Vol. 9

Agricultural Biocatalysis

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Jenny Stanford Series on Biocatalysis Volume 9

Agricultural Biocatalysis

Enzymes in Agriculture and Industry

edited by Peter Jeschke | Evgeni B. Starikov

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Jenny Stanford Series
on Biocatalysis
Volume 9

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Peter Jeschke
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JENNY STANFORD
PUBLISHING

3365.44

Published by

Jenny Stanford Publishing Pte. Ltd.
101 Thomson Road
#06-01, United Square
Singapore 307591

Email: editorial@jennystanford.com
Web: www.jennystanford.com

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Agricultural Biocatalysis: Enzymes in Agriculture and Industry

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ISBN 978-981-4968-47-8 (Hardcover)
ISBN 978-1-003-31310-6 (eBook)

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Chapter 8

Oxygenase Enzymes for Agricultural Biotechnology Applications

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Agriculture biotechnologies have been an important part of our efforts to provide food, fiber, and fuel to a growing world population. One such application is the development of traits that can be integrated with crop plants to address a specific need. These traits can include an enzyme that performs a specific task in the crop plant. For example, herbicide tolerance traits can employ enzymes that act on selected herbicides to enable the use of a given herbicide during a cropping season. The oxygenase enzymes are an enzyme family that has been proven to be useful in developing several herbicide tolerance traits. In this chapter, monooxygenase and dioxygenase enzymes and their utility in agricultural biotechnology–based herbicide tolerance traits will be reviewed.

Agricultural Biocatalysis: Enzymes in Agriculture and Industry
Edited by Peter Jeschke and Evgeni B. Starikov
Copyright © 2023 Jenny Stanford Publishing Pte. Ltd.
ISBN 978-981-4968-47-8 (Hardcover), 978-1-003-31310-6 (eBook)
www.jennystanford.com

8.3 Dioxygenases for FOP and 2,4-D Tolerance Traits

The aryloxyphenoxy-propionate (FOP) herbicides and 2,4-dichlorophenoxyacetic acid (2,4-D) herbicides have different modes of action (MoA) in a plant. The herbicide 2,4-D was first commercialized in the 1940s and acts as a synthetic auxin (Peterson *et al.*, 2016). This herbicide has been shown to directly interfere with the auxin sensing machinery within a plant cell resulting in an uncontrolled over-stimulation of the hormone signaling pathways and eventual plant death in sensitive plants (especially in the dicotyledonous/broadleaf weeds). The FOP herbicides are one chemical family within a broader Group 1 class of herbicides, which all act to inhibit the enzyme acetyl coenzyme A (CoA) carboxylase (ACCase) in the fatty acid synthesis pathway. The FOP herbicides, such as quizalofop, have been shown to inhibit the ACCase isoform that represents the majority of the enzymatic activity in the chloroplasts of the grasses (*Graminaceae*) resulting in strong herbicide activity in the grasses but not in the dicotyledonous plants, which mostly depend on an alternate insensitive ACCase isoform (Ruizzo and Gorski, 1988; Dehaye *et al.*, 1994; Herbert *et al.*, 1996). Despite the unique MoAs as herbicides, both herbicide families have shared portions of the chemical molecule, namely a phenoxy ring with an alkanoic acid attached via an ether linkage (Fig. 8.3). It is through these shared chemical moieties that a single enzyme was identified as an herbicide tolerance trait for both herbicides.

With the application or release of agriculture and industrial chemistries to soil and aquatic environments, microbes have acquired the ability to metabolize some of these compounds. One of the first enzymes characterized in metabolizing 2,4-D is a 2,4-D dioxygenase (2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase; TfdA). TfdA was isolated from bacteria capable of utilizing 2,4-D as a carbon source and was characterized as an α-ketoglutarate-dependent dioxygenase that initiates the biodegradation pathway for 2,4-D (Amy *et al.*, 1985; Fukumori and Hausinger, 1993a; Fukumori and Hausinger, 1993b). However, further characterization of TfdA uncovered stereospecific enzymatic activity for this enzyme

family (Saari *et al.*, 1999). TfdA was able to oxidize *(S)*-dichlorprop (a closely related compound to 2,4-D except for the presence of a chiral propionic acid side chain in place of the achiral side chain for 2,4-D), while activity from other isolates was found to be specific for the *(R)*-enantiomer. Additionally, work by Hogan *et al.* (2000) began to uncover a core set of residues characteristic of the α-ketoglutarate dependent dioxygenase enzyme family that help form the metallocenter and coordinate substrate binding.

Around the same time as the work was underway for TdfA, additional bacterial isolates were being uncovered that showed similar activity on 2,4-D or similar synthetic auxin herbicides (Horvath *et al.*, 1990; Wesendorf *et al.*, 2003). Two bacterial strains, *Sphingomonas herbicidovorans* MH and *Delftia acidovorans* MC1, were found to contain two dioxygenase enzymes: α-ketoglutarate dependent *(R)*-dichlorprop dioxygenase (RdpA) and α-ketoglutarate dependent *(S)*-dichlorprop dioxygenase (SdpA) that were able to enzymatically act on several synthetic auxin herbicide compounds of the corresponding enantiomers (Nickel *et al.*, 1997; Kohler, 1999; Schleinitz *et al.*, 2004; Müller *et al.*, 2006). The RdpA enzymes from both strains were found to be identical enzymes. While the overall sequence homology to TfdA was low, both RdpA and SdpA maintained the characteristic α-ketoglutarate dependent dioxygenase motif as proposed in Hogan *et al.* (2000). Detailed characterization of the enzymes demonstrated the α-ketoglutarate and ferrous cofactor-dependent nature of the enzymes and a strict enantiomer selectivity without any observed enzyme inhibition when supplied with the opposing enantiomer suggesting only the correct enantiomer is capable of binding in the active pocket (Müller *et al.*, 2006). Furthermore, substrate selectivity of RdpA was observed to be dependent mostly on the alkanoic acid side chain and relatively indifferent to the substituents on the aromatic ring (Müller *et al.*, 2006). Given that many of the agriculturally relevant herbicides containing this chemical signature (a phenoxy ring with an alkanoic acid attached via an ether linkage) are active as the *(R)*-enantiomer (the notable exception being 2,4-D, which has an achiral sidechain) and the forgiving nature of the enzyme to accept substrates with a variety of additional substituents on the aromatic ring, RdpA became a key target enzyme for agriculture biotechnology applications.

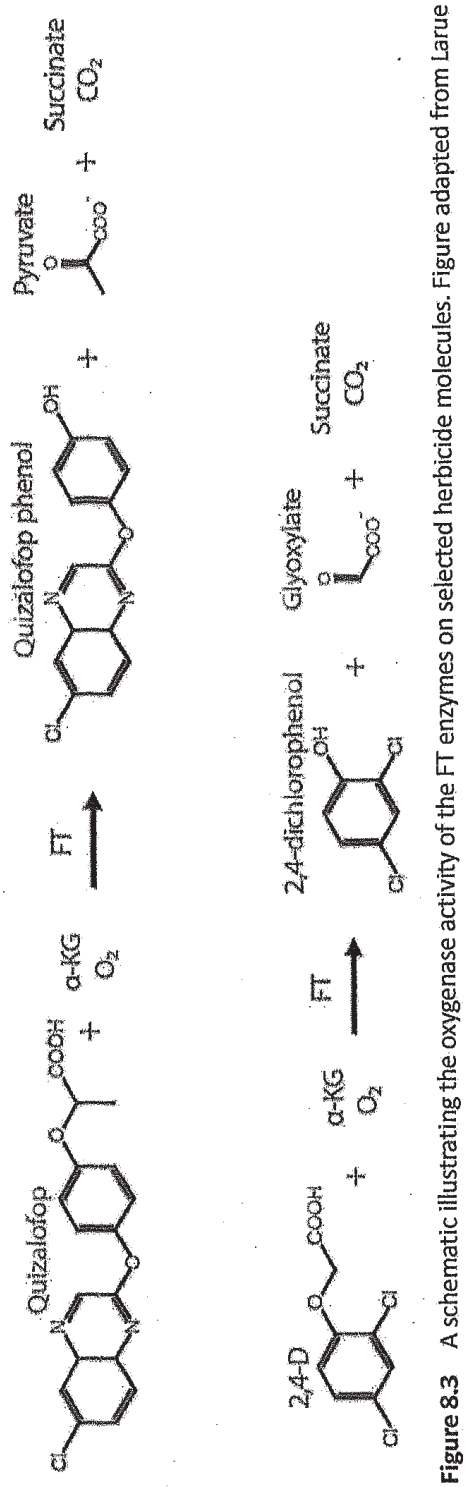

Figure 8.3 A schematic illustrating the oxygenase activity of the FT enzymes on selected herbicide molecules. Figure adapted from Larue *et al.*, 2019.

The use of RdpA and SdpA in agriculture biotechnology was first demonstrated by Wright *et al.* (2010). In this work, RdpA from *S. herbicidivorans* (renamed by Wright *et al.* (2010) as aryloxyalkanoate dioxygenase-1 or AAD-1) and SdpA from *D. acidovorans* (renamed by Wright *et al.* (2010) as AAD-12) were further characterized and expressed in *Arabidopsis thaliana*, corn (maize) and soybean plants. Further characterization of the substrate specificity of these enzymes confirmed that these enzymes were selective based on the nature and chirality of alkanoic acid side chain, but forgiving regarding the substituents on the aromatic ring, including being able to accept members of the FOP herbicides which have additional ring structures as substituents on the aromatic ring (Wright *et al.*, 2010). RdpA was transformed as a transgene into corn plants and plants expressing the transgene were screened with herbicide applications (Wright *et al.*, 2010). These corn plants displayed tolerance to applications of both a FOP herbicide (quizalofop) and 2,4-D herbicide at rates that exceeded the typical field application rates. While 2,4-D injuries dicotyledonous (broadleaf) plants more than monocotyledonous (such as corn), corn will show malformation injury following 2,4-D applications at certain growth stages. SdpA demonstrated better enzymatic activity on 2,4-D relative to RdpA (Wright *et al.*, 2010). Soybeans, being a dicotyledonous plant, are very sensitive to 2,4-D and this likely provided the rationale for using SdpA for the generation of transformed soybean plants. The soybean plants expressing SdpA showed tolerance to 2,4-D applications above normal field use rates (Wright *et al.*, 2010). This work in both corn and soybeans nicely illustrates the potential value these dioxygenase enzymes could bring to agriculture biotechnology.

As mentioned earlier in this chapter, native enzymes, such as oxygenase enzymes, can be excellent starting points for structure-based and computational-assisted enzyme optimization efforts. This was the approach taken by Larue *et al.* (2019). In this work, the authors were interested in engineering optimized dioxygenase enzymes (called FT enzymes) based on the RdpA scaffold which would have robust activity on both members of the FOP herbicides and members of the synthetic auxins such as 2,4-D (Fig. 8.3). However, it was discovered during the optimization process that some engineered versions of the enzyme, and the RdpA starting scaffold, displayed poor enzyme stability at higher temperatures.

This was shown to be problematic when FT expressing plants were exposed to higher temperatures that were selected to mimic hot field environments often found in crop fields in many world geographies and challenged with herbicide applications (Larue *et al.*, 2019). This new finding was used to inform additional enzyme optimization work in which heat stability was included as an optimization target. This large effort resulted in the engineering and identification of several optimized FT enzymes which showed significantly improved temperature stability as well as improved enzymatic parameters for selected herbicide chemistries (Larue *et al.*, 2019). During optimization of the plant expression of the FT enzymes, it was discovered that the addition of a chloroplast targeting peptide to the expressed FT enzyme significantly improved the tolerance *in planta* to the FOP herbicides (such as quizalofop), and thus chloroplast targeting peptides were included for the FT variants tested in corn (Larue *et al.*, 2019). However, the addition of chloroplast targeting peptides to the FT enzymes did not result in large changes in tolerance to 2,4-D herbicide applications in both corn and soybeans expressing these FT variants. The chloroplast targeting peptide is a short amino acid sequence located on the *N*-terminal end of the protein that acts as a cellular signal to import the protein to the chloroplast within the plant cell (Lee and Hwang, 2018). Both controlled environment and field-based testing of corn and soybean plants expressing FT enzyme variants displayed excellent tolerance to applications of quizalofop and 2,4-D herbicides above normal field rates (Larue *et al.*, 2019). In addition, the tolerance remained remarkably robust even at elevated temperatures indicating that optimization efforts to heat stabilize the FT enzymes were successful. This work nicely illustrates that oxygenase enzymes may be amendable to enzyme engineering and optimization efforts to design new oxygenase variants with attractive properties for applied applications such as agriculture biotechnology.

8.4 Monooxygenase Enzyme for Dicamba Tolerance

Dicamba (3,6-dichloro-2-methoxy benzoic acid) is another synthetic auxin herbicide that began use as an herbicide in the 1960s. Similar

to 2,4-D, dicamba disrupts normal hormone signaling pathways in plants resulting in plant death with dicotyledonous (broadleaf) weeds, in general, being more sensitive to the herbicide (Shaner, 2014). However, dicamba is chemically unique relative to some of the other synthetic auxins such as 2,4-D in that it does not contain an alkanoic acid side chain and instead contains a methoxy group.

The soil bacterium, *Pseudomonas maltophilia* DI-6, was characterized to survive on dicamba as a sole carbon source with the likely first step in the metabolism of dicamba to be the removal of the methyl group to produce 3,6-dichlorosalicylic acid (DCSA, Fig. 8.4) with an unidentified demethylase enzyme (Cork and Krueger, 1991; Yang *et al.*, 1994). Further characterization of this strain identified a megaplasmid carrying an *O*-demethylase as a part of a three-component enzyme system (Wang *et al.*, 1997; Herman *et al.*, 2005). This three-component enzyme system included a reductase, a ferredoxin, and an oxygenase enzyme which form a short electron transfer chain from reduced pyridine nucleotides (such as NADH, reduced nicotinamide adenine dinucleotide), through the ferredoxin to the non-heme iron-containing oxygenase. Sequence analysis and enzymatic characterization of the oxygenase revealed the oxygenase to be a Rieske (2Fe-2S) non-heme iron monooxygenase that converts dicamba into DCSA and formaldehyde in the presence of molecular oxygen and an active two enzyme electron handoff partner. This dicamba *O*-demethylase would become known as dicamba monooxygenase (DMO). A similar three-component system with activity on dicamba and vanillate was also uncovered in *Moorella thermoacetica* (Naidu and Ragsdale, 2001).

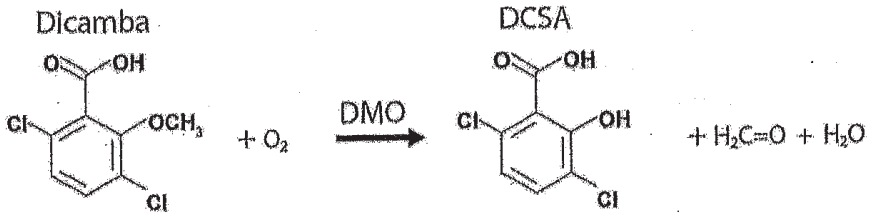

Figure 8.4 A schematic illustrating the oxygenase activity of the DMO enzyme on the dicamba herbicide molecule. Figure adapted from D'Ordine *et al.*, 2009.

Detailed structural enzymatic characterization of DMO was completed (D'Ordine *et al.*, 2009; Dumitru *et al.*, 2009). DMO

was found to organize as a toroidal trimer with a head-to-tail arrangement; the non-heme iron center and the Rieske center are located on roughly opposite ends of the monomer (~48 Å apart) however, the non-heme iron center of one monomer is located in much closer proximity to the Rieske center of the adjacent monomer (~12 Å) likely enabling the electron transfer needed for catalysis. This would enable an electron transfer from the partner ferredoxin to the Rieske center to the non-heme iron center where molecular oxygen is activated to react with dicamba. DMO is unique relative to other Rieske oxygenases that act on aromatic substrates in that DMO oxygenates an exocyclic methyl group instead of directly on the aromatic ring. Additionally, while the domain housing the active site is smaller than other characterized Rieske oxygenases, the active site pocket was found to be larger than expected relative to similar oxygenases that act on larger hydrophobic substrates which may be in part due to the amphipathic nature of the dicamba substrate.

To determine if DMO can function as an herbicide tolerance trait in plants, Behrens *et al.* (2007) transformed Arabidopsis, tomato, tobacco, and soybean plants with a DMO transgene. One of the concerns would be if the DMO would be able to function in a plant cell without the native partner reductase and ferredoxin that would be found in the source bacterial strain. To address this concern, the authors fused a chloroplast targeting peptide to the *N*-terminal end of DMO to take advantage of the reduced ferredoxin naturally found in the plant chloroplasts. Although it was unknown if DMO would be able to accept electrons from a plant ferredoxin. However, plants expressing DMO in all four plant species showed tolerance to dicamba applications above normal field rates, suggesting that DMO could accept electrons from plant ferredoxins in the chloroplasts (Behrens *et al.*, 2007). Additionally, tobacco plants with DMO transformations directed to the chloroplast (DNA integration into the chloroplast genome instead of the nuclear genome) and selected to produce homoplastidic DMO-transformed chloroplasts also displayed robust dicamba tolerance, indicating that DMO can function if it is exclusively localized to the chloroplasts (as opposed to being transported into the chloroplasts posttranslation). Enzyme assays with recombinant DMO enzyme also demonstrated that DMO can function with isolated ferredoxin from spinach or a different bacterium, *Clostridium pasteurianum*, suggesting that DMO can

accept electrons from a variety of ferredoxin partners (Behrens *et al.*, 2007). However, surprisingly, DMO transformations without a chloroplast targeting peptide into the nuclear genome also provided some tolerance to dicamba applications in tobacco (Behrens *et al.*, 2007). It is unclear what partner enzyme could provide electrons to DMO or if DMO was somehow translocated to the chloroplast via an unknown mechanism. While monocotyledonous plants such as corn are not as sensitive as dicotyledonous plants to dicamba applications, Cao *et al.* (2011) also demonstrated that corn expressing DMO showed less injury to dicamba applications illustrating that this enzyme can function, like the RdpA and FT enzymes, in both dicotyledonous and monocotyledonous plants as a robust herbicide tolerance trait.

8.5 Dioxygenase Enzyme for HPPD Inhibitor Tolerance

The 4-hydroxyphenyl-pyruvate dioxygenase (HPPD)-inhibitor herbicides inhibit the activity of the enzyme HPPD, an enzyme in the plastoquinone biosynthetic pathway (Shaner, 2014). Inhibition of plastoquinone biosynthesis disrupts the function of downstream enzymes in the carotenoid biosynthetic pathway, resulting in bleaching of new plant growth and eventual plant death in susceptible species. One family of HPPD-inhibitor herbicides includes the triketone HPPD-inhibitor herbicides which all maintain a conserved chemical scaffold. The HPPD-inhibitor class of herbicides represents a relatively new family of herbicides and research into new chemistries in this family of herbicides remains active (Ndikuryayo *et al.*, 2017). While this chapter focuses on oxygenase enzymes used as deactivation enzymes for herbicide tolerance traits, it is of interest to note that HPPD is a plant dioxygenase and overexpression of HPPD enzymes, which includes HPPD enzymes with reduced sensitivity to herbicide inhibition, has been successful in generating HPPD-inhibitor herbicide-tolerant plants.

HPPD-inhibitor herbicides are used in many cropping systems including rice. In rice, some elite lines have shown sensitivity to some triketone HPPD-inhibitor herbicides, such as benzobicyclon. Benzobicyclon is a pro-herbicide with its hydrolysate (BBC-OH)

being the active molecule *in vivo*. To characterize the rice gene(s) responsible for the sensitivity to benzobicyclon, Maeda *et al.* (2019) undertook a map-based cloning effort with benzobicyclon tolerant and sensitive rice lines. In this effort, a single gene was identified and named HPPD-Inhibitor Sensitive 1 (HIS1). HIS1 contained conserved motifs suggesting it was an α-ketoglutarate-dependent dioxygenase. Additional mutant analysis, genotyping, and breeding cross-analysis confirmed that this gene was able to restore tolerance in sensitive, rice-breeding lines to benzobicyclon, and a short deletion in this gene in the modern *indica* variety parent cultivars is responsible for the herbicide sensitivity observed in some breeding lines (Maeda *et al.*, 2019). HIS1 is a member of a clade of plant α-ketoglutarate dependent dioxygenases with a currently uncharacterized function and members of this clade are found throughout the plant kingdom (Maeda *et al.*, 2019).

Transformation of benzobicyclon sensitive rice-breeding lines and *A. thaliana* (which is naturally sensitive to HPPD-inhibitor herbicides) confirmed that expression of HIS1 yielded plants with a tolerance to benzobicyclon and several other triketone HPPD-inhibitor herbicides such as mesotrione (Maeda *et al.*, 2019). Characterization of these HIS1 transformed rice lines showed an absence of BBC-OH which accumulated in sensitive lines. Further enzymatic characterization of HIS1 uncovered a ring hydroxylation on BBC-OH in an α-ketoglutarate and Fe^{2+} dependent manner (Fig. 8.5) (Maeda *et al.*, 2019). Further characterization by the authors demonstrated similar hydroxylation by HIS1 on several other triketone HPPD-inhibitor herbicides suggesting that HIS1 deactivates triketone HPPD-inhibitor herbicides as an α-ketoglutarate dependent dioxygenase via ring hydroxylation of the active herbicide molecule and thus can be a useful herbicide tolerance trait.

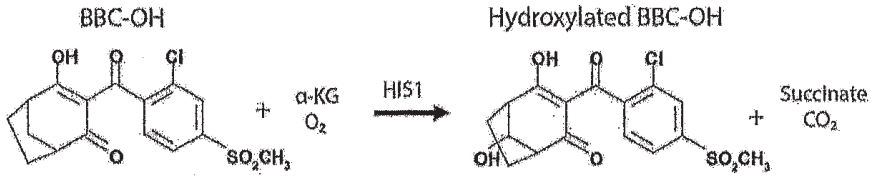

Figure 8.5 A schematic illustrating the oxygenase activity of the HIS1 enzyme on the benzobicyclon (BBC) herbicide molecule. Figure adapted from Maeda *et al.*, 2019.

8.6 Conclusion

In this chapter, three unique oxygenase families were discussed that had demonstrated utility as herbicide tolerance traits via a deactivation approach. These families represent both dioxygenases and monooxygenase and are enzymatically active on unique sets of herbicides. Some of the oxygenases discussed here were originally isolated from microbes but were found to function well in a plant cell. While this illustrates nicely that these enzymes can function in two very different cellular contexts, it is prudent to caution the reader that this should not be taken as a rule and should be carefully investigated with each new oxygenase enzyme. It is also prudent to caution the reader that it is also possible that a given oxygenase may enzymatically act on a given herbicide, but the product produced by this enzymatic activity may still retain activity (or retain partial activity) as an herbicide and thus full-plant tolerance to the herbicide may not be achieved despite good enzymatic activity within the plant cell. Again, this is something that must be carefully investigated during scientific endeavors. However, given the diversity of oxygenase enzymes that can be sourced from both prokaryotes and eukaryotes and given a broad range of substrates and reactions on these substrates that can be performed, it is likely that the oxygenases will remain a rich and fruitful source of enzymes for a variety of biotechnology applications within agriculture and beyond.

While this chapter is focused on the oxygenase enzymes used in herbicide tolerance traits, it is important for the reader to understand the utility and application in agriculture. While it is not possible to review or summarize the complex and continually evolving weed management practices here, a few key points will be made that directly relate to herbicide tolerance traits. The introduction of chemical herbicides for weed control in the mid-20th century brought a powerful new tool to weed control in agriculture. However, as with any new tool, weeds were able to adapt to the new selective pressure and weed resistance to many of these herbicides developed with time and continues to grow (Heap, 2021). The introduction of herbicide tolerance traits in the late 20th century added another tool

to weed management programs for several crops in certain world regions and has been shown to bring value to agriculture (Carpenter, 2010). Often agriculture biotechnology traits are "stacked" in which one or more herbicide tolerance traits may be combined with one or more biotechnology traits for a different goal (such as insect control) within the same plant. This further adds value since it enables better control of multiple pests (such as weeds and insects) or within weed control may add value due to enabling different herbicides to be used on a single crop to address problematic weeds or enable better weed resistance management. However, as the rate of the commercial release of new herbicides with unique MoAs has slowed while the development of weed resistance continues, this has placed increasing emphasis on preserving the value of herbicide chemistries and integrated weed management programs. Weed management programs should include best management practices to reduce the risks of weed resistance to herbicides (Norsworthy *et al.*, 2012). These management practices directed for weed resistance should be a component of a holistic weed management practice that is tailored to a farmer's cropping systems, growing conditions, weed pressures, and sustainability goals.

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