Ectopic expression of a rice triketone dioxygenase gene confers mesotrione tolerance in soybean

Shunhong Dai, Nikolaos Georgelis, Mohamed Bedair, Yun-Jeong Hong, Qungang Qi, Clayton T Larue, Bikram Sitoula, Wei Huang, Brian Krebel, Michael Shepard, Wen Su, Keith Kretzmer, Jiaxin Dong, Thomas Slewinski, Sarah Berger, Christine Ellis, Agoston Jerga and Marguerite Varagona

Abstract

BACKGROUND: Herbicide-resistant weeds pose a challenge to agriculture and food production. New herbicide tolerance traits in crops will provide farmers with more options to effectively manage weeds. Mesotrione, a selective pre- and post-emergent triketone herbicide used in corn production, controls broadleaf and some annual grass weeds via hydroxyphenylpyruvate dioxygenase (HPPD) inhibition. Recently, the rice HIS1 gene, responsible for native tolerance to the selective triketone herbicide benzobicyclon, was identified. Expression of HIS1 also confers a modest level of mesotrione resistance in rice. Here we report the use of the HIS1 gene to develop a mesotrione tolerance trait in soybean.

RESULTS: Conventional soybean is highly sensitive to mesotrione. Ectopic expression of a codon-optimized version of the rice HIS1 gene (TDO) in soybean confers a commercial level of mesotrione tolerance. In TDO transgenic soybean plants, mesotrione is rapidly and locally oxidized into noninhibitory metabolites in leaf tissues directly exposed to the herbicide. These metabolites are further converted into compounds similar to known classes of plant secondary metabolites. This rapid metabolism prevents movement of mesotrione from treated leaves into vulnerable emerging leaves. Minimizing the accumulation of the herbicide in vulnerable emerging leaves protects the function of HPPD and carotenoid biosynthesis more generally while providing tolerance to mesotrione.

CONCLUSIONS: Mesotrione has a favorable environmental and toxicological profile. The TDO-mediated soybean mesotrione tolerance trait described here provides farmers with a new option to effectively manage difficult-to-control weeds using familiar herbicide chemistry. This trait can also be adapted to other mesotrione-sensitive crops (e.g. cotton) for effective weed management.

© 2022 Bayer Crop Science. Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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

Keywords: triketone dioxygenase (TDO); mesotrione; herbicide tolerance trait; hydroxyphenylpyruvate dioxygenase (HPPD); phytoene desaturase (PDS); carotenoids

1 INTRODUCTION

Increasing sustainable crop production is crucial to meet the needs of food for the growing global human population, feed for increased demand for animal-based diets in developing nations, and the expanded use of crop products to produce biofuel, fiber, and other agricultural product-based commodities, while using limited natural resources.1 Weed control is one of the major challenges to sustainable crop production. Herbicide application is an important method to reduce weed pressure, improve productivity, and increase security for global crop production. Selective herbicides were major contributors to improve crop production before the deployment of herbicide-tolerant (HT) crops.2,3 The cultivation of HT crops coupled with the application of corresponding herbicides can effectively manage weeds, improve crop productivity and quality, enhance the content of soil organic matter, improve soil health, and reduce fuel consumption and emission by reducing tillage.2-5
Deployment of glyphosate tolerant (GT) crops, alongside application of glyphosate, revolutionized modern agriculture by providing an effective alternative to using traditional selective herbicides for weed control.\(^6\) GT crops were the most rapidly adopted technology in the history of agriculture, which was primarily due to the nonselective, very effective, and relatively inexpensive nature of glyphosate.\(^6,7,8\) Better weed control in GT crops improved crop production efficiency, e.g. growing GT soybean resulted in an average yield increase of 7% in developed countries and 21% in developing countries with smallholder farmers.\(^6,8\)

Due to the intensive use of herbicides for general weed control, the number of weed species resistant to one or more herbicide sites of action (SOA) has steadily increased (http://www.weedscience.org/).\(^9\) Broad adoption of GT crops coupled with the concurrent application of glyphosate has also caused an increased number of glyphosate-resistant weed species.\(^10\) Herbicide resistance increases the cost of weed management and limits herbicide options to control some resistant weeds.\(^11,12\) Developing and deploying crops with tolerance to diverse herbicide SOAs as trait stacks is essential to enable farmers to effectively manage weeds. Crops with stacked herbicide tolerance traits will also help slow the pace of herbicide resistant weed evolution.\(^13,12,13\)

Hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors are among the newest herbicide SOAs following their introduction in the 1980s.\(^13,14\) HPPD irreversibly converts hydroxyphenylpyruvic acid (HPPA) into homogentisic acid (HGA) in plants. HGA is the substrate to produce α-tocopherols (vitamin E, important for protecting biological membranes against oxidative stress and the photosynthetic apparatus against photo-inactivation), plastquinone [PQ, a cofactor of phytoene desaturase (PDS), important for carotenoid biosynthesis and protection of photosynthesis], and acetoacetaldehyde for central metabolism.\(^15,17\) HPPD-inhibiting herbicides abrogate tyrosine degradation in nonresistant plants, which subsequently stops the production of PQ and vitamin E, negatively affects carotenoid biosynthesis, causes leaf bleaching symptoms, and ultimately leads to plant death.\(^14,18\)

Mesotrione, a β-triketone herbicide, is an extremely potent competitive inhibitor of HPPD. In weed species it is rapidly absorbed following foliar application, translocated acropetally and basipetally, and causes severe bleaching symptoms and plant death.\(^18\) Originally developed as a selective herbicide for controlling weeds in corn, mesotrione provides pre- and post-emergence control of important broad-leaf and some annual grass weeds.\(^18\) Mesotrione has pre-emergence activity ranging from 4.5 to 32 days, depending on soil pH, and shows no risk of carry-over in rotational crops.\(^18,19\) Because of the favorable environmental and toxicological profiles, mesotrione and other HPPD inhibitors were considered ‘reduced risk pesticides’ by the US Environmental Protection Agency.\(^21\)

While useful in corn, mesotrione cannot be used to manage weeds in many other crops (e.g. soybean) due to the sensitivity to this herbicide.\(^18\) Maeda \textit{et al.} identified the rice \textit{HPPD INHIBITOR SENSITIVE 1 (HIS1)} gene, which is responsible for the tolerance to benzobicyclic, based on varietal variation in sensitivity to this selective herbicide.\(^22\) \textit{HIS1}, encoding a 351 amino acid Fe(II)/2-oxoglutarate-dependent oxygenase, can also confer a modest level of tolerance to other β-triketone herbicides in rice, including mesotrione.\(^22\) In this study we report our efforts on utilizing the rice \textit{HIS1} gene to develop a mesotrione tolerance trait in soybean. The rice \textit{HIS1} was codon optimized for better expression in soybean, and the optimized version of \textit{HIS1} is designated as \textit{TDO} (Triketone dioxygenase gene). TDO transgenic soybean plants showed strong tolerance to mesotrione (up to 16× the commercial application rate). Ectopic expression of \textit{TDO} in soybean showed no other effects on plant growth and development. Ectopically expressed \textit{TDO} rapidly converts mesotrione into noninhibitory compounds at the point of application that are further catabolized into metabolites that are similar to known classes of plant secondary metabolites. Rapid and local turnover of mesotrione in \textit{TDO} transgenic soybean plants prevents mesotrione moving from treated leaves to vulnerable emerging leaf tissues and inhibiting the function of HPPD. This rapid metabolism of mesotrione protects the function of PDS and carotenoid biosynthesis and prevents mesotrione-induced herbicide injury. Ectopic expression of \textit{TDO} in soybean resulted in commercial-level mesotrione tolerance for an HT trait that will provide growers with more options in managing difficult-to-control weeds.

\section{2 MATERIAL AND METHODS}

\subsection{2.1 Cloning of \textit{TDO} transformation constructs and developing \textit{TDO} transgenic soybean plants}

The coding sequence of \textit{TDO} (GenBank accession number: ON092642) was amplified via PCR with the primers Dna-HIS1-F\(^5\)-CTGGTTGTGATGTGAGGATCC-ATGCTGACGAGTCTATGGAGGC-3 and Nos-HIS1-R\(^5\)-GCCAAATGTTTGAACGATGGGGAGTCGATGTACC-3\(^\text{'}) using a pE728-based \textit{TDO} protein expression vector as the template.\(^23\) The amplified PCR fragment was cloned into a plant expression binary vector via the BamHI site using hot-fusion cloning, which resulted in p35S::TDO. p35S::TDO carries two cassettes: one with \textit{TDO} driven by an enhanced 35S promoter (e35STDO:Nos) and another one for streptomycin selection. Transgenic soybean events carrying T-DNA of p35S::TDO were developed through \textit{Agrobacterium}-mediated transformation as previously described.\(^12\) The \textit{TDO} copy number was determined via a Taqman assay. Events carrying a single copy \textit{TDO} insertion were used for herbicide tolerance and biochemical assays in this study.

\subsection{2.2 Mesotrione tolerance assay}

Plants from homozygous lines of p35S::TDO transgenic events 116, 125, and 129, and the conventional soybean control, were grown in 3.5\(^{\text{°}}\) pots using soybean potting media in a growth chamber under the following conditions: 28 \textdegree C/24 \textdegree C (light/dark) and 16 h light at 700 \text{μE} \text{m}^{-2} \text{h}^{-1} dark photoperiod and fertilized with Peters general purpose fertilizer. Uniform V4 growth stage plants were sprayed with mesotrione (diluted from Callisto with 1\% crop oil) at 1×, 2×, 4×, 8×, and 16× the commercial rate (1× = 105 g ha\(^{-1}\) of active ingredient) to evaluate mesotrione tolerance. Eight plants per entry were used in each treatment. Crop injury was rated 13 days after mesotrione treatments.

\subsection{2.3 Biochemical quantification of \textit{14C}-mesotrione absorption and movement}

Plants from transgenic event 125 of p35S::TDO and the control were grown under the conditions described above. V1 growth stage plants were first sprayed with 1X nonradiolabeled mesotrione and followed by application of 12 \times 2 \text{μL} droplets of \textit{14C}-mesotrione solution, formulated from [phenyl-U-\textit{14C}] mesotrione (4.627 MBq mg\(^{-1}\)) (Institute of Isotopes Co., Ltd, Izotop, Budapest, Hungary) in 1\% crop oil, to the adaxial surface of the first trifoliate of each plant (1.8 \text{μg} per plant). Plants were sampled at 1, 3, 6, 24,
and 72 h after treatment (HAT), four plants/entry/time point. Four samples were collected from each plant: treated first trifoliate, apex (all tissues above the first trifoliate), un trifoliate (all above ground tissues under the node of the first trifoliate), and roots. The treated first trifoliate was washed with 80% acetonitrile to remove unabsorbed herbicide and air dried. Root samples were separated from soil, washed, and dried with paper towels. All samples were weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis.

Samples were milled on dry ice and extracted by adding 80% acetonitrile in a 1:10 ratio of sample weight to extraction solution, homogenized using a Geno/Grinder 2010 (SPEX SamplePrep) for 20 min and centrifugated. After transferring supernatants to new tubes, each pellet was resuspended in 50% acetonitrile for a second extraction as described above. Supernatants from both extractions were combined, and radioactivity in each sample was quantified using a Liquid Scintillation Analyzer (PerkinElmer Tri-Carb 2900TR) with Ultima Gold scintillation liquid (PerkinElmer). The percentage radioactivity of each sample type was calculated by dividing the radioactivity recovered in the sample type by the total radioactivity recovered in all samples of the plant.

2.4 Radiography of the translocation of 14C-mesotrione and its derivatives

V1 growth stage plants of event 125 of p35S::TDO and the control (six plants/entry) were sprayed with 1× nonradiolabeled mesotrione followed by applying 40 μg of 14C-mesotrione droplets to the first trifoliate using a 0.5 μg μL−1 14C-mesotrione solution in 1% of crop oil. Plants were pulled out of pots at 30 HAT and, after soil was washed from the roots, placed in cassettes, imaged and exposed to phosphoscreens. After 2–3 weeks of exposure in −80 °C, the phosphoscreens were scanned at 50 μm resolution using a Personal Molecular Imager (PMI)-FX (Bio-Rad).

2.5 Biochemical analysis of mesotrione movement and its inhibition of HDPD using nonradiolabeled mesotrione

V1 growth stage plants of p35S::TDO events 125 and 129, and the control were used for mesotrione treatments. Plants with their apex (all tissues above the node of the first trifoliate) covered by aluminum foil were sprayed with 2× mesotrione. After the leaves were air dried, the aluminum foil was removed and the first trifoliate and apex (covered by aluminum foil at spray) of each plant were sampled separately at 1, 3, 6, 24, and 72 HAT, respectively. Tissues from two plants were pooled as one sample and three replicates/entry/time point were collected, frozen in liquid nitrogen, and stored at −80 °C until analysis.

The frozen samples collected above were milled frozen and lyophilized. On average, about 20 mg of each lyophilized sample was extracted in 1 mL of 80% methanol for 7 h at 4 °C and centrifuged. Then 200 μL of supernatant from each sample was diluted to 450 μL with 20% methanol for analysis. Mesotrione, hydroxy-mesotrione, 4-(methylsulfonyl)-2-nitrobenzoic acid (MNBA), 2-amino-4-(methylsulfonyl) benzoic acid (AMBA), HPPA, and HGA were analyzed using an ultra-performance liquid chromatography–tandem mass spectrometer (UPLC–MS/MS) system consisting of a Shimadzu UPLC system and an AB Sciex 5500 triple quad mass spectrometer. Separation was achieved using an AQUITY UPLC BEH C18 column, 1.7 μm, 2.1 × 100 mm (Waters) maintained at 40 °C, with an injection volume of 5 μL. The mobile phase consisted of solvent A (0.1% vol/vol aqueous formic acid) and solvent B (0.1% vol/vol formic acid in acetonitrile). The mobile phase gradient was initially set at 5% B and increased linearly to 60% B in 8 min at a flow rate of 0.3 mL min−1. Mesotrione and hydroxy-mesotrione were analyzed in positive MRM mode, precursor to products ions were 340.0 to 228.0 Da for mesotrione, and 356.0 to 228.0 Da for hydroxy-mesotrione. MNBA, AMBA, HPPA, and HGA were analyzed in negative MRM mode, precursors to product ions were 244.0 to 200.0 Da for MNBA, 214.0 to 170.0 Da for AMBA, 179.0 to 107.0 Da for HPPA, and 167.0 to 123.1 Da for HGA. Data was processed, including peak picking and integration, using Analyst Software (SCIex). All compounds measured were quantified using an external calibration curve of their respective standard except hydroxy-mesotrione, which was quantified using the external calibration curve of the mesotrione standard.

2.6 Identification and quantitation of mesotrione-derived metabolites

Two experiments were conducted to identify and quantify mesotrione-derived metabolites. To identify mesotrione derivatives, the first trifoliates of event 125 of p35S::TDO and the control plants at the V1 growth stage were treated with 60 μg/plant of radiolabeled mesotrione solution droplets containing 13C-mesotrione (WuXi AppTec Co., Ltd) and 14C-mesotrione (Izotop) at a ratio of 0.55 in 28% acetonitrile and 1% crop oil, three replicates/entry/time point. Treated trifoliates were sampled at 3, 24, and 74 HAT, washed with 80% acetonitrile, air dried, weighed, and stored at −80 °C until analysis. Samples were ground using a Geno/Grinder 2010 at 1500 rpm for 10 min after adding 10 mL of 80% acetonitrile extraction solution and three 4-mm stainless ball bearings to each sample and centrifugated at 3500 rpm (Eppendorf centrifuge 5810R) for 10 min after grinding. Supernatants were transferred to new tubes and pellets were extracted two more times (three extractions in total) by repeating the above steps using 10 mL of 80% acetonitrile solution and 10 mL of 50% acetonitrile extraction solution, respectively. Supernatants from all three extractions of the same sample were combined and all samples were evaporated to dryness using a Genevac EZ-2.3 Elite (HCl compatible, SP scientific) with option 08: High + Low BP at 40 °C maximum temperature. Each dried sample was resuspended in 1.5 mL of LC/MS grade water. Finally, 100 μL of each sample was filtered through a 0.45 μm modified nylon centrifugal filter (VWR) and loaded into an amber glass HPLC vial for analysis.

To quantify mesotrione-derived metabolites, V1 growth stage plants were sprayed with 1X nonradiolabeled mesotrione, immediately followed by application of 40 μg of 14C-mesotrione droplets on the adaxial surface of the first trifoliate of each plant using a 0.5 μg μL−1 14C-mesotrione solution in 1% of crop oil. 14C-mesotrione-treated first trifoliate samples were collected at 1, 6, 24, and 72 HAT, washed with 80% acetonitrile, and stored at −80 °C, four replicates/entry/time point. Samples were extracted using the same method as for the 13C-/14C-mesotrione experiment for biochemical analysis presented above.

An Agilent 1290 Infinity HPLC system with temperature-controlled autosampler (15 °C) and a Waters XBridge Shield RP18 column (3.5 μm, 4.6 × 250 mm) at 30 °C were used to separate the extracts (see Supporting Information for details about the mobile phase and injection volumes). A valve (Analytical Scientific Instruments Model: 600-PO10-04) was used to split the outcoming flow to a radioactivity (RAD) detector (β-RAMmodels) and a Q-Exactive HF High Resolution mass spectrometer system at a ratio of 2:1. The flow that went into the RAD detector was mixed with 2.1 mL min−1 of Flowlogic U liquid (LabLogic Systems Inc.).
For mass spectrometer detection, details of MS parameters are listed in the Supporting Information. In addition to the FullScan/ddMS2-positive mode, a set of selected samples was run in the negative mode for mass detection (see Supporting Information for MS parameters). To obtain more complete MS2 data for selected candidate precursor ions, a Full Scan-PRM mode was run on selected $^{13}$C-$^{14}$C-mesotrione-treated samples using the same tuning parameters as for positive mode (details of MS parameters are presented in the Supporting Information). The post-column flow after sample injection was collected for the full 60 min gradient, and the radioactivity was measured and compared with the radioactivity before injection using a Liquid Scintillation Analyzer (PerkinElmer Tri-Carb 2900TR) with Ultima Gold scintillation liquid (PerkinElmer). The recovery rate of $^{14}$C radioactivity after injection was >90% (data not shown).

In the $^{13}$C- and $^{14}$C-mesotrione study to identify metabolites, raw data was processed using the Expected and Unknown Met ID Workflow with MMDF for complex matrix samples within Compound Discoverer 3.1 (Thermo Fisher Scientific). Candidate mesotrione derivatives were identified by taking advantage of their enriched $^{13}$C/$^{12}$C ratio and alignment of HPLC/RAD and HPLC/MS–MS signals. In the $^{14}$C-mesotrione study for metabolite quantification, mesotrione derivatives were quantified by expressing the radioactivity count area of the individual metabolite as a percentage of the total radioactivity count of the whole sample in the HPLC/RAD/MS–MS analysis. Quantitation of count areas and generation of radioactivity graphs was done using Laura software 4.2.6.79 (LabLogic). MS peak areas were measured using Quant Browser within XCalibur software 4.3 (Thermo Fisher Scientific). The list of mesotrione derivatives was narrowed down to the compounds that contributed 1–2% or more to the total radioactivity counts of $^{14}$C-mesotrione-treated samples. Strong positive correlation between radioactivity count and MS signal areas of individual mesotrione-related compounds provided additional support for the identity of the compounds. Putative identities of mesotrione derivatives were proposed based on accurate MS1 and MS2 similarities among compounds in this study as well as comparisons with published MS2 spectra on mesotrione derivatives. Commercial standards for AMBA (AMBEED) and MNBA (ACCELA), and 5-hydroxy-mesotrione, produced by TDO protein mediated in-vitro reaction and verified by NMR analysis, were used to confirm the identification of the specific metabolites.

### 2.7 Quantitation of phytoene, carotenoids, and chlorophylls

V1 growth stage plants were sprayed with 2x mesotrione. The apex and first trifoliate of each plant were sampled at 30 HAT, three replicates/entry, frozen in liquid nitrogen and stored at −80 °C until analysis. After milling and lyophilization, ~20 mg of each milled sample was transferred into a 2-mL vial and weighed. Next 1 mL of extraction solvent (acetone:ethyl acetate, 3:2 v/v) was added to each vial, and the vials were then capped and shaken at room temperature for 1 h. After shaking, 0.8 mL of water was added to each vial and they were capped, mixed vigorously, and centrifuged at 1000 rpm for 10 min. Finally, 200 μL of the supernatant in each vial was transferred to an HPLC vial with a fused insert, capped immediately, and stored with light protection prior to analysis. An Agilent HPLC system with the column temperature set at 35 °C and the autosampler temperature at 10 °C was used to analyze carotenoids, chlorophylls, and phytoene using a YMC Carotenoid C30 column (5.0 μm, 4.6 × 250 mm) with different mobile phases and gradient profiles.

For the analysis of phytoene, the sample injection volume was 20 μL (see Supporting Information for details of mobile phase, flow rate, and gradients). Phytoene was detected at 286 nm and eluted at 5.0 min. An (E/Z)-phytoene standard (Sigma-Aldrich) was dissolved/diluted with ethyl acetate to establish a calibration range of 0.04–5.0 ng μL.

For the analysis of carotenoids and chlorophylls, the sample injection volume was 10 μL (see Supporting Information for details of mobile phase, flow rate, and gradients). Solutions of standards (from Chromadex and Sigma-Aldrich) were prepared using ethyl acetate. The detection wavelengths, retention times, and calibration ranges for each compound are listed in the Supporting Information.

### 3 RESULTS

#### 3.1 Ectopic expression of TDO in soybean confers a commercial level of mesotrione tolerance

It was previously shown that the rice HIS1 gene confers tolerance to multiple triketone herbicides in rice, including mesotrione, although the level of tolerance was not evaluated to determine whether HIS1 is a viable option for HT trait development. To test whether ectopic expression of HIS1 in soybean can confer sufficient tolerance to mesotrione for an HT trait, transgenic soybean plants were developed from a set of binary vectors carrying codon optimized HIS1 (TDO) driven by different promoters. Among events tested, transgenic events carrying p35S::TDO with TDO driven by an enhanced 35S promoter conferred a commercial level of mesotrione tolerance. As shown in Fig. 1(A),(C), events with similar levels of TDO (Fig. S1) can tolerate up to 16× the commercial application rate of mesotrione while conventional soybean plants were severely injured by mesotrione at any rates applied, from 1× to 16× ((A) versus (B) in Fig. 1). The herbicide injury ratings of all TDO transgenic events were lower than 10% when plants were treated with 1× to 8× mesotrione (Fig. 1(C)). The herbicide injury ratings of p35S::TDO events remained below 15% after plants were treated with an ultra-high dose of mesotrione (16×) (Fig. 1(C)). Because these TDO transgenic events had similar levels of TDO expression and effects on mesotrione tolerance (Figs S1 and 1(C)), events 125 and 129 were used in non-radiolabeled biochemical studies, and only event 125 was used in the radiolabeled biochemical experiments described in the following.

#### 3.2 Systemic movement of mesotrione is limited in soybean plants overexpressing TDO

Mesotrione moves acropetally and basipetally in susceptible weeds. To evaluate the effect of overexpression of TDO on mesotrione movement, droplets of $^{14}$C-mesotrione solution were applied to the adaxial epidermis of the first trifoliate at the V1 growth stage. Systemic movement was observed in conventional soybean plants, but not TDO transgenic plants in radiographs of $^{14}$C-mesotrione-treated plants (Fig. 2(A)). In conventional soybean plants, translocation and accumulation of radioactivity were detected in leaf tissues above the treated trifoliate, especially in new emerging leaves, at 30 HAT, while trace amounts of radioactivity moved to the hypocotyl and roots (Fig. 2(A)). In contrast, no translocation of radioactivity to other body parts was detected in TDO transgenic plants, and all detectable radioactivity was retained in the treated first trifoliate (Fig. 2(A)).

To quantify the relative amount of mesotrione and its derivatives that moved out of the treated leaf, a time course study was...
conducted using $^{14}$C-mesotrione. As shown in Fig. 2(C), the percentage of radioactivity translocated from the treated first trifoli-ate to the apex (all tissues above the first trifoliate) kept increasing in conventional soybean plants and reached 28% of total radioactivity absorbed by the plants at 72 HAT. Radioactivity in the treated first trifoliate continued decreasing and reduced to 64% of total absorbed radioactivity at 72 HAT (Fig. 2(D)). A small percentage of absorbed radioactivity was also moved basipetally to unifoliates and roots in the conventional soybean plants (Fig. S2). In contrast, only a basal level of radioactivity was detected in the apexes, unifoliates, and roots of TDO transgenic plants at 72 HAT (Figs 2(C) and S2), and nearly all radioactivity was retained in the treated first trifoliate (Fig. 2(D)).

These two experiments (Figs 2 and S2) demonstrate that, similar to the movement in susceptible weeds, mesotrione moves systemically in conventional soybean plants, especially towards emerging young leaf tissues, and causes herbicide injury. Overall, these data show that overexpression of TDO in soybean prevents the systemic movement of mesotrione and overaccumulation of the herbicide in emerging young leaf tissues, which protects the plant from injury.

### 3.3 Ectopic expression of TDO protects HPPD from mesotrione-induced inhibition

Inhibition of HPPD by mesotrione results in the destruction of chlorophyll and classical bleaching symptoms. A time-course study was conducted to evaluate the effect of TDO on HPPD protection in the apex. V1 growth stage plants with apices covered by aluminum foil were sprayed with a 2× rate of mesotrione. Samples collected at specified time points after the treatment were analyzed using UPLC-MS/MS to detect and quantify mesotrione, hydroxy-mesotrione (mesotrione-OH), HPPA, HGA, AMBA, and MNBA. Similar to results from our radiolabeled study (Fig. 2(C)), the amount of mesotrione translocated to the apexes of TDO transgenic plants was at basal level at all time points (Fig. S3(A)). However, accumulation of mesotrione in the apexes of conventional soybean plants reached 10.2 nmol g$^{-1}$ at 24 HAT and remained at 2.8 nmol g$^{-1}$ at 72 HAT (Fig. S3(A)). Slow reduction of mesotrione in the apexes of conventional soybean plants at 72 HAT may be partially attributed to the slow catabolism of mesotrione in the apex and, potentially, some level of growth-related titration. Mesotrione-OH in the apexes of conventional soybean plants became detectable at low levels at 24 HAT (0.35 nmol g$^{-1}$) and increased to 1.55 nmol g$^{-1}$ at 72 HAT (Fig. 3(B)). In contrast, there was no detectable mesotrione-OH in the apexes of TDO transgenic plants. This is most likely due to the quick turnover of mesotrione in the leaves exposed to the herbicide preventing it from translocating to apexes (Fig. 3(B)).

Due to slow catabolism, mesotrione mostly remains as an unaltered active form to inhibit HPPD when it translocates through the plant and accumulates in apical tissues of conventional soybean plants. The inhibition of HPPD in apexes can be evaluated by tracking the amount of HPPA and HGA. Starting from 3 HAT until 24 HAT, HPPA levels kept increasing in the apexes of conventional soybean plants and remained at elevated levels up to the last time point of this study, 72 HAT (Fig. 3(C)). Likewise, HGA levels quickly decreased to 0.65 nmol g$^{-1}$ at 3 HAT in the apexes of conventional soybean plants and remained at this low level at all later time points (Fig. 3(D)). The observed changes of HPPA and HGA levels indicated that HPPD was inhibited by translocated mesotrione in the apexes of conventional soybean plants. In contrast, due to the lack of detectable translocation of mesotrione to apexes in TDO transgenic plants, the level of HPPA and HGA in the apexes was not perturbed, thus the function of HPPD in apexes of TDO transgenic plants was not affected (Fig. 3).

### 3.4 Ectopic expression of TDO converts mesotrione into metabolites similar to known classes of plant secondary metabolites

Converting mesotrione into AMBA via MNBA and other intermediates was reported as a path to metabolize mesotrione in corn plants, which are naturally resistant to the herbicide, and in soil, which is mediated by bacteria using enzymes such as nitro reductases. However, only basal to low amounts of MNBA and AMBA were detected in the apexes of conventional soybean plants (Fig. S3). In addition, only low amounts of mesotrione-OH were detected in the apexes of conventional soybean plants which contained a much higher amount of translocated mesotrione at 24 and 72 HAT (Fig. S3(A),(B)). Slow deactivation of
mesotrione, either by converting it to AMBA via MNBA or mesotrione-OH, which has previously been shown to be mediated by cytochromes P450s and other enzymes in weeds, makes conventional soybean very sensitive to mesotrione inhibition.

To better understand how mesotrione was metabolized in leaf tissues of conventional and TDO transgenic plants exposed to the herbicide, additional isotope tracing studies were conducted. First, a study using a mixture of $^{13}$C- and $^{14}$C-mesotrione to treat plants and identify mesotrione derivatives was conducted. Samples of treated first trifoliate leaves of TDO transgenic event 125 and conventional soybean plants were analyzed using HPLC/RAD/MS–MS to detect expected compounds (e.g. mesotrione) as well as compounds with an elevated $^{13}$C/$^{12}$C ratio (Fig. S4). A list of identifiable compounds including mesotrione and its derivatives was developed from this experiment (Table 1) and quantified in the following experiment.

To quantify the dynamic change of compounds listed in Table 1, a time-course study was conducted using $^{14}$C-mesotrione-treated first trifoliate samples. Results from HPLC/RAD/MS–MS analysis demonstrated that mesotrione was rapidly catabolized in TDO transgenic plants (Fig. 4(B),(D),(F),(H)). This degradation process was confirmed in TDO transgenic plants developed from pCon::TDO, carrying TDO driven by a different constitutive promoter (Fig. S5(B),(D)). Metabolites with known or putative IDs in the samples were quantified. In treated trifoliate leaves of TDO transgenic plants, mesotrione accounted for an average of 41% of the total absorbed radiation at 1 HAT, which quickly lowered and stabilized between 8.7% and 10.5% at 6 HAT and afterwards (Fig. 5(A)). In contrast, the percentage of absorbed radiation accounted for by mesotrione was 86% in treated trifoliate leaves of conventional soybean plants at 1 HAT and slowly reduced with time but remained at 27% by 72 HAT (Figs 4(A),(C),(E),(G) and 5(A)).

Furthermore, the total percentage of radiation in extracts accounting for mesotrione and other identifiable metabolic derivatives was maintained at a similar level among samples of TDO transgenic plants collected from 1 to 72 HAT (70–77%). TDO-mediated biochemical processes are major paths to rapidly oxidize the majority of mesotrione applied and generally further convert oxidized mesotrione derivatives into tractable compounds in transgenic plants. In contrast, the total percentage of radiation in samples of conventional soybean plants attributed to mesotrione and other identifiable metabolic derivatives started at 89% of total radiation (86% mesotrione plus 3% of other metabolic derivatives) at 1 HAT and gradually reduced to 55% at 72 HAT. This suggests that, even though conventional soybean plants can metabolize mesotrione, it is an inefficient and complex process that leads to low-abundance unidentifiable derivatives (accounting for 45% of the total radiation at 72 HAT) and some identifiable metabolites such as 4-hydroxy-mesotrione (Fig. 5(A)).

Among the identifiable mesotrione derivatives in samples of conventional plants, MNBA and AMBA were observed at low abundances, 0.6–2.0% and 1.0–3.3%, respectively. This confirms that the conversion of mesotrione to AMBA via MNBA is a minor path to catabolize mesotrione in conventional soybean plants (Figs 5 and S5). Three additional identifiable mesotrione derivatives in conventional soybean samples were 4-hydroxy-mesotrione, 5-hydroxy-mesotrione, and xanthone; their levels reached 9.4%, 10.8%, and 6.7% of total radiation counts in extracts at 72 HAT, respectively (Figs 4 and 5(A)).
In addition to metabolic derivatives detected in conventional soybean plants, acridone-OH, xanthone-OH, and their glucosyl- and glucosyl-malonyl conjugates were detected in TDO transgenic samples collected at various time points. Acridone-OH, xanthone-OH, and conjugates of acridone and xanthone were likely formed after the TDO-mediated second oxidation of 5-hydroxy-mesotrione and, potentially, 4-hydroxy-mesotrione. These metabolites detected in TDO transgenic plants are similar to xanthone- and acridone-related plant secondary metabolites.\textsuperscript{31,32} Conjugated xanthone and acridone continued to accumulate and reached 52% of the total radiation counts in samples of TDO transgenic plants at 72 HAT (Fig. 5(A)). A literature review, in combination with identifiable mesotrione derivatives in the datasets described above, allowed us to propose mesotrione metabolism pathways in conventional and TDO transgenic soybean plants (Figs 5(B) and S6).

Table 1. Mass and retention times of mesotrione derivatives with known and putative identifications in conventional soybean and TDO transgenic plants

| #  | Metabolites               | Monoisotopic mass (m/z) | Retention time (min) | Formula          |
|----|--------------------------|-------------------------|----------------------|-----------------|
| 0  | Mesotrione               | 339.0413                | 36.35                | C14H13NO7S      |
| 1  | Mesotrione-OH (5OH)      | 355.0362                | 22.31                | C14H13NO8S      |
| 2  | Mesotrione-OH (4OH)      | 355.0362                | 24                   | C14H13NO8S      |
| 3  | Xanthone-OH              | 306.0198                | 47.74                | C14H10O6S       |
| 4  | Acridone-OH              | 305.0358                | 37.99                | C14H11NO5S      |
| 5  | Xanthone-OH-glucosyl     | 468.0726                | 25.24                | C20H20O11S      |
| 6  | Acridone-OH-glucosyl     | 467.0886                | 24.7                 | C20H21NO10S     |
| 7  | Xanthone-OH-glucosyl-mal  | 554.073                | 31.47                | C23H22O14S      |
| 8  | Acridone-glucosyl-malonyl | 553.089                | 30.44                | C23H23NO13S     |
| 9  | Xanthone                 | 290.0249                | 50.43                | C14H10O5S       |
| 10 | AMBA                     | 215.0252                | 15.76                | C8H9NO4S        |
| 11 | MNBA                     | 244.9994                | 9.97                 | C8H7NO6S        |
3.5 Ectopic expression of TDO prevents mesotrione-mediated inhibition of carotenoid biosynthesis

Mesotrione-induced inhibition of HPPD causes depletion of HGA, which subsequently leads to depletion of PQ and impairs the function of PDS and, thus, carotenoid biosynthesis. Impaired carotenoid biosynthesis will ultimately lead to bleaching symptoms. However, leaves of conventional soybean plants fully expanded prior to mesotrione treatment stayed green for a much longer period of time, as shown in Figs 1 and 6(A), and in a previous report, while bleaching symptoms were visible in emerging leaves in just a couple of days after application. To better understand the heterogenic responses among leaves at different developmental stages to the mesotrione treatment in conventional soybean plants and TDO-mediated protection, key metabolites of the carotenoid biosynthesis pathway (Fig. 6(A)) and chlorophyll a and b were quantified in samples of the first trifoliates (fully expanded prior to treatment) and apexes (emerging tissues at treatment) collected 3 days after a 2× mesotrione spray of V1 growth stage plants.

In the apexes of mesotrione-treated plants, all quantified metabolites showed significant differences \( (P < 0.01) \) between conventional and TDO transgenic plants (Fig. 6(B)–(J), column Apex). Accumulation of phytoene increased ∼ 5.5-fold in conventional plants compared to TDO transgenic plants due to the negative effect of PQ depletion on the function of PDS (Fig. 6(B), column Apex). \( \beta \)-Carotene was almost fully depleted (0.45 ppm) in the apexes of conventional plants while it was 256 ppm in TDO transgenic plants (Fig. 6(C), column Apex). Similarly, other xanthophylls were almost depleted and chlorophyll a and b were dramatically reduced in the apexes of conventional plants at 3 days after treatment (Fig. 6(D)–(J), column Apex).

In contrast to the apex, the phytoene level in the first trifoliate of conventional plants was not significantly different from that of TDO transgenic plants (Fig. 6(B)). Although \( \beta \)-carotene in treated first trifoliates of conventional plants was significantly lower than that of TDO plants (Fig. 6(C)), it was still ∼ 2 times higher than that of untreated apex. One interesting observation was that zeaxanthin and antheraxanthin were significantly increased in the first...
trifoliate of conventional plants treated with mesotrione compared with that of TDO transgenic plants (Fig. 6(D),(E)). No significant difference of violaxanthin, neoxanthin, lutein, and chlorophyll a and b levels was observed between the treated first trifoliate of conventional and TDO transgenic soybean plants (Fig. 6(F)–(J)).

4 DISCUSSION

While the development and deployment of HT crops, especially GT crops, has revolutionized modern agriculture, broad adoption of HT crops with a single HT mode of action has contributed to the rapid rise of herbicide-resistant weeds.6,10 In response to the growing pressure of herbicide-resistant weeds, crops carrying two to three HT traits have been developed.3 Developing crops with additional modes of action of HT traits will provide farmers with more options to manage herbicide-resistant weeds and help slow the pace of weed species evolving resistance. HPPD-inhibiting herbicides including mesotrione have favorable environmental and toxicological profiles,21 making them excellent candidates for transgenic tolerance trait development.

The TDO-mediated mesotrione tolerance trait presented here is different from those traits previously developed using herbicide insensitive HPPDs.33–36 TDO directly deactivates mesotrione molecules by converting them into noninhibitory metabolites that are further converted into metabolites similar to known classes of plant secondary metabolites in soybean plants (Figs 5 and S6). Similar to insensitive HPPD, which confers tolerance to multiple HPPD-inhibiting herbicides in transgenic plants,33 expression of HIS1 in transgenic rice plants showed tolerance to multiple triketone herbicides.22 In this study, overexpression of TDO in soybean also confers tolerance, in addition to mesotrione, to two other triketone HPPD inhibitors, tembotrione and sulcotrione (Fig. S7).

Conventional soybean is highly susceptible to mesotrione even though it is capable of catabolizing mesotrione into a few detectable derivatives at a slow rate. Among these mesotrione derivatives, AMBA and MNBA are minor constituents compared to 4-hydroxy-mesotrione and 5-hydroxy-mesotrione, which are potentially formed through oxidation by unidentified soybean cytochrome p450 monoxygenases and perhaps other unknown enzymes, as has been previously reported in weeds.27–30 At low levels overall, accumulation of 4-hydroxy-mesotrione in conventional soybean increased with time (Figs 3(B) and 5(A)). This suggests that conventional soybean lacks a suitable dioxygenase to further oxidize these two mesotrione derivatives.

Conversely, TDO in transgenic plants first oxidizes mesotrione on the 5-carbon to convert mesotrione into 5-hydroxy-mesotrione, subsequently introduces a second oxidation, and leads to the generation of oxy-mesotrione (Figs 5(B) and S6).23 However, unlike data from conventional soybean plants, 4-hydroxy-mesotrione was not detected in samples of TDO transgenic plants (Fig. 5(A)). One possible explanation is that TDO can rapidly introduce a second oxidation at the 5-carbon position of 4-hydroxy-mesotrione, causing this intermediate to be undetectable. After the second oxidation of mesotrione, oxy-mesotrione is generated.

Figure 5. Relative abundance of mesotrione derivatives and the putative metabolic paths for detoxifying mesotrione in 14C-mesotrione-treated first trifoliate of TDO transgenic plants and conventional soybean control plants. Metabolites with putative identities were analyzed and quantified using HPLC-RAD. (A) Relative abundance of metabolites with putative identities in 14C-mesotrione-treated first trifoliate. Stacked bars show the percentage abundance of metabolites with putative identities in control plants (left panel) and TDO transgenic event 125 (right panel) at 1, 6, 24, and 72 h after 14C-mesotrione treatment. Each data point is the mean of four replicates. (B) Putative metabolic paths for turning over mesotrione in TDO and control plants. The thickness of the arrows indicates the relative strength of the path. Solid arrows point to detected metabolites with the expected molecular mass while dashed-line arrows point to hypothetical intermediates. Metabolites highlighted in green were only detected in the p35S::TDO transgenic event. *Note: mesotrione-4OH was only detected in control plants.
after losing a water molecule and forms xanthone-OH in the TDO transgenic plants (Figs 5(A),(B) and S6). Similarly, TDO is hypothesized to convert the mesotrione-derived MW291.06 intermediate into acridone-OH after the second oxidation (Figs 5(A),(B) and S6). Xanthone-OH and acridone-OH are conjugated with glucosyl and malonyl groups (Figs 5(A),(B) and S6). Most likely due to the poor
aqueous solubility of these metabolites,\textsuperscript{32,37–39} xanthone and acridone derivatives are immobilized in tissues where these metabolites are formed (Fig. 2). There is also a minor hypothetical path to convert mesotrione into xanthone via a MW292.04 intermediate (Figs 5B and 56). Overall, the rapid conversion of mesotrione into noninhibitory metabolites through double oxidations in tissues exposed to the herbicide is the key feature of the TDO-mediated mesotrione tolerance in soybean.

Rapid degradation of mesotrione in TDO transgenic plants minimized the effect of the herbicide on carotenoid biosynthesis and chlorophyll accumulation in vulnerable newly emerging tissues in the apex (Fig. 6). Conversely, mesotrione caused classical bleaching phenotypes in young emerging tissues of conventional soybean plants due to the depletion of PQ caused by depletion of HGA (Figs 1B, 3D, and 6A). Similar to the effect of isoxaflutole-mediated inhibition of HPPD,\textsuperscript{40} mesotrione treatment impairs the function of PDS. The level of phytoene, the substrate of PDS, significantly increased in the herbicide-treated apexes of conventional soybean plants while the level of carotenoids and chlorophylls was greatly reduced (Fig. 6).

In contrast to the emerging leaf tissues, fully expanded leaves of conventional soybean plants are less responsive to mesotrione treatment and slowly develop herbicide-induced bleaching symptoms (Fig. 1). This can be, partially, attributed to the temporospatial expression profile of soybean PDS genes. There are two PDS genes in soybean: Glyma.11G253000, which is highly expressed in young emerging leaves, flowers and developing pods but not in matured leaves, and Glyma.18G003900, which is mostly expressed in flowers (Soybase.org). An unexpected accumulation is that occurrence of zeaxanthin and antheraxanthin significantly increased in the treated first trifoliate of conventional soybean plants (Fig. 6D,E). This is most likely the result of xanthophyll cycle-mediated rebalancing, which involves the npq1-like genes among others.\textsuperscript{42} Mature leaves of conventional soybean plants are slow to respond to mesotrione treatment due to the minimal expression of PDS genes in those leaves in combination with the slow formation of noninhibitory 4-hydroxy-mesotrione and 5-hydroxy-mesotrione,\textsuperscript{43,27–28} a relatively high level reserve of xanthophylls, and the rebalance of xanthophylls.

5 CONCLUSION

Overall, mesotrione has a favorable environmental and toxicological profile,\textsuperscript{21} is familiar to farmers as a selective herbicide in corn production for both pre- and post-emergence weed control,\textsuperscript{18} and has limited known resistance among weed species.\textsuperscript{43} (WeedsScience.org). TDO rapidly deactivates mesotrione in leaf tissues exposed to the herbicide and confers tolerance to up to 16× the commercial application rate (Fig. 1). The chemical and agronomic characteristics of mesotrione in combination with TDO-mediated mesotrione tolerance via deactivation makes the mesotrione tolerance trait described here a new option for farmers to effectively manage difficult-to-control weeds in soybean acres. Moreover, this TDO-mediated mesotrione tolerance trait can potentially be adapted to other important dicot crops (e.g. cotton) for effective weed management.

ACKNOWLEDGEMENTS

We thank Drs Adewale Adio, Brent Brower-Toland, Stephen Duff, Graeme Garvey, Jeff Hass, Kang Liu, Mingsheng Peng, Jonathan Philips, Martin Ruebelt, Aihua Shao, Eden Tesfu, Gregory Tilton, Patrick Videau, Yanfei Wang, Brianna White, and Bosong Xiang for their input.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

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

REFERENCES

1. Popp J, Petö K and Nagy J. Pesticide productivity and food security. A review. Agron Sustain Dev 33:243–255 (2013).
2. Green JM, The benefits of herbicide-resistant crops. Pest Manag Sci 68:1323–1331 (2012).
3. Nandula VY, Herbicide resistance traits in maize and soybean: current status and future outlook. Plants 8:337 (2019).
4. Van Deynze B, Swinton SM and Hennessy DA, Are glyphosate-resistant weeds a threat to conservation agriculture? Evidence from tillage practices in soybeans. Am J Agric Econ 104:645–672 (2022).
5. Yu Z, Lu C, Hennessy DA, Feng H and Tian H, Impacts of tillage practices on soil carbon stocks in the US corn-soybean cropping system during 1998 to 2016. Environ Res Lett 15:014008 (2020).
6. Dill GM, Galjacob CA and Pagnette SR, Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag Sci 64:326–331 (2008).
7. Green JM and Owen MDK, Herbicide-resistant crops: utilities and limitations for Herbicide-resistant Weed Management. J Agric Food Chem 59:5819–5829 (2011).
8. Carpenter JE, Peer-reviewed surveys indicate positive impact of commercialized GM crops. Nat Biotechnol 28:319–321 (2010).
9. Peterson MA, Collavo A, Ovejero R, Shirvain V and Walsh MJ, The challenge of herbicide resistance around the world: a current summary. Pest Manag Sci 74:2246–2259 (2018).
10. Beckie HJ, Herbicide resistance in plants. Plants 9:435 (2020).
11. Pannell DJ, Tillie P, Rodriguez-Cerezo E, Ervin D and Frisvold GB, Herbicide resistance: economic and environmental challenges. AgBioForum 19:136–155 (2016).
12. Larue CT, Ream JE, Zhou X, Moshiri F, Howe A, Goley M, et al., Microbial HemG-type protoporphyrinogen IX oxidase enzymes for biotechnology applications in plant herbicide tolerance traits. Pest Manag Sci 76:1031–1038 (2020).
13. Beckie HJ, Ashworth MB and Flower KC, Herbicide resistance management: recent developments and trends. Plants 8:161 (2019).
14. van Almsick A, New HPPD-inhibitors – a proven mode of action as a new hope to solve current weed problems. Outlooks Pest Manag 20:27–30 (2009).
15. Norris SR, Shen X and Delta Penna D, Complementation of the Arabidopsis pds1 mutation with the gene encoding p-hydroxyphenylpyruvate dioxygenase. Plant Physiol 117:1317–1323 (1998).
16. Stacey MG, Cahoon RE, Nguyen HT, Cui Y, Sato S, Nguyen CT, et al., Identification of homogentisate dioxygenase as a target for vitamin E biofortification in oilseeds. Plant Physiol 172:1506 (2016), 1518.
17. Norris SR, Barrette TR and DeltaPenna D, Genetic dissection of carotenoid synthesis in arabidopsis defines plastoquinone as an essential component of phytoene desaturation. Plant Cell 7:2139–2149 (1995).
18. Mitchell G, Bartlett DW, Fraser TEM, Harkes TR, Holt DC, Townson JK et al., Mesotrione: a new selective herbicide for use in maize. Pest Manag Sci 57:120–128 (2001).
19. Carles L, Joly M, Joly P and Herbicide M, Efficienty, effects, and fate in the environment after 15 years of agricultural use. CLEAN – Soil Air Water 45:1700011 (2017).
20. Dyson JS, Beulke S, Brown CD and Lane MCC, Adsorption and degradation of the weak acid mesotrione in soil and environmental fate implications. J Environ Qual 31:613–618 (2002).
21. Meaza G, Scheffler BE, Tellez MR, Rimando AM, Romagni JG, Duke SO, et al., The inhibitory activity of natural products on plant
p-hydroxyphenylpyruvate dioxygenase. *Phytochemistry* **60**: 281–288 (2002).

22 Maeda H, Murata K, Sakuma N, Takei S, Yamazaki A, Karim MR, et al., A rice gene that confers broad-spectrum resistance to β-triketone herbicides. *Science* **365**: 393–396 (2019).

23 Duff S, Zheng M, Taylor C, Chen D, Mamanella P, Duda D et al., Structural and functional characterization of tri-ketone dioxygenase from Oryza sativa, in preparation. Forthcoming.

24 Durand S, Sancelme M, Besse-Hoggan P and Combournieu B, Biodegradation pathway of mesotrione: complementarities of NMR, LC–NMR and LC–MS for qualitative and quantitative metabolic profiling. *Chemosphere* **81**: 372–380 (2010).

25 Carles L, Besse-Hoggan P, Joly M, Vigouroux A, Moréra S and Batisson J, Functional and structural characterization of two *Bacillus megaterium* nitroreductases biotransforming the herbicide mesotrione. *Biochem J* **473**: 1443–1453 (2016).

26 Alferness P and Wiebe L, Determination of mesotrione residues and metabolites in crops, soil, and water by liquid chromatography with fluorescence detection. *J Agric Food Chem* **50**: 3926–3934 (2002).

27 Kaundun SS, Hutchings S-J, Dale RP, Howell A, Morris JA, Kramer VC, et al., Mechanism of resistance to mesotrione in an *Amaranthus tuberculatus* population from Nebraska, USA. *PLoS ONE* **12**: e0180095 (2017).

28 Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinness DL, Hager AG et al., Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. *Plant Physiol* **163**: 363–377 (2013).

29 Nakka S, Godar AS, Wani PS, Thompson CR, Peterson DE, Roelofs J et al., Physiological and molecular characterization of hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor resistance in *Palmer amaranth* (*Amaranthus palmeri* S.Wats.). *Front Plant Sci* **8**: 555 (2017).

30 Küpper A, Peter F, Zoller L, Lorenz L, Tranel PJ, Beffa R et al., Temo-bromine detoxification in 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor-resistant *palmer amaranth* (*Amaranthus palmeri* S. wats.). *Pest Manag Sci* **74**: 2325–2334 (2018).

31 Alhassan A, Abdullahi M, Uba A and Umar A, Prenylation of aromatic secondary metabolites: a new frontier for development of novel drugs. *Trop J Pharm Res* **13**: 8 (2014).

32 Vieira LMM and Kijjoa A, Naturally-occurring xanthones: recent developments. *Curr Med Chem* **12**: 2413–2446 (2005).

33 Siehl DL, Tao Y, Albert H, Dong Y, Heckert M, Madrigal A, et al., Broad 4-hydroxyphenylpyruvate dioxygenase inhibitor herbicide tolerance in soybean with an optimized enzyme and expression cassette. *Plant Physiol* **166**: 1162 (2014), 1176.

34 Dreesen R, Capt A, Oberdoerfer R, Coats I and Pallett KE, Characterization and safety evaluation of HPPD W336, a modified 4-hydroxyphenylpyruvate dioxygenase protein, and the impact of its expression on plant metabolism in herbicide-tolerant *MST-FG072-2* soybean. *Reul Toxicol Pharmacol* **97**: 170–185 (2018).

35 Hawkes TR, Langford MP, Viner R, Blain RE, Callaghan FM, Mackay EA, et al., Characterization of 4-hydroxyphenypyruvate dioxygenases, inhibition by herbicides and engineering for herbicide tolerance in crops. *Pestic Biochem Physiol* **156**: 9–28 (2019).

36 Kramer CM, Launis KL, Traber MG and Ward DP, Vitamin E levels in soybean (Glycine max (L.) Merr.) expressing a p-hydroxyphenylpyruvate gene from oat (Avena sativa L.). *J Agric Food Chem* **62**: 3453–3457 (2014).

37 Taguchi G, Uubukata T, Nozue H, Kobayashi Y, Takahi M, Yamamoto H et al., Malonylation is a key reaction in the metabolism of xenobiotic phenolic glucosides in Arabidopsis and tobacco. *Plant J* **63**: 1031–1041 (2010).

38 Ramesh Kumar MK, Chemistry of acridone and its analogues: a review. *J Chem Pharm Res* **3**: 114 (2011).

39 Ho LY, Lim YY, Tan CP and Siow LF, Comparison of physicochemical properties and aqueous solubility of xanthone prepared via oil-in-water emulsion and complex coacervation techniques. *Int J Food Prop* **21**: 789–798 (2018).

40 Pallett KE, Little JP, Sheekey M and Veerasakaran P, The mode of action of isoxalflutole I: physiological effects, metabolism, and selectivity. *Pestic Biochem Physiol* **62**: 113–124 (1998).

41 Du H, Zeng X, Zhao M, Cui X, Wang Q, Yang H et al., Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *J Biotechnol* **217**: 90–97 (2016).

42 Niyoji KK, Grossman AR and Björkman O, Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* **10**: 1121–1134 (1998).

43 Ndikuryayo F, Moosavi B, Yang W-C and Yang G-F, 4-Hydroxyphenylpyruvate dioxygenase inhibitors: from chemical biology to agrochemicals. *J Agric Food Chem* **65**: 8523–8537 (2017).