the presence of dicamba (Fig. 2B). Arabidopsis transformed with SoIAA2Δ27 (lines R1 and R4) exhibited resistance to 2,4-D and dicamba, with root elongation of 24% and 36% relative to the ethanol control for 2,4-D and root elongation of 25% and 42% relative to the ethanol control for dicamba (Fig. 2B). R4 SoIAA2Δ27 was resistant to IAA, with 77% root elongation compared to the ethanol control. All other lines were sensitive to IAA at 0.5 μM, including Col-0, empty vector lines, lines expressing SoIAA2 (S1 and S7), and line R1. SoIAA2Δ27-expressing lines were also resistant to 2,4-D and dicamba at the whole-plant level with foliar herbicide applications (SI Appendix, Fig. 5 and Table 3). The auxin-responsive genes AILAA19 and AIGH3.3 exhibited an increase in expression levels following 2,4-D foliar treatment in Col-0, null vector (Ø2), and SoIAA2 (S1 and S7) lines, while lines transformed with SoIAA2Δ27 (R1 and R4) showed no increase in expression of AILAA19 and AIGH3.3 following 2,4-D treatment (Fig. 2C). AILAA2 expression was similar to that of Col-0 and null vector lines. Taking all these results together, SoIAA2Δ27 is sufficient to confer resistance to both 2,4-D and dicamba when expressed in Arabidopsis.

**SoIAA2 and SoIAA2Δ27 Protein Structure.** The Aux/IAA DT has pre-
by the deletion mutation, full-length SoIAA2 and SoIAA2Δ27 (the protein product of SoIAA2Δ27) were expressed and purified to perform circular dichroism (CD) spectrometry and size-exclusion chromatography. Size-exclusion chromatography showed that both mutant and wild-type proteins purify as oligomers of varied composition depending on protein concentration (SI Appendix, Fig. 7 A and B), which has also been seen for other Aux/IAA proteins (22, 23). While the deletion did not disrupt or enhance oligomer formation, it did enhance the overall stability of the protein.

During the purification process, the wild-type protein displayed a much-lower maximum-concentration threshold than the deletion mutation (SI Appendix, Fig. 7A). CD spectrometry data are nearly identical for both proteins (SI Appendix, Fig. 7D). A BeStSel analysis of the data (24) results in an alpha:beta:other content of 13%:38%:49%, which agrees with a partial structure from the PB1 domain and about half of the protein being disordered or “other.” This suggests the nine–amino acid deletion does not impact the overall folding of the protein (SI Appendix, Fig. 7D).
Binding-Affinity Analysis of TIR1/AFB with SoIAA2 and SoIAA2-9. To determine the mechanism by which the DT deletion in SoIAA2 interferes with synthetic auxin herbicide–binding, a surface plasmon resonance (SPR) assay was performed (25). The assay measures the auxin-induced assembly of TIR1/AFB receptors with degron peptides, which is representative of the coreceptor complex (26). We used a biotinylated 24-aa peptide (SoIAA2) consisting of the degron core (9 aa), DT (9 aa), a small fraction of PB1 (6 aa), and a biotinylated 15-aa peptide lacking the DT segment (SoIAA2-9) (SI Appendix, Table 4). Both peptides bound to TIR1 and to AFB5 in the presence of IAA (Fig. 3A and B). However, the assay showed weaker binding by SoIAA2-9 to TIR1 with IAA compared to WT SoIAA2 and similarly weaker binding with both synthetic auxins 2,4-D and dicamba (Fig. 3). Binding kinetic experiments showed that weaker binding translates as lower concentrations of each auxin. (8) The same analysis with AFB5, which characteristically displays faster kinetics for both association of the complex, and dissociation is shown. Auxins were mixed with the TIR1 or AFB5 preparation in advance of injection over the biotinylated SoIAA2 peptides using standard Biacore double-baseline subtraction with single-cycle kinetic routines. RU, resonance units. Tables show kinetic data for the binding of TIR1 (Left) and AFB5 (Right) to SoIAA2 and SoIAA2-9 in the presence of natural and synthetic auxins. Values were calculated using a global fit for a Langmuir 1:1 kinetic model using the manufacturer's Biacore Evaluation software.

![Fig. 3](image)

**Fig. 3.** The mutant SoIAA2-9 binds to the receptors TIR1 and AFB5 with poorer affinity than the WT SoIAA2. SPR was used to measure the binding of purified receptor protein to each degron peptide using single-cycle kinetics. (A) Typical sensorgrams showing binding and dissociation of TIR1 with each degron peptide at a series of rising concentrations of each auxin. (B) The same analysis with AFB5, which characteristically displays faster kinetics for both association of the complex, and dissociation is shown. Auxins were mixed with the TIR1 or AFB5 preparation in advance of injection over the biotinylated SoIAA2 peptides using standard Biacore double-baseline subtraction with single-cycle kinetic routines. RU, resonance units. Tables show kinetic data for the binding of TIR1 (Left) and AFB5 (Right) to SoIAA2 and SoIAA2-9 in the presence of natural and synthetic auxins. Values were calculated using a global fit for a Langmuir 1:1 kinetic model using the manufacturer’s Biacore Evaluation software.

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Homology-Based Modeling and Docking Analysis for TIR1 with SoIAA2 and SoIAA2-9. Homology-based protein models were used to explore the effect of the DT deletion on the interaction between TIR1 and SoIAA2. The sequence of IAA7 (chain C of Protein Data Bank [PDB]: 2P1Q) (2) had 85% sequence identity to the corresponding sequence of SoIAA2 and included no gaps. The crystal structure of pea (*Pisum sativum*) PB1 domain (PDB: 2M1M) (4) was identified as a crystal homolog of the PB1 portion of SoIAA2 using the search function of Swiss Model (27). Sequence comparison revealed a 72% sequence identity with no gaps. Docking of SoIAA2 and SoIAA2-9 models generated several clusters (SI Appendix, Table 5). Clusters 3 and 1 had the lowest z-score for SoIAA2 and SoIAA2-9, respectively (SI Appendix, Table 5). Docking of SoIAA2 to TIR1 was better than that of SoIAA2-9, with a 25% reduction in affinity for SoIAA2-9 ($\Delta G = -8$ kCal/mol$^{-1}$; SD ± 0.55) relative to SoIAA2 ($\Delta G = -10.63$ kCal/mol$^{-1}$; SD ± 0.64) and a 160-fold decrease in the dissociation constant for SoIAA2-9 ($K_D = 5$ μM; SD ± 1.74) relative to SoIAA2 ($K_D = 0.031$ μM; SD ± 0.005) (Fig. 4A). Docking of SoIAA2 allowed for interaction of PB1 with TIR1 as described by Niemeyer et al. (5), while maintaining the same interaction of the core IAA2 degron toward the center of the barrel described by Tan et al. (2). On the other hand, the deletion of nine residues in SoIAA2-9 caused a dramatic change in the architecture of TIR1 while preventing the proper interaction of PB1 with its binding domain on the outside of TIR1 (Fig. 4B). Overall, we propose that in the presence of auxin, the WT version of SoIAA2 binds to TIR1 by interacting with the degron and the PB1 domain. In this conformation, the IDR is displayed across the top surface of TIR1 with all lysine residues accessible for ubiquitination. In SoIAA2-9-resistant individuals, the reduction of the molecular distance between the core degron and the PB1 domain likely precludes binding of this domain to TIR1. Lower overall affinity for SoIAA2-9 may reduce the lifetime of the complex and reduce the likelihood of polyubiquitination (Fig. 4C).
At the resistance phenotype. Equivalent residues in the DT of SoIAA2, which is a mechanism of target-site mutation in the auxin coreceptor associated with resistance to auxin herbicides in weeds. Our results confirm the importance of the DT as a contributor to Aux/IAA stability (5) and for perception of synthetic auxin herbicides in planta. Homology modeling indicated that the SoIAA2Δ9 protein is only able to engage one of the two domains (PB1 and the degron) at a time when binding to TIR1/AFB (Fig. 3). A poorer binding affinity associated with the two domains (PB1 and the degron) at a time when binding association with the TIR1/2,4-D/IAA2 protein complex. (Δ) Computational characterization of the thermodynamic profiles of SoIAA2 and SoIAA2Δ9 docking to TIR1. Binding free-energy ΔG and dissociation constant Kd were calculated. (B) Docking of SoIAA2 and SoIAA2Δ9 to TIR1. Side view of the interaction between TIR1 (white) and I) SoIAA2 and II) SoIAA2Δ9 is shown (proteins are displayed as cartoons). Bird’s eye view of the interaction between TIR1 (white) and III) SoIAA2 and IV) SoIAA2Δ9 is shown (proteins are displayed as surfaces). The different parts of SoIAA2 are shown as light blue for PB1, blue for the nine residues that are deleted in SoIAA2Δ9 (DT), and dark blue for the SoIAA2 core degron. Panels II and IV are drawn with the degron instead of the PB1 domain bound, because it had the more-favorable, calculated binding energy, and this pose is therefore predicted to be more likely. (C) Proposed hypothetical model for the mechanistic basis of 2,4-D resistance caused by the DT deletion in SoIAA2Δ9 compared to wild-type SoIAA2. ARF repression due to increased SoIAA2 protein ubiquitination may also contribute to the resistance mechanism, because protein stability and oligomerization are important factors for transcription repression activity (28).

The DT deletion reduced the binding affinity of the mutant Aux/IAA coreceptor protein for the TIR1 receptor with all auxins tested in SPR binding assays (Fig. 3A). In silico molecular-docking analysis showed similar changes in the calculated binding kinetics to that measured in the biochemical assays (Fig. 4A). SoIAA2 bound in a similar fashion as that proposed by Niemeyer et al. and Tan et al. (2, 5). The deletion of nine amino acids from the DT in SoIAA2Δ9 caused a dramatic change in the architecture of the protein, permitting the degron to continue to interact with TIR1 while preventing the proper interaction of PB1 (Fig. 4B). Thus, resistance may be associated with reduced binding of the mutant SoIAA2Δ9, resulting in less ubiquitination and subsequent degradation by the proteasome. We propose a hypothetical model to be tested in future work that the SoIAA2Δ9 protein maintains steric hindrance that could limit lysine ubiquitination, since PB1 appears to occlude most of domain II (Fig. 4). Another putative explanation for the loss of sensitivity to auxins is increased oligomerization. SoIAA2Δ9 had increased in vitro stability forming higher oligomers compared to the wild-type SoIAA2. ARF repression due to increased SoIAA2Δ9 oligomerization may also contribute to the resistance mechanism, because protein stability and oligomerization are important factors for transcription repression activity (28).

The target sites for synthetic auxin herbicides consist of complexes of two protein families, the receptor TIR1/AFB and the coreceptor Aux/IAA protein. The deletion of 27 nucleotides in the gene SoIAA2 results in the loss of nine amino acids in the DT of SoIAA2, which is a mechanism of target-site mutation in the auxin coreceptor associated with resistance to auxin herbicides. The deletion of the DT in SoIAA2Δ9, resulting in less ubiquitination and subsequent degradation by the proteasome. Auxin-responsive genes are not induced by synthetic auxin herbicides, which leads to herbicide resistance.

**Discussion**

The target sites for synthetic auxin herbicides consist of complexes of two protein families, the receptor TIR1/AFB and the coreceptor Aux/IAA protein. The deletion of 27 nucleotides in the gene SoIAA2 results in the loss of nine amino acids in the DT of SoIAA2, which is a mechanism of target-site mutation in the auxin coreceptor associated with resistance to auxin herbicides. The deletion of the DT in SoIAA2Δ9, resulting in less ubiquitination and subsequent degradation by the proteasome. Auxin-responsive genes are not induced by synthetic auxin herbicides, which leads to herbicide resistance.
higher transcriptional repression of auxin-responsive genes in the presence of synthetic auxin herbicides than wild-type SoIAA2 (Fig. 4C). SoIAA2 DT deletion leads to cross-resistance to both 2,4-D and dicamba, revealing a pathway to explore introduction of synthetic auxin resistance traits in crop plants by targeting the DT region using gene-editing technologies.

SoIAA2 had low expression under natural conditions; however, it was induced rapidly after exogenous auxin application. A similar response was also observed in Arabidopsis Col-0 seedlings when the expression of AtIAA2 increased sixfold 1 h after 2,4-D treatment (29). The SoIAA2Δ27 allele encoding the SoIAA2Δ protein maintained transcriptional repression of auxin-responsive genes GH3.3 and IAA19 following 2,4-D treatment, showing that the DT is critical for IAA and synthetic auxin herbicide–binding, leading to Aux/IAA protein degradation. SoIAA2 seems to play an important role in auxin perception. Previous reports of mutations that confer higher stability to Aux/IAA proteins have been associated with drastic morphological defects, as for axr1-1/AA1 (30, 31), shy2/IAA3 (32), axr2/IAA7 (31), iaa16 (6), and iaa28 (33). All these mutants were reported to be insensitive or hypersensitive to natural and synthetic auxins. Further, in the weed species S. scoparia, a mutation in the core degron of BslIAA6 conferred dicamba resistance but also resulted in a substantial fitness penalty in the form of reduced growth (8, 9). In our study, overexpression of SoIAA2Δ27 in Arabidopsis induced strong phenotypes in homozygous plants with severely reduced growth (Fig. 24). Future research is needed to determine whether the SoIAA2Δ27 mutation in 2,4-D-resistant S. orientale causes any morphological or reproductive defects or reduction in competitiveness with crops.

Materials and Methods

Plant Material. One population of 2,4-D-resistant S. orientale was collected from a wheat field in Port Broughton (PB-R), and a second 2,4-D-resistant population was collected 92 km away (PB-R2), while the susceptible population originated from Roseworthy (S), Australia (17, 19). Two biparental crosses were made to study inheritance of 2,4-D resistance (18), using PB-R as the male parent for Cross A and PB-R2 as the male parent for Cross B, with S as the female parent for both crosses. The F1 progeny were self-pollinated to produce F2 progeny, and F2 individuals were self-pollinated to produce F3 progeny (18, 19). Homozygous resistant and homozygous susceptible F3 lines were inbred via single-seed descent to create RILs to the F4 generation (Cross A). The F3 generation was used for Cross B.

RNA-sequencing (RNA-seq). RNA-seq (RNA-sequencing) (RNA-seq was performed on untreated plants for a total of six individual RILs, including six individuals from Cross A (three homozygous 2,4-D-resistant and three homozygous 2,4-D-susceptible) and Cross B (two homozygous 2,4-D-resistant and four homozygous 2,4-D-susceptible). RNA from the youngest expanded leaf of four- to five-leaf stage plants was extracted using the RNeasy Plant Mini Kit (Qiagen), and libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit (Illumina). Sequencing was performed on a HiSeq 4000 (Illumina) using bar-coded adapters in two lanes of the Illumina flow cell for 101 cycles, yielding 1.6 billion paired-end reads. Individual library yields ranged from 52 to 75 million paired-end reads. Fastq files were generated and demultiplexed using bcl2fastq version 2.17.13.13 conversion software (Illumina), and adapters were trimmed from the 3′-end reads. The sequence data and processed data files discussed in this publication are deposited in National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO) (34) and accessible through GEO Series accession no. GSE159202 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159202).

Novo reference transcriptome assembly was performed by separately assembling Illumina reads from a Cross A 2,4-D-resistant RIL (R206) and a susceptible RIL (S242) with Trinity (35). The assembled contigs were filtered for minimum contig length of 500 bp. The two assemblies were compared and merged using CDHit version 4.6.6.35 (with a 95% Cl to eliminate redundant contigs and retain all contigs unique to either the R or S assembly. This resulted in a 72-MB transcriptome with 4,997 contigs (available at GEO Series accession no. GSE159202) (BUSCO score of 98.4%, Eukaryota ODB81). Putative annotations were assigned using Trinotate (36) and The Arabidopsis Information Resource (TAIR) 10 protein database (37). Read alignments to the de novo reference transcriptome were conducted with Bowtie2 (38) using the default “end-to-end” mode and the “sensitive” option. The minimum allowed fragment length was set to 100 and the maximum to 800 bp.

Differential Expression and Single-Nucleotide Polymorphism Analysis. Raw read counts were extracted using sequence alignment/map (SAMtools) (39). Counts-per-million (CPM) and gene-expression differences were calculated with the package “edgeR” (40) using the statistical software R version 3.3.41 and an expression threshold of ≥1 CPM in at least two samples. After normalization expression differences were compared between 2,4-D resistant and susceptible RILs within Cross A and B, respectively, as well as between all 2,4-D-resistant and -susceptible RILs. Differentially expressed transcripts were then filtered for a fold change of ≥2 and an FDR adjusted P value ≤0.05. Transcripts annotated as PRR9-2 (10-fold lower in R than S, FDR < 0.0001, similar to AT1G27540 that encodes an auxin efflux transporter) met the criteria for significant differential expression but were excluded from the resistance hypothesis because both PRR9-2 and ABCB13 had at least one 2,4-D-resistant RIL replicate that deviated in expression pattern from the other replicates. Single-nucleotide polymorphism (SNP) calling was performed using SAMtools (version 1.3.1) with the default options and the “mpileup” command. The command “bcftools” was used to retain only SNPs that had a quality score higher than 10 and a minor allele frequency (MAF) of less than 0.1. An additional filtering step was performed for SNPs that were heterozygous or homozygous in at least three individuals of all R-RILs and all S-RILs. Contigs with annotations in the TIR1/AFB family and the Aux/IAA gene family were manually inspected for sequence variants between R-RILs and S-RILs using Integrative Genomics Viewer to view Binary Alignment Map file read alignments (42).

Sequence Verification of IAA2 Deletion, Segregation, and Analysis of Field Populations. The SoIAA2 deleted region was sequenced from samples of R-RILs and S-RILs used in RNA-seq. Complementary DNA (cDNA) was synthesized using Script (Bio-Rad), and a PCR was performed with primers listed in SI Appendix, Table 6 spanning the predicted deletion in IAA2. Amplions were Sanger sequenced. Transcript sequences of SoIAA2 and SoIAA2Δ were deposited in GenBank (accessions OK631624 and OK631625). To analyze the IAA2 deletion in other resistant S. orientale populations collected in South Australia, plants were sprayed with 200 g/ha 2,4-D at the five true-leaf stage using an overhead track sprayer (DeVries Manufacturing) equipped with a flat-fan nozzle tip (Teejet 8002EVS, Spraying System) calibrated to deliver 187 L/ha (Fig. 27) at a spray speed of 172 Kpa. Percent damage was evaluated at 28 DAT, and DNA was extracted by cetrimonium bromide (43) and analyzed using the primers listed in SI Appendix, Table 6.

Progeny test segregation analysis was performed on 219 F3 plants derived from self-pollination of a heterozygous F2 individual from Cross B, identified via progeny test due to segregation of 2,4-D resistance in the F3. Plants were sprayed with 250 g/ha 2,4-D at the five true-leaf stage as described above, and visual injury was evaluated on a scale of 0% (no injury) to 100% complete plant death. Competitive Allele Specific Polymerase Chain Reaction (KASP) genotyping markers were developed using a forward primer specific to the R (HXD) and S (FAM), together with a universal reverse primer (SI Appendix, Table 6).

Herbicide Absorption, Translocation, and Metabolism. S. orientale plants of both R and S genotypes were planted in soil under controlled conditions (60% relative humidity (RH), 21/18 °C, and 168 h light/dark photoperiod) and at the stage of two to three developed leaves, they were transferred to fine sand and irrigated with fertilizer. A total 1 wk later, the second true leaf was marked, covered with aluminum foil, and sprayed with a single nozzle overhead track sprayer (Generation III Research Sprayer; DeVries, Hollandale, MN) with 250 g/ha 2,4-D (2,4-D amine; 455 g L−1; DuPont, Wilmington, DE) in a water volume of 224 L/ha with 0.25% nonionic surfactant (NIS). The aluminum foil was removed, and a solution of [14C]2,4-D was applied in 10 droplets of 1 μL each, for total radioactivity applied per plant of 3.33 KBq (200,000 dpm). Three biological replicates were used for evaluation time points of 3, 6, 12, 24, 48, 96, and 192 h after herbicide treatment. At each time point, the treated leaf was cut and washed in 10% methanol and 1% NIS washing solution, and the rinse solution radioactivity was counted in 10 mL of scintillation mixture (EcoCount XR, National Diagnostics, Atlanta, GA) using liquid scintillation spectrometry (Packard TriCarb 2300TR, Packard Instrument Co., Meriden, CT). Roots were removed from sand and washed with deionized water, and rinsed with sand. Plants were then transferred to 3 L containers, dried in a 60 °C oven for 72 h, and exposed to Phosphor Screen film (GE Healthcare Life Sciences, Pittsburgh, PA), and images were taken 3 d later with a Typhoon Trio Imager (GE Healthcare). Subsequently, plants were separated into treated leaf, untreated leaves, stem, and roots and then oxidized in...
a biological oxidizer (OX500; RJ Harvey Instrument Co., Tappan, NY) followed by radioactivity measurement.

For metabolism, the treated leaf and roots were washed, the entire plant was ground in liquid nitrogen and digested in 10 mL solution of acetic acid for 10 min, and this extraction was done three times. After filtering, the extract was concentrated using solid-phase extraction C18 cartridge, in 4 mL of acetonitrile, evaporated, and resuspended in 225 μL of HPLC. A solvent, filtered, and 200 μL of the solution was injected in the HPLC (Hitachi Instruments, Inc., San Jose, CA, and C18 Column, Zorbax Eclipse XDB-C18; Agilent Technologies, Santa Clara, CA; Mobile phase A: 89.9% water, 10% acetonitrile, and 0.1% formic acid; and phase B: 99.0% acetonitrile and 0.1% formic acid), attached to a radio detector (FlowStar LB 513; Berthold Technologies GmbH & Co., Bad Wildbad, Germany) with a flow-cell YG-150-USD solid-cell YG-Scintillator (150 μL, Berthold Technologies). The proportion of absorbed, translocated, and metabolized herbicide was calculated using the equations from ref. 12, and the data-set was processed using the R package drc (44) to generate the corresponding mathematical models for each performed experiment (SI Appendix, Table 2).

Functional Validation of IAA2 Deletion in Arabidopsis. SoIAA2 alleles were amplified by PCR from cDNA (SI Appendix, Table 6), ligated into the binary vector pGC5341, and transformed into Agrobacterium tumefaciens strain GV22101. Each allele of SoIAA2 was transformed into Arabidopsis thaliana (Col-0) by floral dip method (45). Seeds were plated on 1/2 MS, 1% phytoagar, and 7.5 μg/mL of α-ammonium gluconate for selection of transformed seedlings. Homozygous plants of the subsequent generations containing one copy of the transgene were selected.

Root assays were performed in Plant Nutrient media (46) with 0.5% (w/v) sucrose and 0.6% agar, supplemented with synthetic and natural auxins (2,4-D, dicamba, and IAA, Caisson Labs). Seeds of S. orientale leaf disk was collected from the eighth leaf in watering 21-d-old plants. Homozygous plants of the subsequent generations containing one copy of the transgene were selected.

For gene-expression analysis under auxin treatments, soil-cultivated S. orientale (five to six leaves) and transgenic Arabidopsis lines (preflowering 16 leaves) were sprayed with 250 g/a ha−1 2,4-D, and 6 at 6 h after treatment, a leaf disk was collected from the eighth leaf in Arabidopsis and fourth for S. orientale, cDNA was generated, and gene expression of known Arabidopsis auxin-responsive genes GH3.3, AtIAA19, and AttIAA2 was analyzed by qPCR using primers listed in SI Appendix, Table 6.

In Silico Protein Structure Predictions. Susceptible (SoIAA2) and resistant (SoIAA2Δ9) protein sequences were used for IDR predictions using PrDos (47), IUPred (48), and Spot2 (49) algorithms. Kyte-Doolittle hydropathy maps were calculated by Expasy-linked ProtScale (50). The flexibility of the various portions of SoIAA2 and SoIAA2Δ9 was evaluated using Medusa (https://www.dsmb.inserm.fr/MEDUSA) (51).

IAA2 Protein Purification. The cDNAs corresponding to the two alleles of SoIAA2 were cloned into a pPFN2A (GST) flexi vector (Promega; primers in SI Appendix, Table 6), expressed in Ershcheria coli BL21, and purified using GST beads (Glutathione Sepharose 4B from GE according to the manufacturer manual. The GST-IAA2 proteins were recovered, GST was cleaved using TEV protease (100 μL at 400 μM; New England Biolabs), and GST beads were added to eliminate GST and undigested proteins. The final purified SoIAA2 and SoIAA2Δ9 proteins were then quantified using spectrophotometry and separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis to verify the purity.

SEC Purification and Retention Volume Studies. The cleaved IAA2 proteins were loaded into a GE Superdex 200 10/300 increase column equilibrated in CD buffer (10 mM Tris HCl, pH 7.0 and 150 mM NaClO4) and quantified using a Cary Bio-SD ultraviolet (UV)-Vis spectrophotometer. Proteins were quantified by a UV-280 nm detector. Protein oligomers were analyzed based on their retention volumes and compared to Bio-Rad gel filtration protein standards (15 to 600 kDa).

CD Analysis. CD was performed using a MOS-500 spectrometer (Biologic, France) scanning 185 to 285 nm with a 2-nm slit width at an acquisition rate of 2.1 s/min. Buffer and proteins were scanned in a polytetrafluoroethylene-impregnated 1-mm UV quartz cuvette (FireflySc). Protein concentration was between 1 and 7 μM, and the absorbance and photomultiplier tube voltage targeted were 1 AU and 500 V, respectively. CD measurements were taken three times, and the raw ellipticity values were averaged after buffer subtraction and converted to mean residue ellipticity or micromolar ellipticity values.

SPR Affinity Binding Analysis. SPR experiments were conducted according to the protocols described in Lee et al. (25) with modifications. TIR1/AFBs was expressed in Sf9 insect cell culture using a recombinant baculovirus encoding 10xHis:GFP:FLAG:TEV receptor and 10xHis:ASK1. Initial purification using the His-tag on nickel resin was followed by clean-up using FLAG chromatography. For SPR assays using a Biacore T200 (Cytiva), the purified protein was mixed with the appropriate concentration of each auxin before passing it over a streptavidin chip loaded with biotinylated SoIAA2 and SoIAA2Δ9 degron peptides (Thermo Fisher Scientific) (SI Appendix, Table 7).

The SPR buffer was Hepes-buffered saline (10 mM Hepes, 3 mM ethylenediaminetetraacetic acid, 150 mM NaCl, and 0.05% Tween 20). Binding experiments were run at a flow rate of 30 μL/min−1 using a series of 100 s of injection and dissociation times, followed by a final dissociation of 600 s, according to the manufacturer’s single-cycle kinetic routine. Data from a control channel (loaded with a mutated degron, mIAA7) (25) and from a buffer-only channel were subtracted from each sensogram following the standard double-reference subtraction protocol.

Homology-Based Modeling and Docking Analysis for TIR1 with SoIAA2 and SoIAA2Δ9. Homology models of portions of the sequences of SoIAA2 corresponding to the PB1 domain and degron were aligned to chain C of PDB: 2P1Q (2) and PDB: 2M1M (4), respectively, using Praline (52). These crystal structures were used to generate homology models of the degron and PB1 portions of SoIAA2 using Modeler (version 10.0) (53, 54). Homology models of portions of SoIAA2 corresponding to the PB1 motif and degron motif portions were aligned on TIR1 from PB2:2P1Q using ypmol, and the IDR segment connecting these two models was generated using ModLoop (55, 56). A similar process was followed to generate the SoIAA2Δ9 version of this protein. Amino acids involved in the interaction between SoIAA2 or SoIAA2Δ9 and TIR1 were identified using the InterfaceResidues.py ypmol script (https://pymolwiki.org/index.php/InterfaceResidues). These residues were used as docking parameters in high affinity driven protein-protein docking (HADDOCK) 2.2 (https://wennmr.science.uu.nl/haddock2.4) (57–59). Docking calculations were performed under expert level, deactivating the DNA/RNA functions and activating the Surface Contact Restraints to enforce contact between the molecules. Degron residues KNNN of SoIAA2 were assigned semiflexible properties during docking, whereas the shorter connection in SoIAA2Δ9 was not allowed to be flexible to preserve the structural integrity of PB1. The binding affinities of the SoIAA2/ΔTIR1 and SoIAA2Δ9/ΔTIR1 biological complexes were calculated using protein binding energy prediction (PRODIGY) (https://bianca.science.uu.nl/prodigy) (60, 61) for all top four poses from the best HADDOCK clusters.

Data Availability. RNA-sequencing data, transcript sequences, and protein docking calculations data have been deposited in NCBI GEO, NCBI GenBank, and Mountain Scholar [GEO Series accession no. GSE159202 (62); GenBank accessions OK631624 (63) and OK631625 (64); and Mountain Scholar DOI: https://dx.doi.org/10.25675/10217/234027 (65)].

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