Research Article

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A novel mutation in *IAA16* is associated with dicamba resistance in *Chenopodium album*

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Abstract

BACKGROUND: Resistance to dicamba in *Chenopodium album* was first documented over a decade ago, however, the molecular basis of dicamba resistance in this species has not been elucidated. In this research, the resistance mechanism in a dicamba-resistant *C. album* phenotype was investigated using a transcriptomics (RNA-sequence) approach.

RESULTS: The dose–response assay showed that the resistant (R) phenotype was nearly 25-fold more resistant to dicamba than a susceptible (S) phenotype of *C. album*. Also, dicamba treatment significantly induced transcription of the known auxin-responsive genes, *Gretchen Hagen 3* (*GH3*), small auxin-up RNAs (SAURs), and *1-aminocyclopropane-1-carboxylate synthase* (*ACS*) genes in the susceptible phenotype. Comparing the transcripts of auxin TIR/AFB receptors and auxin/indole-3-acetic acid (AUX/IAA) proteins identified from *C. album* transcriptomic analysis revealed that the R phenotype contained a novel mutation at the first codon of the GWPPV degron motif of *IAA16*, resulting in an amino acid substitution of glycine (G) with aspartic acid (D). Sequencing the *IAA16* gene in other R and S individuals further confirmed that all the R individuals contained the mutation.

CONCLUSION: In this research, we describe the dicamba resistance mechanism in the only case of dicamba-resistant *C. album* reported to date. Prior work has shown that the dicamba resistance allele confers significant growth defects to the R phenotype investigated here, suggesting that dicamba-resistant *C. album* carrying this novel mutation in the *IAA16* gene may not persist at high frequencies upon removal of dicamba application.

Supporting information may be found in the online version of this article.

Keywords: AUX/IAA proteins; dicamba; herbicide resistance; mechanism of resistance; synthetic auxin herbicides

1 INTRODUCTION

Chenopodium album L. is an annual weed species and a member of the Amaranthaceae family,[1] and is particularly troublesome in spring and summer crops.[2] Germination across a wide range of temperatures (5–30 °C) and large phenotypic and physiological plasticity under a wide range of environmental conditions are two primary weediness characteristics enhancing the competitive ability of *C. album* against crops.[3] Crop yield losses due to *C. album* competition can be significant, though its detrimental impacts on the yield loss vary according to crop, weed density and location.[4] In New Zealand, *C. album* is particularly troublesome in maize (*Zea mays* L.) and brassica crops.[5,6] In maize, *C. album* interference can negatively impact growth with up to 100% yield losses.[7,8]

The management of *C. album* involves both chemical and non-chemical approaches.[3] In New Zealand, the current weed management practice in maize involves spring cultivation and application of pre-emergent herbicides (e.g., acetochlor + atrazine), followed by the application of selective post-emergent herbicides such as dicamba.[9] In the early 1980s, resistance to atrazine was recorded for *C. album* in New Zealand,[10] and this was the first case of herbicide resistance ever documented for this country.[5] In 2005, resistance to dicamba in *C. album* was also reported,[11] with the phenotype exhibiting almost 19-fold greater resistance to this

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herbicide than susceptible plants.[12] It was noted that this dicamba-resistant *C. album* was cross-resistant to the synthetic auxins clopyralid, aminopyralid and picloram but not to 2,4-D (2,4-dichlorophenoxyacetic acid) and mecoprop-P.[13]

Dicamba belongs to the synthetic auxin herbicide group (HRAC Group 4), which mimic the natural auxin, indole-3-acetic acid (IAA).[14] Dicamba has been commonly applied alone or in combination with other herbicides for selective control of perennial and annual broadleaved weed species in cereal crops, pastures and turfgrass. Although dicamba has been used for more than five decades, resistance to this herbicide has been documented in only a small number of weed species, including *Sinapis arvensis* L.[15] and *Bassia scoparia* (L.) A.J.Scott[16] (formerly known as *Kochia scoparia*), with dicamba-resistant *C. album* having been reported only in New Zealand.[17]

Resistance to synthetic auxin herbicides can be conferred by non-target site resistance (NTSR)[18] and target-site resistance (TSR)[19] mechanisms. NTSR mechanisms such as impaired herbicide translocation,[20,21] enhanced herbicide metabolism,[22] rapid cell death,[23] and root exudation[24] have been reported in several synthetic auxin-resistant weed species. Recent studies have provided insights into the physiological and molecular basis of impaired herbicide translocation and enhanced herbicide metabolism mechanisms in synthetic auxin herbicide-resistant weeds. For instance, Goggin *et al.*[20] showed that impaired translocation of 2,4-D in *Raphanus raphanistrum* L. could be due to alterations in an adenosine triphosphate (ATP)-binding cassette subfamily B (ABCB) membrane transporter mediating 2,4-D transport. In *B. scoparia*, reduced translocation of dicamba was attributed to overexpression of the *Chalcone synthase* (*CHS*) gene, regulating the synthesis of flavonols.[25] The authors suggested that overexpression of this gene led to overproduction of flavonols which can compete with auxins for intercellular transport via ABCB transporters. Herbicide metabolism can be mediated by one or multiple known herbicide-detoxifying enzymes, such as cytochrome P450 monooxygenases (CYP), glutathione S-transferases (GST), and uridine 5′-diphospho-glucuronosyltransferase (UDP-GT).[18] Enhanced herbicide metabolism has been documented for weeds resistant to 2,4-D,[26] MCPA (2-methyl-4-chlorophenoxyacetic acid),[27] and mecoprop-P[28]; however, the biochemical pathways mediating the detoxification process in most cases are unknown. Recently, a comprehensive study attributed 2,4-D resistance in *Amaranthus tuberculatus* (Moq.) J.D.Sauer to rapid hydroxylation of 2,4-D by CYP followed by sugar conjugation by UDP-GT.[29] The authors noted that susceptible *Amaranthus tuberculatus* plants conjugated 2,4-D to aspartate to form 2,4-D-aspartic acid (2,4-D-Asp), which is still herbicidally active. Resistant *Amaranthus tuberculatus* has however evolved a two-phased detoxification mechanism to efficiently deactivate 2,4-D molecules.[29]

The TSR mechanisms of synthetic auxin herbicides have been shown to be conferred primarily by mutations in the Skp1-Cullin-F-box TIR1/AFB E3 ubiquitin ligase complex ($SCF^{TIR1/AFB}$) and transcriptional repressors (Aux/IAAs).[30] In *Arabidopsis thaliana* (L.) Heynh., mutations in the auxin receptors, transport inhibitor-resistant 1 (TIR1) and auxin F-box (AFB) receptor proteins of the $SCF^{TIR1/AFB}$ complex have conferred resistance to synthetic auxin herbicides, such as 2,4-D, dicamba and picloram.[31,32] Beyond *Arabidopsis thaliana*, however, no naturally occurring mutations have been documented to date in the $SCF^{TIR1/AFB}$ complex for weed species resistant to synthetic auxin herbicides. Target-site modifications of the *IAA* genes have also been implicated in insensitivity to natural and synthetic auxins in *Arabidopsis thaliana*.[33–35] Thus far, dicamba resistance in *B. scoparia*[36] and 2,4-D-resistance in *Sisymbrium orientale* L.[37] have been attributed to target-site modifications of the *IAA16* and *IAA2* genes, respectively.

Given that dicamba is a synthetic auxin herbicide important to agriculture globally, it would be useful to understand the mechanisms by which weeds evolve resistance to this herbicide. Previously, we showed that dicamba resistance in a phenotype of *C. album*, population R (formerly known as population L[12]) was not associated with any known NTSR mechanisms,[38] and dicamba resistance in this phenotype is governed by a single dominant gene.[39] In addition, we showed that the dicamba-resistant phenotype suffers from fitness costs.[40] In this article, we report the molecular basis for the dicamba resistance in this phenotype using a transcriptomic analysis.

2 MATERIALS AND METHODS

2.1 Plant material

One dicamba-resistant (R) population and one dicamba-susceptible (S) population of *C. album* both originated from Waikato, New Zealand that had been characterized previously[12] were used for dose–response experiments and to develop F_2 generations. The F_2 generations in this predominantly self-pollinating species was developed using methods described previously.[39] Briefly, the R phenotype went through six rounds of recurrent selection to ensure the homozygosity of the individuals for the dicamba resistance trait. The F_1 progenies were generated by pair-crossing between R and S individuals. Subsequently, the F_1 progenies were treated with 400 g a.e. ha^{-1} of dicamba (Kamba 500, Nufarm, Auckland, New Zealand as dimethylamine salt), and the individuals ($n = 8$) from S mother plants that survived the treatment were self-pollinated to produce F_2 progenies. These eight individuals manifested the same phenotypic characteristics associated with dicamba resistance as reported previously,[11] and did not develop any symptoms typical of dicamba susceptible plants. The F_2 individuals ($n = 8$) of each R and S phenotype were self-pollinated in separate pollen-proof cages to generate F_3 progenies. One of the resultant F_3 family of each phenotype were used for RNA-sequencing (RNA-Seq) and the remaining F_3 families were used for Sanger sequencing. The R and S individual plants of the F_3 family used for the RNA-Seq were selected based on their phenotypic characteristics reported previously.[39]

2.2 Dose–response experiments

The response of R and S phenotypes of *C. album* to dicamba was evaluated in dose–response experiments. The seeds of each phenotype were pre-germinated as described previously,[12] and the seedlings ($n = 10$) were transplanted in planter bags with a diameter of 18 cm containing potting media [Pacific Pumice (7 mm), 30% fiber, 50% bark and slow-release fertilizer (Woodace, PA, USA)]. Plants were maintained in a glasshouse (maximum/minimum temperatures = 22 °C/17 °C, relative humidity = 50%, photoperiod = 14 h light/8 h dark). At the four-leaf stage, plants were treated with dicamba (Kamba 500; Nufarm) rates of 0, 50, 100, 200, 400, 800, 1600, 3200, 6400 and 12 800 g a.e. ha^{-1} using a track sprayer calibrated to deliver 230 L ha^{-1} at a pressure of 200 kPa. The number of plants that survived at each rate was recorded 4 weeks after treatment. This experiment was conducted in a randomized design with three replicates (i.e., three planter bags) and was repeated in time.

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2.3 RNA-sequencing

The F_3 individuals of R and S phenotypes were pre-germinated[12] and each transplanted to 0.7-L plastic pots containing potting media described earlier. The plants were grown to a height of 7 cm in a glasshouse (daily maximum/minimum temperatures = 25 °C/18 °C, relative humidity = 50%, and photoperiod = 14 h light/10 h dark), before they were treated with 400 g a.e. ha^{-1} of dicamba (Kamba 500; Nufarm) using a precision sprayer calibrated to deliver 230 L ha^{-1} of spray solution at 200 kPa. The experimental design included three harvest times, namely 0 (untreated), 24 and 48 h after dicamba application, with three biological replicates of the R and S phenotype harvested for each time course. The harvested leaf material was snap-frozen using liquid nitrogen. RNA was extracted from leaf material of each sample using a commercial kit (Qiagen RNeasy Plant Mini Kit; Qiagen, Hilden. Germany), following the manufacturer's instructions. RNA sample quality was evaluated using a Qubit HS RNA assay kit (Thermo Fisher Scientific, Dreieich, Germany) and a LabChip RNA assay (Agilent RNA 6000 Nano Labchip kit; Agilent Technologies, Waldbronn, Germany). Non-strand-specific RNA-Seq libraries were prepared from RNA samples using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Hitchin, UK). Libraries were sequenced on an Illumina NovaSeq6000 platform (Novogene, Singapore) to generate 150-bp paired-end reads.

The quality of raw RNA-Seq reads from the samples of both phenotypes was assessed using FastQC tools version 0.11.9,[41] and the low-quality reads were removed and adaptors were trimmed using Trimmomatic (version 0.39).[42] The trimmed data from an R and an S plant were assembled separately using Trinity (version 2.14.0).[43] The two assemblies were merged, and redundant contigs were removed using CD-Hit version 4.8.1,[44] resulting in 74 693 assembled transcripts. These transcripts were functionally annotated by alignment to the National Center for Biotechnology Information (NCBI) protein databases using Diamond (version 0.9.25),[45] with a cut-off of E-value of 10^{-5}. The merged assemblies were used as the reference transcriptome to align reads from R and S phenotypes with STAR (version 2.7.10).[46] The contigs with annotations in the *TIR1/AFB* and the *Aux/IAA* gene families were inspected for single nucleotide variants between R and S phenotypes using Integrative Genomics Viewer (IGV) version 2.12.3.[47] Differential expression was conducted using raw read counts extracted with featureCounts version 2.0.3).[48] Differentially expressed genes (DEGs) were identified with the edgeR package[49] using the statistical software R version 4.2.1.[50] The expression of genes for each phenotype at 24 and 48 h after dicamba treatment was compared to time 0, and genes with a $\log_2$ fold change (FC) > 2, and statistically significant at adjusted $P \leq 0.05$, were considered differentially expressed.

2.4 Sequence verification of the mutation in *IAA16*

The *IAA16* gene was partially sequenced for the 60 individuals of each R and S phenotype of *C. album*. For this, a polymerase chain reaction (PCR) was used to amplify a 320-bp region encompassing the degron region (GWPPV)[36] of the IAA16 protein using a pair of primers, PF11:5′-AGGATACCAAGGCGGAACAAG-3′ and PR11:5′-AGGAGCTGAACATTTTGCCTA-3′. Amino acid substitutions in this degron have been shown to underpin TSR to dicamba/synthetic auxin herbicides. RNA was extracted from freshly collected leaves of each sample using a Tiangene commercial kit (Tiangen Biotech, Beijing, China), following the manufacturer's instructions. The complementary DNA (cDNA) was subsequently synthesized using cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, Nanjing, China), following the manufacturer's instructions. The PCR amplification reaction contained 10 ng of template cDNA, 0.5 μM of each primer, 10 mM of dNTPs, 2.5 μL of 10X Taq Buffer and 0.2 μL of LA Taq Polymerase (TaKaRa, Dalian, China) and nuclease-free water to bring the reaction volume to 25 μL. The PCR cycling program was 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C, 60 s extension at 72 °C, followed by a final extension at 72 °C (one cycle of 5 min). The PCR products were visualized and examined on an agarose 1× lithium borate 2% (*w/v*) gel prior to sequencing by Sangon Biotech Company (Shanghsi, China). The presence of the novel mutation was confirmed by inspecting the sequence data using an online sequence alignment tool, Emboss Needle (https://www.ebi.ac.uk).

2.5 Protein modeling of IAA16 amino acid substitutions

A visual inspection of the effect of amino acid substitutions in the IAA16 degron (GWPPV), described earlier, was performed by generating models of the wild-type (S), the R phenotype, and an asparagine (Asn) mutant at position 131 in the *C. album* IAA16 using the online tool Alphafold2 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=_sztQyz29DlC).[51] The targetable loops/constructs were visualized using PyMOL Molecular Graphics System version 2.0.

2.6 Statistical analysis

The data from the dose–response experiment were fitted to a three-parameter log-logistic model after they were assessed for normality and homogeneity of variance using Shapiro–Wilk test and Levene's test, respectively. A two-stage meta-analysis approached[52] using *medrc* package in R was employed to fit the data to the following model:

$$Y = [d/1 + \exp(b(\log(x) - \log(LD_{50})))] \quad (1)$$

where Y is the percentage of surviving plants relative to untreated control, d is the upper limit (fixed at 100% for these

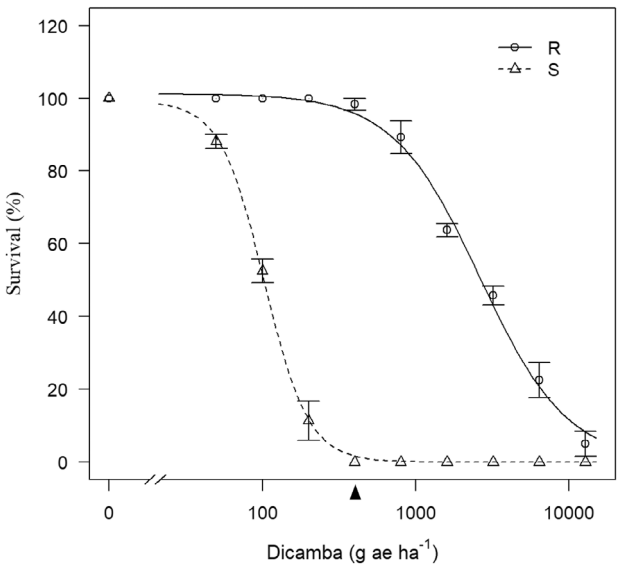

Figure 1. Assessing dicamba resistance in *Chenopodium album* using a dose–response experiment. The response of dicamba-resistant (R) and dicamba-susceptible (S) populations to dicamba was fitted to a three-parameter log-logistic model. The percentage survival of treated plants was used to produce the fitted curves using *medrc* package in R. The recommended rate for dicamba (400 g a.e. ha^{-1}) is denoted with a black triangle.

experiments), and x is the herbicide rate. The LD_{50} denotes the herbicide rate corresponding to a 50% reduction in plant survival and b is the slope around LD_{50}. The LD_{50} values for the R and S phenotypes were statistically compared using the *compParm* function.[52]

3 RESULTS AND DISCUSSION

3.1 Dose–response experiments

The response of R and S phenotypes to dicamba was evaluated in glasshouse dose–response experiments. No mortality was recorded for the R phenotype at the recommended rate of dicamba (400 g a.e. ha^{-1}), while all individuals of the S phenotype were controlled at this rate (Fig. 1). Increasing the dicamba rate, however, gradually increased the mortality rate of the R phenotype, with 100% mortality recorded for the R phenotype at 12 800 g a.e. ha^{-1} of dicamba. The LD_{50} values of the R and S phenotypes were 2591 and 102 g a.e. ha^{-1}, respectively (Table 1). Based on the R/S LD_{50} ratio, the R phenotype was 25.3 times more resistant to dicamba than the S phenotype. Similarly, a high level of dicamba resistance has also been reported for a *B. scoparia* population from Nebraska, and the R population was found to be almost 30 times more resistant to dicamba.[53] By contrast, lower levels of resistance to dicamba have been documented in another *B. scoparia* population from Montana (five-fold)[16] and a population of *Soliva sessilis* Ruiz & Pav. from New Zealand (14-fold).[54] The differences in the resistance level between *C. album* and *B. scoparia* from Montana and *Soliva sessilis* may be due to the presence of different resistance mechanisms.

Table 1. Parameters (see footnote) estimated from the three-parameter log-logistic model analysis of dicamba dose–response experiments for dicamba-resistant (R), dicamba-susceptible (S) of *Chenopodium album* at 4 weeks after treatment

Family	b (± SE)	LD_{50} (± SE)	Resistance index
S	1.5 ± 0.2	102.0 ± 3.2	
R	3.1 ± 0.3	2591.0 ± 125.4	25.3

Note: b represents the slope around the LD_{50}, where LD_{50} is the rate of herbicide (g a.e. ha^{-1}) required to cause 50% mortality. The parameter d (i.e., upper limit) was fixed to 100%. SE, standard error.

Table 2. Mean log_2 fold change (FC) in the expression of auxin-responsive, photosynthetic process and abscisic acid (ABA) biosynthesis genes in response to dicamba application at 24 and 48 h after treatment (HAT) by comparing the expression of genes at each timepoint to time 0 in dicamba-resistant and dicamba-susceptible *Chenopodium album*

		Mean log_2 FC			
		Dicamba-susceptible		Dicamba-resistant	
Gene ID	Annotation	24 HAT	48 HAT	24 HAT	48 HAT
Auxin responsive genes					
MSTRG.8283	Indole-3-acetic acid-amido synthetase GH3	1.98*	2.49*	−1.02	0.29
MSTRG.45521	Auxin-responsive protein IAA29	3.03*	3.09*	0.16	0.95
MSTRG.39391	Auxin-responsive protein SAUR66	1.53*	1.53*	0.38	0.72
MSTRG.45055	Auxin-responsive protein SAUR67	2.38*	2.34*	−0.47	−0.35
MSTRG.7734	Ethylene-responsive transcription factor RAP2	2.60*	3.34*	0.29	0.74
MSTRG.42511	Ethylene-responsive transcription factor 1B	2.90*	2.93*	−0.56	−0.78
MSTRG.48996	1-Aminocyclopropane-1-carboxylate synthase	1.30*	1.91*	0.69	1.18
Photosynthesis genes					
MSTRG.392	Photosystem II D1 precursor processing protein PSB27-H2	−0.55	−0.76*	−0.01	−0.11
MSTRG.20481	Photosystem I P700 apoprotein A1	−0.30	−0.79*	−0.80	−0.74
MSTRG.4058	Early light-induced protein 1	−1.03	−3.07*	−0.01	−1.67*
MSTRG.26593	RuBisCO large subunit-binding protein subunit alpha	−0.62	−1.37*	−0.08	−0.73
MSTRG.7337	Magnesium chelatase subunit H	−0.65	−0.94*	−0.84	−0.53
MSTRG.37712	Omega-3 fatty acid desaturase	−0.78	−1.70*	−0.74	−0.37
MSTRG.37925	Cytochrome b6-f complex iron–sulfur subunit	−2.08*	−1.70*	−1.29	−0.90
MSTRG.35516	Fatty acid hydroperoxide lyase	−1.05	−1.53*	−0.29	−0.94
MSTRG.21832	Zinc metallopeptidase EGY3	−1.48*	−1.40*	−0.44	−1.08
MSTRG.39525	Alpha carbonic anhydrase 1	−0.39	−0.86*	−0.02	−0.64
ABA biosynthesis and inactivation genes					
MSTRG.26797	Abscisic-aldehyde oxidase	−2.19*	−1.28	−0.36	0.28
MSTRG.10528	Abscisic acid 8′-hydroxylase 1	1.26	2.89*	0.77	1.72

Note: Positive and negative values correspond to up-regulated and down-regulated genes, respectively. Significant values (adjusted $P \leq 0.05$) are indicated by asterisks.

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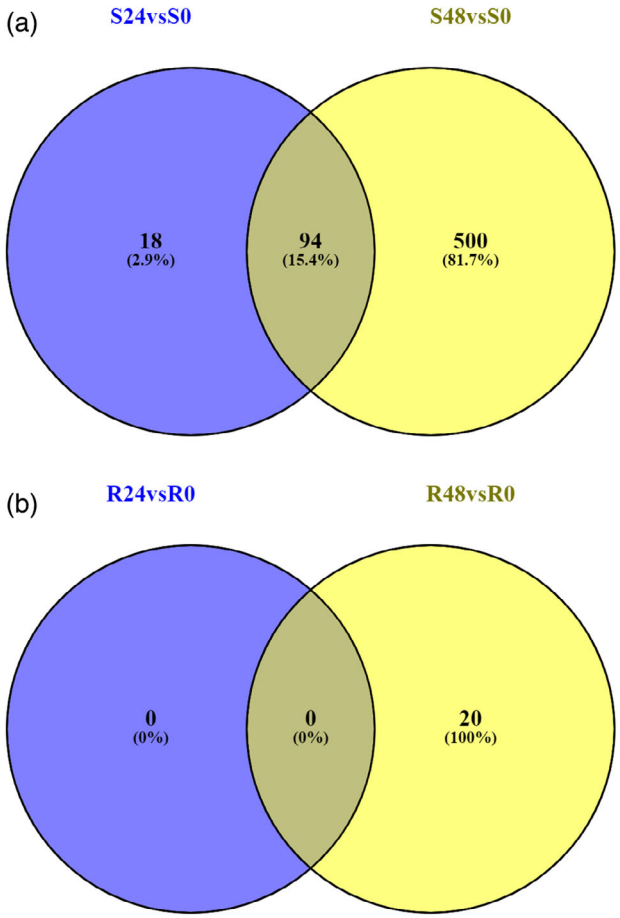

Figure 2. Venn diagrams showing transcripts that were differentially expressed between the untreated condition and either 24 or 48 h after treatment (HAT) with dicamba in (a) dicamba-susceptible (S) and (b) dicamba-resistant (R) populations.

3.2 RNA-Seq gene expression analysis

An average of 11.0 Gb of transcriptome data was generated by the Illumina NovaSeq6000 platform, with an average of 95 000 000 clean reads per sample. A *de novo* reference transcriptome was generated, and the reads were mapped to this reference transcriptome. Following alignment, differential expression between dicamba-resistant and -susceptible phenotypes was evaluated. The results showed that, relative to time 0, there are 112 and 594 DEGs for the S phenotype at 24 and 48 h after treatment (HAT), respectively, with 94 genes being commonly differentially overexpressed at both time points (Fig. 2(a)). Conversely, the number of DEGs in the R phenotype was found to be markedly smaller than that of the S phenotype in response to dicamba treatment, with zero and 20 genes being differentially expressed at 24 and 48 HAT, respectively, relative to time 0 (Fig. 2(b)). Among the DEGs, differential expression of the genes induced by synthetic auxin herbicide application was recorded in both phenotypes (Table 2). The level of expression of the *Gretchen Hagen 3 GH3*, auxin-responsive protein *IAA29*, *small auxin-up RNAs* (*SAURs*), and *1-aminocyclopropane-1-carboxylate synthase* (*ACS*) genes were found to be differentially expressed in both phenotypes after dicamba application. However, in all cases, the relative expression of the earlier-mentioned auxin-responsive genes was significantly greater for the S phenotype (adjusted *P*-value ≤ 0.05), suggesting that dicamba application did not induce typical downstream expression of auxin-responsive genes in the R phenotype. Auxin-responsive genes, such as the *GH3* and *ACS*, are transcribed rapidly in plants treated with high levels of auxin[55] and induction of these auxin-responsive genes has been reported in other susceptible weed species treated with synthetic auxin herbicides.[25,29]

It has been noted that the *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene, a key enzyme involved in the biosynthesis of abscisic acid (ABA), was up-regulated significantly at 1 and 6 HAT in *Erigeron canadensis* following 2,4-D, dicamba and halauxifen-methyl treatment, leading to rapid production of ABA in the treated plants.[56] The research also showed that the increased level of ABA was independent of ethylene levels or a loss of cell turgor and concurrently led to the down-regulation of many key genes associated with photosynthesis. The authors proposed that the primary mode of plant death triggered by synthetic auxin herbicides is rapid ABA biosynthesis and whole-scale down-regulation of photosynthesis genes.[56] In this current research, we also recorded significant down-regulation of ten photosynthesis-related genes in the S phenotype, especially at 48 HAT (Table 2). However, the relative expression level of the *NCED* gene was not significant ($\log_2$ FC < 2.0 and adjusted *P*-value > 0.5) in the S phenotype. In addition, in the S phenotype, the expression of the *abscisic aldehyde oxidase* gene, a key enzyme catalyzing the last step of ABA biosynthesis,[57] was down-regulated significantly at 24 HAT, while the relative expression of the abscisic acid 8′-hydroxylase, which catalyzes the first step in the catabolism of ABA[58] was up-regulated significantly at 48 HAT. The lack of significant expression of the *NCED* gene at 24 and 48 HAT in the current study, in contrast to that observed by McCauley *et al.*[56] at 1 and 6 HAT, suggests that ABA homeostasis in plants treated with synthetic auxin herbicides may not only be maintained by the biosynthesis process but it is coordinated by a regulatory circuit that involves both biosynthesis and catabolism of ABA.[59]

3.3 Evaluating auxin receptors, auxin transporters and auxin-responsive proteins for single nucleotide polymorphisms and differential expression

Candidate genes involved in auxin transport and regulatory response were identified from *C. album* transcriptomic analysis (Table 3) and compared between the R and S phenotypes. According to the results, no significantly differential expression was recorded for any of the genes between both phenotypes at both time points, except for the *IAA29*, which was significantly overexpressed in the S phenotypes at both 24 and 48 HAT. According to Xu *et al.*[60] the expression of the *IAA29* can be induced by ethylene and ABA, which are hormones highly promoted by the application of synthetic auxin herbicides in plant species susceptible to this group of herbicides.

After examining and comparing the transcripts, we detected a non-synonymous single nucleotide polymorphism, substituting a glycine (G) with aspartic acid (D) amino acid as a result of a change from guanine to adenine in the second base of the first codon of the highly conserved degron region (GWPPV) of the *IAA16* gene (Fig. 3). Previously, it was shown that a glycine (G) to asparagine (N) amino acid substitution at this codon caused resistance to various synthetic auxin herbicides, including dicamba in *B. scoparia*.[36] This degron region is essential for auxin binding and interaction with TIR/F-box and AUX/IAA proteins,[61] and mutations within this region result in resistance to synthetic auxin herbicides.[30] For instance, a cytosine to thymine mutation, altering the first proline in the conserved GWPPV degron motif of *IAA16*

Table 3. Mean log_2 fold change (FC) in the expression of auxin receptor and transporter genes in response to dicamba application at 24 and 48 h after treatment (HAT) by comparing the expression of genes at each timepoint to time 0 in dicamba-resistant and dicamba-susceptible *Chenopodium album*

		Mean log_2 FC			
		Dicamba-susceptible		Dicamba-resistant	
Gene ID	Annotation	24 HAT	48 HAT	24 HAT	48 HAT
MSTRG.3974	ABCB1	−0.17	0.44	0.06	0.12
MSTRG.40760	ABP1	0.09	−0.13	0.17	0.01
MSTRG.13778	AFB3	−0.42	−0.18	−0.29	0.07
MSTRG.41298	AFB5	0.40	0.15	−0.21	−0.12
MSTRG.22361	AUX1	−0.19	0.01	−0.27	0.17
MSTRG.3668	AUX4	−0.02	−0.45	0.25	0.22
MSTRG.38462	IAA12	0.59	0.85	−1.4	−0.86
MSTRG.39920	IAA14	−0.11	−0.45	0.25	−0.47
MSTRG.4454	IAA16	0.83	0.91	0.53	0.78
MSTRG.16930	IAA26	1.32	0.95	0.22	0.16
MSTRG.45492	IAA27	−0.37	1.20	1.17	0.31
MSTRG.45523	IAA29	3.41*	3.19*	0.66	1.02
MSTRG.14204	IAA9	0.16	−0.43	−0.06	0.61
MSTRG.18714	IAA33	0.28	−0.20	0.62	0.71
MSTRG.14204	IAA31	0.16	−0.43	−0.06	0.61
MSTRG.47022	LAX2	1.68	0.49	0.59	−0.48
MSTRG.15398	LAX3	0.21	0.23	−1.16	0.22
MSTRG.3643	PIN1	0.73	0.68	0.07	0.39
MSTRG.9061	PIN3	0.94	1.08	0.19	0.35
MSTRG.40025	PIN5	−0.52	−0.59	0.58	0.68
MSTRG.17154	SKP2A	0.41	1.37	0.12	0.89
MSTRG.13781	TIR1	−0.64	−0.88	−0.84	−1.13
MSTRG.6913	ARF18	0.17	0.29	0.34	0.25
MSTRG.17076	ARF2	0.29	0.01	−0.15	−0.07
MSTRG.37545	ARF6	1.03	0.62	0.76	0.88
MSTRG.27729	ARF7	0.08	0.21	0.39	0.61
MSTRG.39601	ARF8	−0.42	0.76	0.53	0.70
MSTRG.41953	ARF9	−0.07	−0.36	0.25	0.26

Note: Positive and negative values correspond to up-regulated and down-regulated genes, respectively. Significant values (adjusted $P \leq 0.05$) are indicated by asterisks.

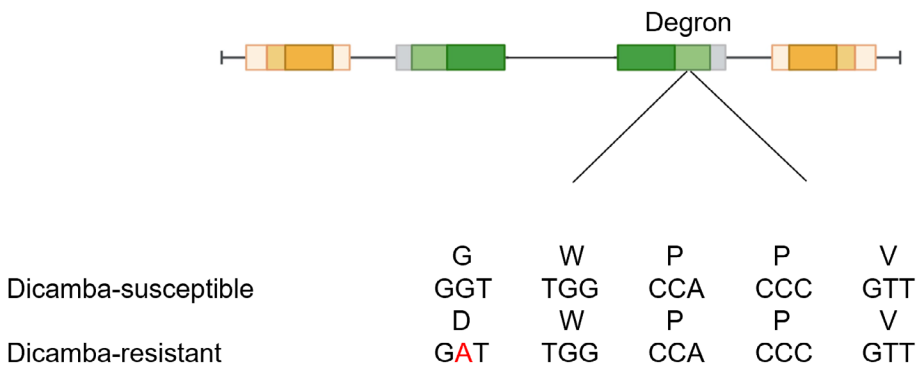

Figure 3. Schematic representation of a glycine (G) to aspartic acid (D) amino acid substitution due to a change from guanine to adenine in the second base of the first codon of the highly conserved degron region (GWPPV) of the *IAA16* gene. This G to D substitution in the degron of the *IAA16* gene confers resistance to dicamba in *Chenopodium album*.

to leucine, has been demonstrated to confer resistance to auxin and synthetic auxin herbicides in *Arabidopsis thaliana*.[35] However, to the best of our knowledge, a G to D substitution at the first codon of the GWPPV degron motif of *IAA16* has not been documented before; thus, dicamba resistance in this *C. album* population is conferred by a novel mutation.

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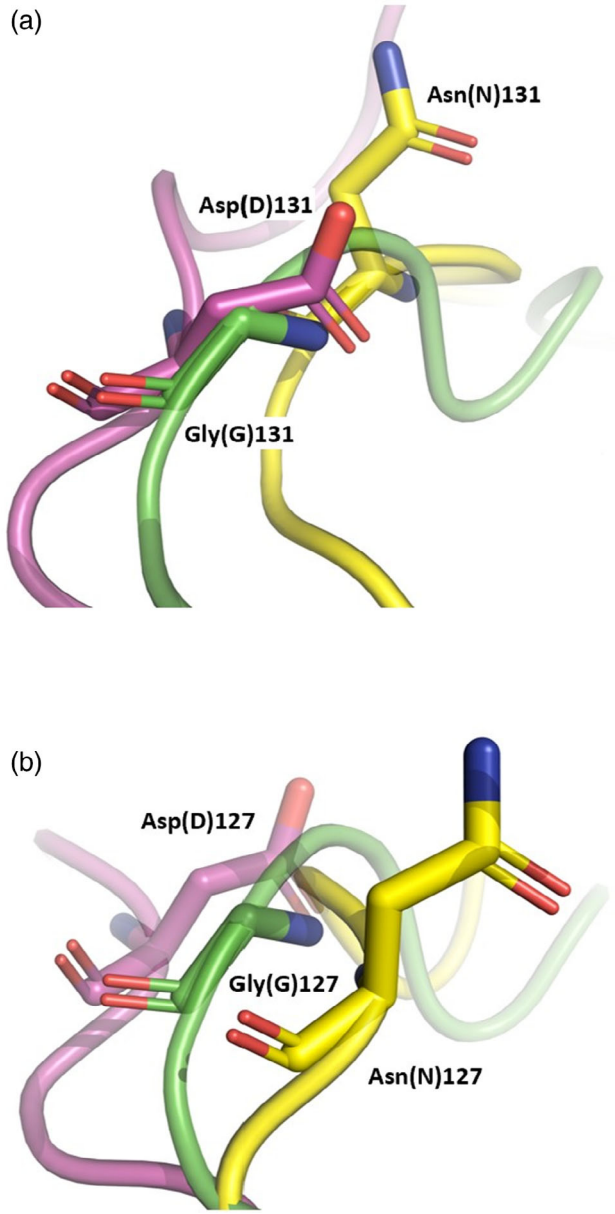

Figure 4. Superimposition of protein models showing the influence of amino acid substitutions in the degron motif in IAA16 proteins. (a) The *Chenopodium album* wild-type (green) dicamba sensitive IAA16 degron motif region [glycine (Gly) position 131] and the influence of substitution with aspartic acid (Asp; magenta) and asparagine (Asn; yellow). (b) The *Bassia scoparia* wild-type (green) dicamba sensitive IAA16 degron motif region [glycine (Gly) position 127] and influence of substitution with aspartic acid (Asp; magenta) and asparagine (Asn; yellow).

3.4 The *IAA16* gene sequencing and protein modeling

To confirm if the G to D substitution is also present in other R individuals and absent in S individuals, the 320-bp region of the *IAA16* messenger RNa (mRNA) encompassing the GWPPV degron motif was amplified and sequenced from 60 individuals each of the R and S phenotype. The results showed that a G to D substitution was present in all R individuals. Conversely, none of the S individuals exhibited this substitution in the degron, indicating that the G to D mutation was associated with dicamba resistance. No other non-synonymous amino acid substitutions were recorded between both phenotypes in the region encompassing the GWPPV degron motif in the *IAA16* gene.

The G to N substitution In *B. scoparia* GWPPV degron resulted in resistance to synthetic auxin herbicides, including dicamba.[36] The N and D amino acids are comparable in the sidechain structure and volume and have almost identical molecular weight (Supporting Information Fig. S1). This suggests the G to D substitution identified in the GWPPV degron of *C. album* IAA16 would experience spatial conformity changes very similar to an N substitution. This was corroborated when modeling these changes in the IAA16 protein (Fig. 4(a)). Focusing on a model of the degron motif of the *B. scoparia* IAA16 protein showed that substituting the G with N, which gave rise to the dicamba resistance,[36] has a very similar conformation when substituted with D (Fig. 4(b)). The structural similarities of N and D strongly suggest that the D underpins the degron alteration giving rise to dicamba resistance in *C. album*. In addition to the structural alteration in the degron induced by the G to N or D substitutions, D has a charged carboxylate on the sidechain which, unlike the neutral N, would introduce electrostatic repulsion between like charges within the receptor and protein. This would further perturb their interaction, interfering with auxin binding and driving dicamba resistance. LeClere *et al.*[36] noted that a G to N substitution in the degron of the IAA16 protein perturbed the interaction of this protein with TIR1 and AFB6 in the presence of dicamba. Here we showed that the causal basis for this perturbation is likely the conformational changes in the structure of the IAA16 protein generated as a result of a G to N replacement in the degron. Given that a G to D substitution can cause similar conformation changes in the degron of the IAA16 protein, it is very likely that the dicamba resistance observed in *C. album* is due to the mutation in the *IAA16* of dicamba-resistant phenotype.

4 CONCLUSION

In New Zealand, synthetic auxin herbicides have been extensively used in pastures, turf, maize and brassica crops,[54,62] leading to the development of unique cases of resistance to this group of herbicides in weed species across sectors. In this research, we report on the dicamba resistance mechanism in *C. album*, which is unique and the only dicamba resistance case reported in this species globally. The results described in this research confirmed that dicamba resistance in *C. album* is associated with a novel mutation in the GWPPV degron of the *IAA16* gene. Previously, it was shown that dicamba resistance in this R population was governed by a single gene,[39] which is in agreement with the findings of this research. It was also shown that the R gene is dominant and can be transmitted via pollen and seeds, suggesting that dicamba resistance in *C. album* can be spread to other farms. However, due to significant growth defects and the lack of competitiveness with crops recorded for the R population,[40] it was hypothesized that dicamba resistance would not persist at high frequencies in New Zealand maize farms upon removal of dicamba application.[5] This hypothesis has been supported by the results from a recent survey in maize grown in the Waikato region where dicamba resistance in *C. album* had been documented for the first time, yet none of the recently evaluated *C. album* samples were dicamba-resistant.[63]

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raw RNA-sequencing data from this article have been deposited with the National Center for Biotechnology Information (NCBI) data libraries under the accession number PP194760 and the BioProject Number PRJNA1066517, respectively. Open access publishing facilitated by Massey University, as part of the Wiley - Massey University agreement via the Council of Australian University Librarians.

DATA AVAILABILITY STATEMENT

The IAA16 sequence and the raw RNA-sequencing data from this article have been deposited with the National Center for Biotechnology Information (NCBI) data libraries under the accession number PP194760 and the BioProject Number PRJNA1066517, respectively.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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