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Agricultural Biocatalysis

Enzymes in Agriculture and Industry

edited by Peter Jeschke | Evgeni B. Starikov



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Peter Jeschke
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Contents

<i>Preface</i>	xv
----------------	----

Part I Agricultural Enzymes Market

1. Metagenomics as a Tool to Isolate New Enzymes for Application in Hydrolysis and Synthesis Reactions: The Case of Lipolytic Enzymes	3
<i>Rocío Cuaspa Ropaín, Robson Carlos Alnoch, Patrícia Gruening de Mattos, Janafna Marques de Almeida, David Alexander Mitchell, and Nadia Krieger</i>	
1.1 Introduction	4
1.2 Metagenomics as an Enzyme Prospecting Tool	6
1.3 Functional Screening in a Metagenomic Library	19
1.4 Sequence-Based Screening in a Metagenomic Library	21
1.5 Lipolytic Enzymes Isolated from Metagenomic Libraries	23
1.6 The Lipases LipC12, LipG9, and LipMF3: A Case Study	24
1.7 Conclusions	31
2. Plant Pectin Methylesterase: An Insight into Agricultural and Industrial Applications	39
<i>Zeba Khan, Rakesh Srivastava, Sumit K. Bag, and Praveen C. Verma</i>	
2.1 Introduction	40
2.2 Pectin Methylesterases Classification	41
2.3 Pectin Methylesterases: An Insight into Their Functions	45
2.3.1 Role of PME's in Different Aspects of Plant Development	46
2.4 Role of PME's in Plant Defense-Related Mechanism	49

2.5	Agriculture and Industrial Applications of PMEs	51
2.6	Conclusion	53
3.	Induced Polyphenol Oxidases Are Associated with Laccase Activity in Different Genotypes of Resistant Chickpea Cultivars Infected by <i>Fusarium oxysporum</i> f.sp. <i>ciceris</i> and Salicylic Acid	59
	<i>Shivashankar Gayatri Devi, Kuruba Sreeramulu, and Senigala K. Jayalakshmi</i>	
3.1	Introduction	60
3.2	PPO and Laccase Activity	62
3.3	Detection of PPO and Laccase Isozymes in Native PAGE	64
3.4	Conclusion	69
Part II Plant Enzymes		
4.	Classification and Evolutionary Landscape of Acid Phosphatase–Encoding Gene Families in Plants	75
	<i>Amir Feizi, Mohammad Ali Malboobi, Javad Zamani, Tahmineh Lohrasebi, and Shahrokh Kazempour-Osaloo</i>	
4.1	Introduction	76
4.2	Plant APase Sequences Retrieval and Comparison	79
4.3	Sequence Alignments, Clustering, and Phylogenetic Analysis	89
4.4	PAP Family	90
4.4.1	PAP I Subfamily	93
4.4.2	PAP II Subfamily	95
4.4.3	PAP III Subfamily	96
4.4.4	PAP IV Subfamily	96
4.4.5	PAP V Subfamily	96
4.5	Halo Acid Dehalogenases (HAD)-Related APase Family	97
4.5.1	HRP I Subfamily	97
4.5.2	HRP II Subfamily	100
4.6	Phospholipid Phosphatase (PLP) Family	100
4.7	Histidine Acid Phosphatase Family	102

4.8	Protein E (SurE)-Related Acid Phosphatase Family	104
4.9	Structural Comparisons of APase Families	106
4.10	Concluding Remarks	108
5.	Acid Phosphatases Roles in Plant Performance	117
	<i>Mohammad Ali Malboobi, Mohammad Sadegh Sabet, Katayoun Zamani, Tahmineh Lohrasebi, Zahra Fathi, and Javad Zamani</i>	
5.1	Introduction	118
5.2	Phosphate Ion and Its Significance	119
5.3	Stepwise Plant Response to Available Pi	121
5.4	APases Types and Functions	122
5.4.1	Purple Acid Phosphatase (PAP) Family	123
5.4.2	Haloacid Dehalogenase (HAD)-Related Acid Phosphatase (HRP) Family	124
5.4.3	Phospholipid Phosphatases (PLP) Family	125
5.4.4	Histidine Acid Phosphatase (HAP) Family	126
5.4.5	SurE-Related Acid Phosphatase (SAP) Family	127
5.5	Multiple Isoforms and Broad Substrates Range of APases	127
5.6	Tissue-Specific Expression of APases	132
5.7	Responsiveness of APases to Pi Status	135
5.8	Roles of APases in Pi Homeostasis	137
5.9	APases Responsive to Other Stresses	140
5.10	Interplay between APases to Keep Pi Homeostasis	141
5.11	Concluding Remarks	145
6.	Superoxide Dismutases in Plants: New Insights Into Regulation and Functioning	159
	<i>Ravi Prakash Sanyal, Abiraami T. V., Sabiha Perween, Satish B. Verulkar, Hari S. Misra, and Ajay Saini</i>	
6.1	Introduction	160

6.2	Impact of Great Oxygenation Event (GOE) on Cellular Metabolism	161
6.3	Reactive Species: Generation, Molecular Targets, and Scavenging	162
6.3.1	Superoxide Radical ($O_2^{\cdot -}$)	166
6.3.2	Hydrogen Peroxide (H_2O_2)	166
6.3.3	Hydroxyl Radical ($\cdot OH$)	166
6.3.4	Singlet Oxygen ($^1O_2$)	167
6.3.5	Other Reactive Species (RNS, RCS, RSS)	167
6.4	Reactive Species and Important Sites of Generation in Plants	168
6.5	Reactive Species: Detrimental and Beneficial Effects	170
6.6	Cellular Antioxidant Defense Systems	172
6.6.1	Nonenzymatic Antioxidants	173
6.6.2	Enzymatic Antioxidant Defense System	174
6.7	Multiple SOD Isoforms and Their Need in Biological Systems	176
6.8	Nickel Superoxide Dismutase (Ni SOD)	177
6.9	Cambialistic Superoxide Dismutase (Fe/Mn SOD)	178
6.10	Iron Superoxide Dismutase (Fe SOD)	179
6.11	Manganese Superoxide Dismutase (Mn SOD)	183
6.12	Copper Zinc Superoxide Dismutases (CuZn SODs)	185
6.13	Dynamics of Regulation and Functioning of SODs	188
6.13.1	<i>cis</i> Elements–Mediated Regulation of SODs	188
6.13.2	MicroRNA miR398–Mediated Regulation of CuZn SODs	191
6.13.3	Alternative Splicing in Regulation and Functioning of SODs	192
6.13.4	Copper Chaperone for Superoxide Dismutase (CCS)–Mediated Regulation and Functioning of CuZn SODs	195

6.13.5	Role of Posttranslational Modifications (PTMs) in SOD Functioning	198
6.13.6	Genome Duplication-Mediated Copy Number Increase of Plant SOD Genes	201
6.14	Stress Responsiveness of SODs	203
6.15	Biotechnological Applications of SODs for Stress Tolerance Enhancement	204
6.16	Industrial Applications of Plant SODs	208
6.17	Conclusions	208
7.	Peroxisomes from Higher Plants and Their Metabolic Diversity	225
	<i>Francisco J. Corpas and José M. Palma</i>	
7.1	Introduction	226
7.2	Photorespiration	228
7.3	Fatty Acids β -Oxidation, Glyoxylate Cycle, and Auxin Metabolism	229
7.4	Biosynthesis of Jasmonic Acid and Polyamine Metabolism	231
7.5	Metabolism of ROS and RNS	232
7.6	Pexophagy	235
7.7	Conclusions and Further Perspectives	235
Part III	Herbicide-Tolerant Traits	
8.	Oxygenase Enzymes for Agricultural Biotechnology Applications	245
	<i>Clayton T. Larue</i>	
8.1	Introduction	246
8.2	Discovery and Early Development of Herbicide Tolerance Traits	249
8.3	Dioxygenases for FOP and 2,4-D Tolerance Traits	253
8.4	Monooxygenase Enzyme for Dicamba Tolerance	257
8.5	Dioxygenase Enzyme for HPPD Inhibitor Tolerance	260
8.6	Conclusion	262

Part IV Plant Viruses

9. P1 Leader Proteinases from the <i>Potyviridae</i> Family	269
<i>Fabio Pasin and Hongying Shan</i>	
9.1 Introduction	270
9.2 P1 Diversity of the <i>Potyviridae</i> Genomes	275
9.3 Structural Properties and Proteolytic Activity of P1	276
9.4 P1 Proteins as Viral Suppressors of RNA Silencing	278
9.5 P1 Proteins as Host-Range and Symptom Determinants	280
9.6 Additional P1 Functions	287
9.7 Biotechnologies of P1 Proteinases	288
9.8 Conclusions	290

Part V Soil Enzymes

10. Soil Enzymes: Distribution, Interactions, and Influencing Factors	303
<i>Sesan Abiodun Aransiola, Femi Afolabi, Femi Joseph, and Naga Raju Maddela</i>	
10.1 Introduction	304
10.2 Source, Distribution, and Abundance of Soil Enzymes	305
10.3 Ecological Stoichiometry of Plant-Soil-Enzyme Interactions	310
10.3.1 Role of Plant in Soil Health	311
10.3.2 Plant-Soil-Enzyme Relationship and Soil Health	312
10.3.2.1 Soil urease	313
10.3.2.2 Soil invertase	314
10.3.2.3 Soil phosphatase	314
10.4 Soil Chemical Properties Versus Soil Enzyme Activities	314
10.5 Impact of Anthropogenic Factors on Soil Enzyme Activities	317
10.6 Conclusions	325

11. Carbon-, Nitrogen-, Phosphorus-, and Sulfur-Cycling Enzymes and Functional Diversity in Agricultural Systems	335
<i>Avijit Ghosh, Ranjan Paul, Abhijit Sarkar, M. C. Manna, Sudeshna Bhattacharjya, Khurshid Alam, Sourav Choudhury, and Prithusayak Mondal</i>	
11.1 Introduction	336
11.2 Carbon-Cycling Enzymes and Their Mechanisms	337
11.2.1 Amylase	337
11.2.2 β -Glucosidase	338
11.2.3 Cellulase and Hemicellulase	339
11.2.4 Ligninase	341
11.2.5 Invertase	342
11.2.6 Laccase	342
11.2.7 Pectinase	342
11.3 Nitrogen-Cycling Enzymes and Their Mechanisms	344
11.3.1 Soil Protease	344
11.3.2 Urease	344
11.3.3 Chitinase	345
11.4 Phosphorus-Cycling Enzymes and Their Mechanisms	345
11.4.1 Phosphatase Enzymes	345
11.4.2 Phosphomonoesterases	348
11.4.3 Phosphodiesterases	349
11.4.4 Phosphotriesterases	350
11.4.5 Polyphosphates	351
11.4.6 Phosphoamidase	353
11.5 Sulfur-Cycling Enzymes and Their Mechanisms	353
11.5.1 Aryl Sulfatases	353
11.6 Microbial Functional Diversity in Agrosystems	355
11.7 Conclusions and Future Prospects	355

Part VI Bioremediation

12. Bioremediation: Removal of Polycyclic Aromatic Hydrocarbons from Soil	367
<i>Zdeněk Košnář, Johanka Wernerová, Petr Frühbauer, and Pavel Tlustoš</i>	
12.1 Introduction	368

12.1.1	General Description of Polycyclic Aromatic Hydrocarbons (PAHs)	368
12.1.2	Basic Description of 16 Individual US EPA PAHs	371
12.1.3	Sources of PAHs in the Environment	375
12.1.4	Emissions of PAHs in the Environment	377
12.1.5	Soil Contamination by PAHs	378
12.1.6	Impact of PAHs on Animal and Human Health	379
12.2	Conventional Remediation of Soils Contaminated by PAHs	381
12.3	Bioremediation of Soil Contaminated by PAHs	382
12.3.1	Phytoremediation of Soil Contaminated by PAHs	384
12.3.2	Bacterial Remediation of Soil Contaminated by PAHs	391
12.3.3	Mycoremediation of Soil Contaminated by PAHs	395
12.4	Conclusions	403

Part VII Biochemical Conversion

13. Enzymatic Saccharification of Lignocellulosic Biomass 413

Madhavi Latha Gandla, Chaojun Tang, Carlos Martín, and Leif J. Jönsson

13.1	Introduction	414
13.2	Lignocellulosic Biomass	415
13.3	Biodegradation of Lignocellulose in Nature	420
13.4	Fungal Enzymes	422
13.4.1	Glycoside Hydrolases	422
13.4.2	Polysaccharide Lyases	431
13.4.3	Carbohydrate Esterases	434
13.4.4	Auxiliary Activities	436
13.4.5	Associated Modules	439
13.5	Bacterial Enzymes	441
13.6	Determination of Cellulolytic Activity	443
13.7	Biorefining of Lignocellulosic Feedstocks	445
13.8	Pretreatment to Facilitate Enzymatic Saccharification	448

13.9	Inhibition of Enzymatic Saccharification	454
13.10	Process Configurations	455
13.11	Future Outlook	459
14.	Biological Biorefineries Based on Orange Peel Wastes	471
	<i>Alberto García-Martín, Itziar A. Escanciano, V. Martín-Domínguez, Álvaro Lorente-Arévalo, Jorge García-Montalvo, Jesús Esteban, Juan M. Bolívar, Victoria E. Santos, and Miguel Ladero</i>	
14.1	Introduction	472
14.2	Upstream Processes in Biological Biorefineries from OPW	476
14.2.1	Pretreatment of OPWs	476
14.2.2	Enzymatic Saccharification of OPWs	481
14.3	Biological Processes to Platform Chemicals and Materials	484
14.3.1	Bioethanol and Superior Alcohols	484
14.3.2	Gas Energy and Material Vectors from OPW	487
14.3.3	Monomers and Other Organic Compounds	496
14.3.4	Production of Enzymes from OPWs	499
14.3.5	Biopolymers, Exopolysaccharides, and High Molecular Weight Active Ingredients	502
14.4	Techno-Economic and Environmental Impact Studies	504
14.5	Conclusions	506
	<i>Index</i>	515

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.

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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 TfdA, 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 alkanolic 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 alkanolic 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.

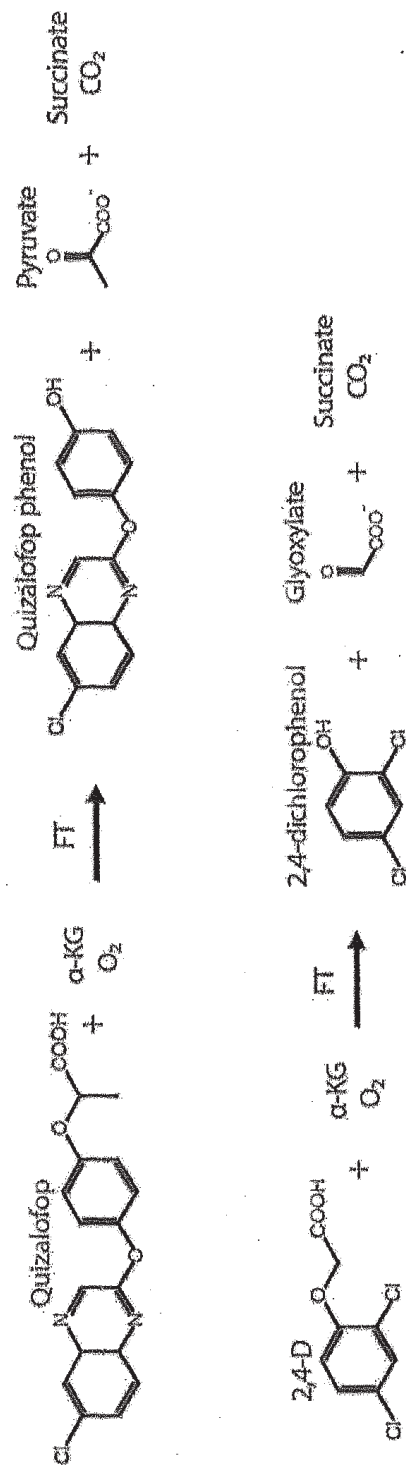


Figure 8.3 A schematic illustrating the oxygenase activity of the FT enzymes on selected herbicide molecules. Figure adapted from Larue *et al.*, 2019.

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 alkanolic 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 alkanolic 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).

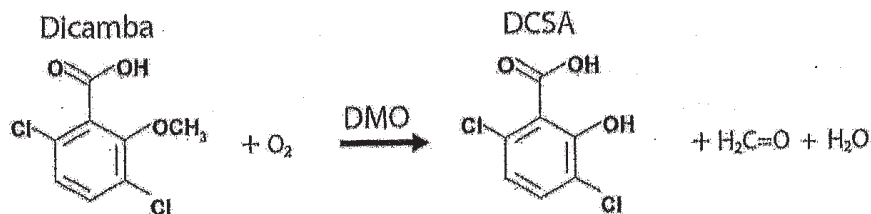


Figure 8.4 A schematic illustrating the oxygenase activity of the DMO enzyme on the dicamba herbicide molecule. Figure adapted from D'Ordine *et al.*, 2009.

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.

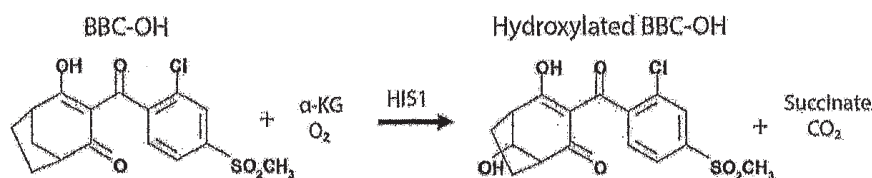


Figure 8.5 A schematic illustrating the oxygenase activity of the HIS1 enzyme on the benzobicyclon (BBC) herbicide molecule. Figure adapted from Maeda *et al.*, 2019.

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.

References

- Amy P. S., Schulke J. W., Frazier L. M., and Seidler R., *J. Appl. Environ. Microbiol.* **49**(5), (1985), 1237–1245.
- Behrens M. R., Mutlu N., Chakraborty S., Dumitru R., Jiang W. Z., LaVallee B. J., Herman P. L., Clemente T. E., and Weeks D. P., *Science* **316** (2007), 1185–1188.
- Bugg T. D. H., *Tetrahedron* **59** (2003), 7075–7101.
- Cao M., Sato S. J., Behrens M., Jiang W. Z., Clemente T. E., and Weeks D. P., *J. Agric. Food Chem.* **59** (2011), 5830–5834.
- Carpenter J. E., *Nat. Biotechnol.* **28**(4), (2010), 319–321.
- Cork D. J., and Krueger J. P., *Adv. Appl. Microbiol.* **36** (1991), 1–66.
- Dehaye L., Alban C., Job C., Douce R., and Job D., *Eur. J. Biochem.* **225** (1994), 1113–1123.
- Dumitru R., Jiang W. Z., Weeks D. P., and Wilson M. A., *J. Mol. Biol.* **392** (2009), 498–510.

- D'Ordine R. L., Rydel T. J., Storek M. J., Sturman E. J., Moshiri F., Bartlett R. K., Brown G. R., Eilers R. J., Dart C., Qi Y., Flasiński S., and Franklin S. J., *J. Mol. Biol.* **392** (2009), 481–497.
- Fukumori F., and Hausinger R. P., *J. Bacteriol.* **175**(7), (1993a), 2083–2086.
- Fukumori F., and Hausinger R. P., *J. Biolog. Chem.* **268**(32), (1993b), 24311–24317.
- Guengerich F. P., *J. Biol. Chem.* **290**(34), (2015), 20700–20701.
- Heap I. *The International Survey of Herbicide Resistant Weeds*. Available: www.weedscience.org. (Accessed 2021).
- Herbert D. Price L. J., Alban C., Dehay L., Job D., Cole D. J., Pallett K. E., and Harwood J. L., *Biochem. J.* **318** (1996), 997–1006.
- Herman P. L., Behrens M., Chakraborty S., Chrastil B. M., Barycki J., and Weeks D. P., *J. Biol. Chem.* **280**(26), (2005), 24759–24767.
- Hogan D. A., Smith S. R., Saari E. A., McCracken J., and Hausinger R. P., *J. Biolog. Chem.* **275**(17), (2000), 12400–12409.
- Horvath M., Ditzelmüller G., Loidl M., and Streichsbier F., *Appl. Microbiol. Biotechnol.* **33** (1990), 213–216.
- Kohler H. P. E., *J. Ind. Microbiol. Biotechnol.* **23** (1999), 336–340.
- Larue C. T., Goley M., Shi L., Evdokimov A. G., Sparks O. C., Ellis C., Wollacott A. M., Rydel T. J., Halls C. E., Van Scoyoc B., Fu X., Nageotte J. R., Adio A. M., Zheng M., Sturman E. J., Garvey G. S., and Varagona M. J., *Pest Manag. Sci.* **75** (2019), 2086–2094.
- Larue C. T., Ream J. E., Zhou X., Moshiri F., Howe A., Goley M., Sparks O. C., Voss S. T., Hall E., Ellis C., Weihe J., Qi Q., Ribeiro D., Wei X., Guo S., Evdokimov A. G., Varagona M. J., and Roberts J. K., *Pest Manag. Sci.* **76** (2020), 1031–1038.
- Lee D. W., and Hwang I., *Mol. Cell* **41**(3), (2018), 161–167.
- Maeda H., Murata K., Sakuma N., Takei S., Yamazaki A., Karim R., Kawata M., Hirose S., Kawagishi-Kobayashi M., Taniguchi Y., Suzuki S., Sekino K., Ohshima M., Kato H., Yoshida H., and Tozawa Y., *Science* **365**(6451), (2019), 393–396.
- Mall T., Gupta M., Singh Dhadialla T., and Rodrigo S. Overview of biotechnology-derived herbicide tolerance and insect resistance traits in Plant Agriculture in Transgenic Plants: Methods and Protocols, *Methods in Molecular Biology*, Volume 1864 (Kumar S., et al. eds), Springer Nature, London, 2019.
- Müller T. A., Fleischmann T., van der Meer J. R., and Kohler H.-P. E., *Appl. Environ. Microbiol.* **72**(7), (2006), 4853–4861.

- Naidu D., and Ragsdale S. W., *J. Bacteriol.* **183**(11), (2001), 3276–3281.
- Ndikuryayo F., Moosavi B., Yang W.-C., and Yang G.-F., *J. Agric. Food Chem.* **65** (2017), 8523–8537.
- Nickel K., Suter M. J.-F., and Kohler H.-P. E., *J. Bacteriol.* **179**(21), (1997), 6674–6679.
- Norsworthy J. K., Ward S. M., Shaw D. R., Llewellyn R. S., Nichols R. L., Webster T. M., Bradley K. W., Frisvold G., Powles S. B., Burgos N. R., Witt W. W., and Barrett M., *Weed Science* **12**(sp1), (2012), 31–62.
- Padgett S. R., Kolacz K. H., Delannay X., Re D. B., LaVallee B. J., Tinius C. N., Rhodes W. K., Otero Y. I., Barry G. F., Eichholtz D. A., Peschke V. M., Nida D. L., Taylor N. B., and Kishore G. M., *Crop Science* **35** (1995), 1451–1461.
- Pazmiño D. E. T., Winkler M., Glieder A., and Fraaije M. W., *J. Biotechnol.* **146** (2010), 9–24.
- Peterson M. A., McMaster S. A., Riechers D. E., Skelton J., and Stahlman P. W., *Weed Technol.* **30**(2), (2016), 303–345.
- Ruizzo M. A., and Gorski S. F., *Weed Science* **36** (1988), 713–718.
- Schleinitz K. M., Kleinstaub S., Vallaeys T., and Babel W., *Appl. Environ. Microbiol.* **70**(9), (2004), 5357–5365.
- Shaner D. L. *Herbicide Handbook*, 10th edn, Weed Science Society of America, Lawrence, KS, 2014.
- Stowe E., and Dhingra A. *Development of the Arctic® Apple in Plant Breeding Reviews*, Vol. 44 (Goldman I., ed), John Wiley and Sons, New York, 2020.
- Wang X.-Z., Li B., Herman P. L., and Weeks D. P., *Appl. Environ. Microbiol.* **63**(4), (1997), 1623–1626.
- Wehrmann A., Van Vliet A., Opsomer C., Botterman J., and Schulz A., *Nat. Biotechnol.* **14** (1996), 1274–1278.
- Westendorf A., Müller R. H., and Babel W., *Acta Biotechnol.* **23**(1), (2003), 3–17.
- Wright T. R., Shan G., Walsh T. A., Lira J. M., Cui C., Song P., Zhuang M., Arnold N. L., Lin G., Yau K., Russell S. M., Cicchillo R. M., Peterson M. A., Simpson D. M., Zhou N., Ponsamuel J., and Zhang Z., *Proc. Nat. Acad. Sci.* **107**(47), (2010), 20240–20245.
- Wu L.-F., Meng S., and Tang G.-L., *Biochim. Biophys. Acta* **1864** (2016), 453–470.
- Yang J., Wang X.-Z., Hage D. S., Herman P. L., and Weeks D. P., *Analyt. Biochem.* **219** (1994), 37–42.