MECHANISM OF RESISTANCE IN COMMON RAGWEED TO PPO-INHIBITING HERBICIDES

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Crop Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

Synthetic herbicides have been reliable tools to help growers produce a healthy and profitable crop for several decades but their efficacy is under attack from the evolution of herbicide-resistant weeds. Almost all commercially available herbicidal modes of action have documented cases of herbicide-resistant weeds. One particular herbicidal class is known as the protoporphyrinogen oxidase (PPO, Protox) inhibiting herbicides. Only four weed species have been documented with resistance to this herbicide class. Waterhemp was the first weed species documented with PPO-resistance, which was due to a single codon deletion in a dual-targeting gene known as *PPX2L*. Common ragweed, from Delaware, was the third documented weed species that evolved PPO-inhibiting herbicide resistance. This thesis provides information on the resistance mechanism of this common ragweed biotype to PPO-inhibiting herbicides. Chi-square goodness-of-fit analysis on a segregating F_2 population of common ragweed sprayed with a PPO-inhibitor, fomesafen, revealed that data were consistent with resistance conferred by a single, dominant gene. Sequences of the target-site genes were obtained through the screening of a sensitive common ragweed cDNA library and 5'-RACE. Analysis of sequence from the target-site genes revealed multiple polymorphisms between the parental alleles of an F_1 plant that could be responsible for resistance. Molecular marker analysis of the F_2 common ragweed population revealed that the *PPX2* gene co-segregated with PPO-inhibiting herbicide resistance. One of the polymorphisms, R98L, was suspected of being responsible for conferring resistance since it was at a conserved location and functionally involved in substrate coordination within the active site.

Complementation of a mutant bacterial system with a construct containing a R98L mutation conferred resistance while in the presence of a PPO-inhibiting herbicide, lactofen. Growth curves were performed with the same complemented mutant bacterial system while in the presence of lactofen to quantitatively confirm the role of the mutation in resistance. Currently, the R98L mutation of common ragweed is the second documented resistance mechanism to PPO-inhibiting herbicides. Both of the resistance mechanisms of waterhemp and common ragweed are due to mutations present in the *PPX2* gene. Resistance in waterhemp can be obtained by a deletion, but for common ragweed only a single base pair change is sufficient to confer resistance to PPO-inhibiting herbicides.

To My Father, Mother, and Brother

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Patrick J. Tranel, for his support, guidance, attention to detail, and expertise throughout the course of my degree. I am grateful for the opportunity to work on such a dynamic project that has given me the necessary tools to succeed as a scientist and developed my interest in weed science. I thank my committee members: Dr. Stephen P. Moose, for all his scientific insights and advice; and Dr. Marty Williams, for his valuable input and agreeing to serve on my committee. I wish to thank Dr. Ryan M. Lee, in particular, for mentoring me during my project; his encouragement, technical assistance, and patience made this possible. To the rest of the Tranel lab past and present, Sukhvinder Singh, Michael S. Bell, Kate Thinglum, Dr. Danman Zheng, Dr. Chance Riggins, and Dr. Jianyang Liu; I appreciate all they have done and I could not have made it through graduate school without their friendship, support, advice, and coffee breaks. To AJ Woodyard, I am forever thankful for his unwavering support, advice, practical weed science knowledge, and friendship. He is such a wonderful soul and I have enjoyed being a part of his life. I would like to thank the herbicide evaluation team; Doug Maxwell, Lisa Gonzini, Josh Kunkel, and Dr. Aaron Hager for their willingness to take me under their wings to teach me the applied side of weed science and for this I am truly grateful. I also owe thanks to Dr. Fred Kolb for encouraging me throughout my entire college career. To Sharon Conatser, and Edhilva Campos, I thank you both for your wonderful friendships, support, and our countless discussions over many great dinners. My deepest gratitude goes to my father, mother, and brother for their tremendous support, guidance, and unconditional

love, which they have unselfishly given throughout my entire life. The successes in my life would not have been possible without them.

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CHAPTER 1

INTRODUCTION

1.1 Herbicide Resistance

Herbicides are an excellent tool that agriculture possesses today but they can also be a culprit in generating many challenges for the entire industry. One challenge in particular, is the selection of herbicide-resistant weeds. There are multiple factors that contribute to the evolution of an herbicide resistance trait in plant populations. There are several mechanisms of resistance in weeds: insensitive site of action, enhanced metabolism, compartmentalize/sequestration, or decreased uptake or translocation. Aiding in the evolution of this trait are two precursors: the occurrence of heritable variation for the trait and natural selection (Maxwell and Mortimer 1994). Herbicide applications exert an immense amount of selection intensity on a plant population and therefore influence the evolution rate of resistance. Three characteristics of herbicides generally contribute to selection pressure for herbicide resistance: the efficacy of the herbicide, frequency of use, and duration of effect (Maxwell and Mortimer 1994). Repetitive use of similar herbicidal modes of action and reduced discovery of new herbicides (Cole et al. 2000) has made it exceedingly difficult to control herbicide-resistant weeds. All of these multiple factors have lead to the global evolution of 345 resistant weed biotypes in 194 weed species and over 340,000 fields (Heap 2010).

The first documented case of herbicide resistance was reported in Washington during 1970 with common grounsel *(Senecio vulgaris* L.) to the photosystem II (PSII) inhibiting herbicides (Ryan 1970). PSII-inhibiting herbicides contain several chemical

families but one in particular is the triazine herbicides. The most common mutation conferring triazine resistance is S264G of the psbA gene (Hirschberg and McIntosh 1983). This particular mutation causes reduced binding of the triazine herbicides which results in a decrease of photosynthesis efficiency and a fitness penalty for resistant plants. Less common mechanisms of resistance to these herbicides are differential absorption or translocation, or herbicide detoxification (Hess 2000). For example, in a triazine-resistant velvetleaf (*Abutilon theophrasti* Medik.) biotype, resistance is due to enhanced glutathione S-transferase activity for triazines which results in an increased capacity to detoxify the herbicide via glutathione conjugation (Anderson and Gronwald 1991). There are currently over 60 resistant weed biotypes world-wide to PSII inhibitors (Heap 2010).

The first acetolactate synthase (ALS) inhibiting herbicides were introduced in the 1980s. Reports of resistance started surfacing around 1990, which was not long after this chemistry was launched into the market. In most cases, resistance to ALS-inhibiting herbicides is caused by an altered ALS enzyme and less commonly due to enhanced metabolism by the cytochrome P450s, such as in rigid ryegrass (*Lolium rigidum* Gaudin) (Christopher et al. 1991, 1992) and wild oat (*Avena fatua* L.). There are multiple single point mutations which alter the binding pocket of the herbicide and therefore confer resistance to ALS-inhibitors in natural weed populations: A122, P197, A205, D376, W574, S653, and G654 (Tranel et al. 2009). Resistance easily occurred via natural selection in this chemistry for multiple reasons: mutations in the ALS enzyme confer little if any fitness penalty to the plant, widespread usage of these herbicides, repetitive usage, and the strong selection pressure exerted. These herbicides wield strong selection

pressure because of their high activity on sensitive biotypes at the rates used and because of their soil residual activity (Tranel and Wright 2002). There are currently over 100 resistant weed biotypes world-wide to ALS-inhibiting herbicides (Heap 2010).

Acetyl-CoA Carboxylase (ACCase) inhibitors are a class of herbicides with the third most cases of resistance present with over 30 resistant weed biotypes in the world (Heap 2010). Resistance to this class of herbicides was first documented in the 1980s. There are two mechanisms of resistance present for ACCase inhibitors: increased metabolism or insensitive site of action. In nearly all weed biotypes analyzed, resistance is governed by a single, dominant or semi-dominant, nuclear gene (Seefeldt et al. 1998; Tardif et al. 1996). There also appears to be little or no fitness penalty associated with resistant biotypes.

Another herbicide for which resistance issues have gained much recent interest is glyphosate [N-(phosphonomethyl)glycine]. Ever since the creation of glyphosate-resistant crops, there has been a heavy overreliance and usage of this herbicide. Not surprisingly, resistant weed biotypes have emerged and there are currently 18 biotypes world-wide (Heap 2010). Although resistance has been present for numerous years, the resistance mechanism has been somewhat elusive and arduous to determine. Two resistance mechanisms have been identified in naturally occurring glyphosate-resistant weeds, reduced target-site affinity for glyphosate and reduced translocation of glyphosate to meristematic regions (Brewer and Oliver 2009). Recently, a new mechanism of glyphosate resistance was discovered: *EPSPS* gene amplification in Palmer amaranth (*Amaranthus palmeri* S. Wats.) from Georgia resulted in high levels of EPSPS expression which produced high-level glyphosate resistance (Gaines et al. 2010).

1.2 PPO-Inhibiting Herbicides: Mode of Action

One particular class of herbicides that has only a few documented cases of natural weed resistance is protoporphyrinogen oxidase (PPO, Protox) inhibitors. There are three main chemical classes that share Protox as a site of action: diphenylethers, N-phenylpthalimides, and aryl triazinones. PPO-inhibiting herbicides are important for weed control in turfgrass, soybean [*Glycine max* (L.) Merr.], maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), sunflower (*Helianthus annus* L.), peanut (*Arachis hypogaea* L.), sugarcane (*Saccharum officinarum* L.), sweet corn (*Zea mays var*. *rugosa* Bonaf.), and rice (*Oryza sative* L.).

Protox is a fundamental enzyme in the tetrapyrrole biosynthetic pathway. It oxidizes protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX) as the last common step in the production of both heme and chlorophyll (Beale and Weinstein 1990). Tetrapyrroles in the form of hemoproteins and chlorophylls are present in almost all forms of life. These tetrapyrrole derivatives function primarily in energy metabolism, as carriers of oxygen (hemoglobins or leghempglobin), as electron carriers (cytochromes), or as agents for trapping radiant energy (chlorophylls) (Lascelles 1964). Chlorophyll belongs to a family of pigments that are present in the green light-harvesting tissues of a plant responsible for carrying out photosynthesis. During photosynthesis, chlorophylls are able to capture energy they absorb from the sun. Energy, which was previously obtained from chlorophylls, facilitates the conversion of carbon dioxide and water into oxygen and carbohydrates. Heme is a crucial compound for respiration and an essential cofactor in catalases, cytochromes, oxygenases, and peroxidases. Other important compounds produced from this pathway are poryphyrins and vitamin B_{12}.

Under optimal conditions, meaning no herbicide is present, the tetrapyrrole pathway starts with glutamate in the chloroplast and through a series of reactions is converted into δ-aminolevulinic acid. During another sequence of reactions, protoporphyrinogen IX is produced and then converted to protoporphyrin IX. Depending on a subsequent reaction, with either iron chelatase with Fe^{2+} or magnesium chelatase with Mg^{2+}, heme or chlorophyll production will ensue respectively.

Diphenylether (DPE) herbicides inhibit Protox by competing for the substrate binding site. Since Protox is blocked, protoporphyrinogen IX accumulates and leaks from the plastid into the cytoplasm, and surrounding cellular membranes, where an enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX rapidly occurs (Jacobs and Jacobs 1993; Jacobs et al. 1991; Lehnen et al. 1990). The enzyme involved in the oxidation of protoporphyrinogen IX is unknown but it appears to be a type of peroxidase linked with the plasma membrane, endoplasmic reticulum, or microsomes (Lee and Duke 1994; Retzlaff and Böger 1996; Yamato et al. 1994). The end products of the pathway, heme and chlorophyll, do not accumulate and eventually are depleted which deregulates the pathway. This creates large quantities of protoporphyrin IX and since the conversion of protoporphyrinogen IX to protoporphyrin IX is occurring in the cytoplasm there are no protective antioxidant mechanisms present, which exists in the chloroplast, to prevent cell damage. Interaction of protoporphyrin IX with oxygen and light causes production of massive amounts of singlet oxygen and other oxidative species that trigger lipid peroxidation, cell membrane damage, and rapid necrosis.

1.3 PPO-Inhibiting Herbicides: Symptoms and Crop Injury

Herbicides targeted to Protox cause susceptible plants to develop symptoms rapidly in the presence of light. Due to this rapid nature, these herbicides are known as contact burning herbicides. Depending on which herbicide is used, applications can be made preplant, preemergence, and/or postemergence. Most broadleaf weeds are controlled and some suppression of perennial grasses and other weeds are possible but thorough spray coverage of the weed is crucial for control.

Temporary crop injury can be expected after a postemergence application, even when used at the proper rates. Application of PPO-inhibiting herbicides prior to prolonged cool periods or during hot humid conditions will result in crop injury because of reduced ability of the crop to metabolize the herbicide, decreasing herbicide tolerance (Hartzler 2004; Owen and Hartzler 2008). Soybean leaves that are open during the time of application may have some bronzing, burn, or speckling. Trifoliate soybean leaves that have emerged but are not yet open can have leaf cupping, malformation, and crinkled leaf margins (Anonymous 2007). Soybean yields are typically not adversely affected from these symptoms and the plants will usually grow out of them. In severe situations herbicide injury has resulted in the death of the terminal growing point in crops resulting in malformed plants (Owen and Hartzler 2008). Exposure of susceptible plants to a postemergence application of these herbicides results in a water soaked appearance, which is a product of membrane damage, followed by necrosis in those affected regions (Owen and Hartzler 2008). With preemergence applications, tissue necrosis is initiated when plants emerge above the soil surface and are exposed to light. Crop injury also can happen if heavy rains occur when the plants are emerging through the soil surface. The

splashing, from rainfall, causes high concentrations of the herbicide to come into contact with the hypocotyls, cotyledons, and growing points to cause tissue necrosis (Hartzler 2004).

1.4 PPO-Resistance: Synthetic

Target-site genes for PPO-inhibiting herbicides have been studied in several crop species, model plant species, weed species, and bacterium for various purposes. PPO proteins are integral membrane-bound proteins present in the chloroplast and associated with both chloroplast membranes: envelope and thylakoid (Lee et al. 1993; Matringe et al. 1992). Activities of the last two enzymes involved in the heme synthesizing pathway, protoporphyrinogen oxidase and ferrochelatase, were found in the mitochondria which demonstrate that protoporphyrinogen IX is distributed between the plastidic pathway and the mitochondrial heme synthesis pathway (Lermontova and Grimm 2000). There are at least two nuclear PPO genes present in higher organisms, *PPX1* and *PPX2*, which encode plastid- and mitochondrial-targeted PPO isoforms respectively.

Synthetic resistance to PPO-inhibitors has been created in many different ways by using multiple genes from various sources or via selection. An herbicide-resistant tobacco (*Nicotiana tabacum* L.) cell line was selected by conventional tissue culture while in the presence of a PPO-inhibitor (Ichinose et al. 1995). It was concluded that the herbicide resistance in this cell line was due to over expression of the mitochondrial PPO gene (*PPX2*) (Ichinose et al. 1995; Watanabe et al. 1998; Watanabe et al. 2002). A resistant soybean cell line was created by stepwise selection methods to oxyfluorfen [2-

chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] as a result of the overproduction of mitochondrial PPO (*PPX2*) mRNA (Warabi et al. 2001).

In an alga species, *Chlamydomonas reinhardtii*, resistance was obtained by selection and several co-transformation protocols (Randolph-Anderson et al. 1998). The resistant mutant contained a V389M substitution in the plastidic PPO gene (*PPX1*) which may have caused a change in the conformation of the active site and decreased its affinity for herbicides (Randolph-Anderson et al. 1998).

The wild-type *Arabidopsis thaliana* L. plastidic PPO gene isoform (*PPX1*) was placed under the control of the cauliflower mosaic virus 35S promoter and over expressed in tobacco (Lermontova and Grimm 2000). Resistance to acifluorfen {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid} was obtained because the over expression of the PPO gene neutralized herbicidal action by preventing the accumulation of the substrate, protoporphyrinogen IX, which prevented the light-dependent damage from occurring (Lermontova and Grimm 2000).

Creation of a highly resistant double mutant occurred when Y426M and S305L in the plastidic PPO gene isoform (*PPX1*) were combined and placed under the control of a native *Arabidopsis* PPO promoter and transformed back into *Arabidopsis* (Hanin et al. 2001; Li et al. 2003). This double mutant was also used to develop transgenic PPO-resistant crops. PPO-resistant maize was created using the *Arabidopsis* double mutant and a two-step screen, involving the greenhouse and field, with field rates of a PPO-inhibiting herbicide (Li et al. 2003). Resistant PPO genes and the process of using those genes to create herbicide-resistant maize were given the trade name of Acuron® technology (Holmberg 2000).

A dual-targeting *Myxococcus xanthus* Protox gene was placed under the control of the cauliflower mosaic virus 35S promoter, over expressed in rice, and conferred high levels of oxyfluorfen resistance (Jung et al. 2004). This transgenic rice line, named M4, was found to be cross-resistant to multiple PPO-inhibiting herbicides and resistant to field level herbicide applications (Jung et al. 2010). However a minor yield drag could potentially occur with the transgenic plants in certain environments (Jung et al. 2010).

1.5 PPO Resistance: Natural

Multiple methods have been utilized to synthetically manufacture PPO-resistance but there are only a few documented cases that exist in nature. DPEs were first introduced as commercial herbicides in 1962 (Matsunaka 1976). It took until 2001 before there were any documented cases of resistant weed species (Heap 2009). To date there are four weed species in the world that have evolved resistance to PPO-inhibitors: waterhemp (*Amaranthus rudis* Sauer), common ragweed (*Ambrosia artemisiifolia* L.), wild poinsettia (*Euphorbia heterophylla* L.), and pigweed (*Amaranthus quitensis* Kunth) (Heap 2010).

The first weed to develop resistance to PPO-inhibiting herbicides and in which the mechanism of resistance identified was waterhemp. Resistance to PPO-inhibiting herbicides in waterhemp was shown to result from a unique mechanism: a single codon deletion in a gene that encoded PPO (Patzoldt et al. 2006). Specifically, resistance is a result of a deletion in gene *PPX2L*, which encodes both plastid- and mitochondrial-targeted PPO gene isoforms. This dual-targeting phenomenon has been previously reported for both spinach (*Spinacia oleracea* L.) and maize (*Zea mays* L.) *PPX2* gene

(Watanabe et al. 2001) and by analyzing protein sequence data, it has been determined that sorghum [*Sorghum bicolor* (L.) Moench] contains this unique dual-targeting gene as well. An alignment of the amino acid sequences from *PPX2L* and *PPX2* from various plant species distinguishes the plants with the rare dual-targeting transit peptide from plants where it is absent (Figure 1.1). The *PPX2* gene has another unique aspect to it in that the mitochondrial transit peptide is important for the functionality of the protein and was found in tobacco to be transported to the mitochondria without any detectable size reduction (Lermontova et al. 1997) meaning that it is not processed. In the case of *PPX2L*, the chloroplast transit peptide is processed after transport into chloroplasts and the mitochondrial transit peptide stays intact.

It was originally thought that waterhemp contained several *PPX* genes: *PPX1*, *PPX2S*, and *PPX2L*. The resistant biotype contained *PPX2L* with the 3-bp deletion, ΔG210, but *PPX2S*, which was predicted to only encode the mitochondrial PPO isoform, was absent. Recently, after further allele testing and examination of gene sequence data, it has been determined that there are likely only two *PPX* genes in waterhemp, *PPX1* and *PPX2L* (Lee et al. 2008).

Multiple PPO-inhibiting herbicide resistant waterhemp populations from Illinois have been characterized and ΔG210 was the only mechanism of resistance (Lee et al. 2008). Resistant waterhemp populations from Missouri, Kansas, and more from Illinois have also been characterized and the ΔG210 deletion is still the only mechanism of resistance found in waterhemp to confer PPO-inhibiting herbicide resistance (Thinglum et al. 2009). Due to the prevalence of this mutation in resistant populations, a PCR-based assay was utilized as a diagnostic tool for resistance to PPO inhibitors in waterhemp (Lee

et al. 2008). To date there is no published literature or explanation of the resistance mechanism to PPO-inhibiting herbicides in common ragweed, wild poinsettia, or pigweed (quitensis).

1.6 Common Ragweed: Characteristics and Interspecific Competition

Common ragweed is a native annual, dicot, and diploid with a chromosome number of 2n=36 (Payne et al. 1964). Common ragweed is the most abundant of the ragweed species and is present in every state in the United States as well as in numerous other countries. Wind is the primary mode of pollen flow. This species is monoecious and highly outcrossing with a low capacity for self-pollination (Friedman and Barrett 2008). Common ragweed only spreads by seeds and smaller plants produce about 3,000 seeds per plant, while larger plants can produce up to 62,000 seeds per plant (Dickerson and Sweet 1971). Seeds that are buried in the soil can survive there for years and remain viable until the proper conditions for germination occur.

Common ragweed can be a hard-to-control weed for many farmers in their fields. Researchers from numerous states claim common ragweed on their lists of the worst weeds to control. It has been reported that common ragweed is more competitive with soybean than with corn (Weaver 2001). Growth of common ragweed is drastically reduced by decreased light quantity and increased far red to red light ratios as might occur under a corn canopy (Raynal and Bazzaz 1975). In a study performed in Ontario, the estimated yield loss at high common ragweed density was 65% to 70% in soybean (Weaver 2001). It has also been shown that as common ragweed densities increase in

soybean there are several negative effects: soybean yield decreases, soybean seed moisture increases, and dockage increases (Cowbrough et al. 2003).

1.7 Common Ragweed Resistance

An additional factor which contributes to common ragweed being difficult to control for producers is its resistance to different herbicide modes of action. Worldwide, common ragweed is resistant to five different modes of action: PSII-inhibitors, ALS-inhibitors, glycines, PPO-inhibitors, and ureas and amides (Heap 2010). Not much is known about the resistance mechanism of common ragweed to any of these herbicide classes except for the ALS-inhibitors. Resistance to ALS-inhibitors in an Indiana population is due to an altered target-site, specifically a W574L mutation (Patzoldt et al. 2001). This biotype from Indiana has a resistance level of greater than 10 fold to sulfonylureas, imidazolinones, and triazolopyrimidines (Tranel et al. 2009). It has not been determined if there is resistance to the pyrimidinylthiobenzoates or sulfonylaminocarbonyltriazolinones (Tranel et al. 2009). Resistance mechanisms of PSII-inhibitors, glycines, PPO-inhibitors, and ureas and amides have not been documented to date.

Another biotype of common ragweed, which is researched in this thesis, represents the third weed to develop resistance to PPO-inhibiting herbicides and was discovered in Sussex County Delaware in 2005 (Moreira et al. 2006). It was found infesting soybean fields that had been previously treated with various PPO-inhibitors and ALS-inhibitors. Resistance was suspected so common ragweed seeds were collected from the field, grown, and subjected to greenhouse trials with numerous herbicides from

both of these modes of action. Comparisons of the GR_{50} values from the resistant and a sensitive biotype indicated the resistance levels to the PPO-inhibiting herbicides used in the study (Table 1.1). This biotype of common ragweed had multiple resistances to PPO- and ALS-inhibiting herbicides and cross resistance to multiple herbicides within these two sites of action (Moreira et al. 2006).

1.8 Research Objectives

With the occurrence of herbicide-resistance for various modes of action, PPO-inhibiting herbicides may be a practical option for control of herbicide-resistant weeds, assuming PPO-resistance is not already present. Resistance in this class of herbicides has been a rare and relatively slow phenomenon to develop. Understanding the mechanism of resistance and its genetic basis is important, so that the commonalities and differences between resistance cases can be unraveled (Neve 2007). The mechanism of resistance in waterhemp has been well documented but no work has been done with common ragweed. With this in mind, we can use the resistance mechanism in waterhemp as a model to understand PPO-inhibiting herbicide resistance in common ragweed.

Once the mechanism is discovered in common ragweed, perhaps new methods to overcome resistance can be developed and thus control resistant weeds (Tharayil-Santhakumar 2010). For example, if the resistance mechanism in common ragweed is understood, perhaps this knowledge could be put to practical use by creating an assay to test for presence of resistance in a suspected field to then make proper management decisions based on the results. Another possible application is to transform the herbicide resistance genes into crops, to produce herbicide resistance, and allow the use of

alternative herbicides in crops (Tharayil-Santhakumar 2010). The resistance trait could also be utilized as a tool to understand basic plant biochemical processes and fundamental mechanisms by which they defend themselves from toxic xenobiotic chemicals (Tharayil-Santhakumar 2010). Therefore the need to elucidate the resistance mechanism of common ragweed to PPO-inhibiting herbicides is vital for proper management, future possibilities of crop development, and knowledge.

Resistance of common ragweed to this class of herbicides raises many questions. Is the resistance mechanism target-site based and, if so, what are the genes responsible? Is the resistance mechanism of common ragweed similar to that of waterhemp? Does the common ragweed genome contain a *PPX2L* gene like waterhemp? How many genes are controlling this herbicide resistance trait and is it dominant or recessive? Can a DNA based assay be developed to diagnose resistance, similar to that in waterhemp? All of these questions are addressed in this thesis to ascertain common ragweed's resistance mechanism.

1.9 Attributions

Some of this research was initiated by Dr. Ryan M. Lee before I had joined the lab as a graduate student and, therefore, part of the work cited in this thesis was performed by him. In Chapter 2, Dr. Ryan M. Lee performed the genetic crosses of the F_1 and F_2 common ragweed lines; evaluated the F_1 line; assisted me with the evaluation of the F_2 common ragweed line; amplified the partial *PPX2* sequence from the cDNA library with primers created from waterhemp, which were later utilized by me to create the homologous *PPX2* probe; and designed the CRag-PPX2-F and CRag-PPX2-R

primers. He also created the waterhemp wild-type *PPX2* construct, which was in the pET28a(+) DNA expression vector as cited in Chapter 3. In Chapter 2, the Del-R and DV1-S initial common ragweed populations were stratified in the wet sand by Dr. Dean Volenberg, Sukhvinder Singh, and Michael S. Bell before I had joined the lab. In Chapters 2 and 3, I performed the initial sequencing reactions and then they were sent to the W.M. Keck Center for Comparative and Functional Genomics as ready-to-load reactions to be purified and sequenced.

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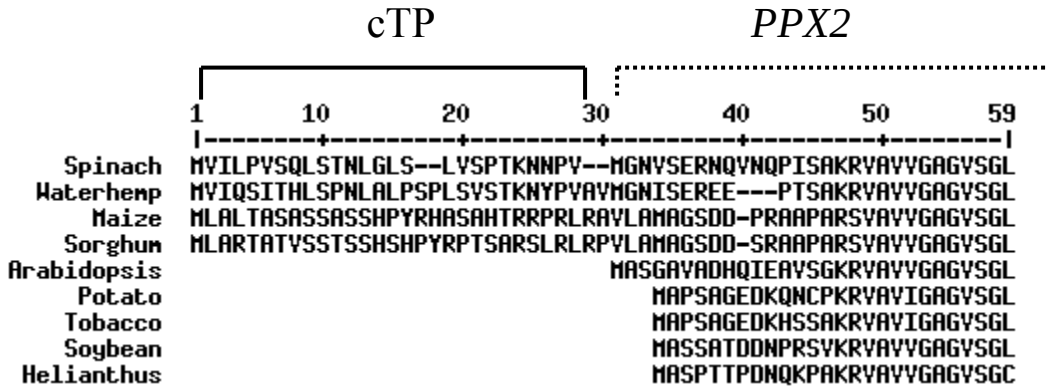
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1.11 Tables and Figures

Table 1.1. Resistance levels of the common ragweed biotype to various PPO-inhibiting herbicides. (Data from Moreira et al. 2006)

PPO-Inhibiting Herbicide	Resistance Level (X)
acifluorfen	32
fomesafen	28
lactofen	14
flumioxazin	12
carfentrazone	6-10
flumiclorac	6-10
oxyfluorfen	6-10
pyraflufen	6-10
sulfentrazone	6-10

Figure 1.1. Partial alignment of *PPX2L* and *PPX2* proteins from various plant species.



Sequences are from spinach (AB046993), waterhemp (DQ386117), maize (NP_001105004), sorghum (XP_002446710), *Arabidopsis* (NM_121426), potato (CAA12401), tobacco (Y13466), soybean (BAA76348), and *Helianthus* (HELI_7CDS.CSA1.5882).

CHAPTER 2

ISOLATION OF *PPX* GENES IN COMMON RAGWEED

2.1 Abstract

Herbicides are an essential facet of agriculture to manage weeds, except if the weeds have evolved resistance then options for control decrease dramatically. Resistance to protoporphyrinogen oxidase (PPO, Protox) inhibitors has been relatively slow to evolve, with only four resistant weeds world-wide, compared to other herbicidal modes of action. Common ragweed was previously reported to be resistant to multiple herbicidal modes of action, including PPO-inhibiting herbicides, making it the third documented case of such resistance. Resistance to PPO-inhibiting herbicides in waterhemp previously was demonstrated to be due to a single codon deletion, designated as ΔG210, in the *PPX2L* gene, which encodes both plastid- and mitochondrial-targeted PPO isoforms. With the resistance mechanism in waterhemp already documented, we used this information as a model for resistance in common ragweed to PPO-inhibiting herbicides. Evaluation of the common ragweed biotype revealed that data were consistent with resistance conferred by a single, dominant gene when performing a chi-square goodness-of-fit test on a segregating F_2 population. Partial to full-length sequences of the target-site genes were obtained through the screening of a sensitive common ragweed cDNA library and 5'-RACE. Analysis of sequence from the target-site genes revealed multiple polymorphisms between the parental alleles of an F_1 plant that could be responsible for PPO-inhibiting herbicide resistance in common ragweed.

2.2 Introduction

Herbicides are a very important aspect of crop production and plant health but they also can create challenges, such as the selection of herbicide-resistant weeds. Several resistance mechanisms exist in weeds including insensitive site of action, enhanced metabolism, compartmentalize/sequestration, or decreased uptake or translocation. When an herbicide is applied it exerts strong selection pressure on a plant population and therefore influences the evolution rate of resistance. The lack of discovery of new herbicidal modes of action and the repetitive use of similar herbicidal modes of action has greatly increased the difficultly to control herbicide-resistant weeds (Cole et al. 2000). Multiple factors have led to the global evolution of a startling 345 resistant weed biotypes in 194 weed species in over 340,000 fields (Heap 2010), and these numbers will only increase as time goes on. Therefore, it has become crucial to understand the mechanisms of resistance to perhaps develop new methods to overcome resistance and thus control resistant weeds (Tharayil-Santhakumar 2010).

A class of herbicides with only a few documented cases of natural weed resistance is the protoporphyrinogen oxidase (PPO, Protox) inhibitors. Protox oxidizes protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX) as the last common step in heme and chlorophyll biosynthesis in the tetrapyrrole biosynthetic pathway (Beale and Weinstein 1990). Chlorophyll is crucial for the capture and utilization of energy from the sun and heme is an important compound in respiration and an essential cofactor for key enzymes.

One particular class of PPO-inhibitors is diphenylether (DPE) herbicides. When DPEs are applied they cause susceptible plants to develop symptoms rapidly in the

presence of light. Rapid nature of these herbicides dubs them as contact burning herbicides. DPEs compete for the substrate binding site thereby inhibiting Protox. Since Protox is blocked, protoporphyrinogen IX accumulates and leaks from the plastid into the cytoplasm, and surrounding cellular membranes, where an enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX rapidly occurs (Jacobs and Jacobs 1993; Jacobs et al. 1991; Lehnen et al. 1990). Large quantities of protoporphyrin IX are produced in the cytoplasm where no protective antioxidant mechanisms are present to prevent cell damage. Interaction of protoporphyrin IX with oxygen and light causes the production of singlet oxygen and other oxidative species which trigger lipid peroxidation, cell membrane damage, and rapid necrosis of the susceptible plant.

Two types of resistance to PPO-inhibiting herbicides have been researched: synthetic and natural. At least two nuclear PPO genes are present in higher organisms, *PPX1* and *PPX2*, which encode plastid- and mitochondrial-targeted PPO isoforms respectively. Various methods were utilized in manipulating these target-site genes in different plant and bacterial species to obtain synthetic resistance. Methods of manipulation for the gene encoding the enzyme mitochondrial targeted involved the over expression of *PPX2* in tobacco (*Nicotiana tabacum* L.) and soybean [*Glycine max* (L.) Merr.] (Ichinose et al. 1995; Warabi et al. 2001). Synthetic resistance was obtained in the plastidic targeted enzyme by introducing a single mutation, double mutation, or over expression of *PPX1* in an alga species, maize (*Zea mays* L.), *Arabidopsis thaliana* L, or rice (*Oryza sative* L.) (Randolph-Anderson et al. 1998; Holmberg 2000, Lermontova and Grimm 2000, Hanin et al. 2001; Jung et al. 2010).

Natural resistance to PPO-inhibiting herbicides has been currently identified in four weed species to date: waterhemp (*Amaranthus rudis* Sauer), common ragweed (*Ambrosia artemisiifolia* L.), wild poinsettia (*Euphorbia heterophylla* L.), and pigweed (*Amaranthus quitensis* Kunth) (Heap 2010). The first weed to develop resistance in which the mechanism was identified was waterhemp. Resistance to PPO-inhibiting herbicides in waterhemp was shown to result from a unique mechanism: a single codon deletion in the *PPX2L* gene, which encodes both plastid- and mitochondrial-targeted PPO gene isoforms (Patzoldt et al. 2006). Multiple PPO-inhibiting herbicide resistant waterhemp populations from Illinois, Missouri, and Kansas have been characterized and the 3-bp deletion, named ΔG210, was the only mechanism of resistance (Lee et al. 2008; Thinglum et al. 2009). At this time, there is no explanation of the resistance mechanism to PPO-inhibiting herbicides in the other three resistant weed species.

Common ragweed is a native annual dicot, diploid with a chromosome number of 2n=36 (Payne et al. 1964), monoecious, and highly outcrossing with a low capacity for self-pollination (Friedman and Barrett 2008). Common ragweed has evolved resistance to five different herbicidal modes of action world-wide (Heap 2010). A particular biotype of common ragweed, which is researched in this thesis, represents the third weed to evolve resistance to PPO-inhibiting herbicides (Moreira et al. 2006). This biotype of common ragweed had multiple resistances to PPO- and ALS-inhibiting herbicides and cross resistance to multiple herbicides within these two sites of action (Moreira et al. 2006).

The objective of this research was to elucidate the resistance mechanism of common ragweed to PPO-inhibiting herbicides. Since the mechanism of resistance in

waterhemp has been well documented, we used this as a model to understand PPO-inhibiting herbicide resistance in common ragweed. In this chapter several questions are addressed which aid in the discovery of the resistance mechanism. How many genes are controlling this herbicide resistance trait and is it dominant or recessive? Is the resistance mechanism target-site based and, if so, what are the genes responsible and their sequence? Does the common ragweed genome contain a *PPX2L* gene as seen in waterhemp? Do the target-site genes in the R biotype contain polymorphisms relative to wild-type genes that could be responsible for resistance?

2.3 Materials and Methods

2.3.1 Analysis of the F_2 Population

2.3.1.1 Plant Material:

PPO- and ALS-resistant (Del-R) common ragweed (Delaware origin) was acquired from Dr. Mark VanGessel at the University of Delaware in Newark, WI, and PPO- and ALS-sensitive (DV1-S) common ragweed (Illinois origin) was obtained from the University of Illinois in Urbana, IL. Seeds from Del-R and DV1-S were placed between layers of moistened sand in a 10 cm sq pot. Pots were sealed with a polypropylene bag and placed in a cold chamber maintained at 4 C for seven weeks. Sand was then washed from the seeds with water and the seeds were stored moist at 4 C until needed (Baskin and Baskin 1980; Patzoldt et al. 2001).

2.3.1.2 Plant Growth:

Common ragweed seeds were sown in flats containing 3 : 1 : 1 : 1 mixture of commercial potting mix[1] : sand : soil : peat. Seedlings were transplanted at the two-leaf stage into 12-cm-diam pots with the same soil mixture accompanied with a slow release 13–13–13 fertilizer[2]. Plants were grown in a greenhouse at the University of Illinois in Urbana, Illinois where the conditions were set to 28/22 C day/night temperature with 16-h photoperiod and light threshold of 600 watts/m^2. Natural light was supplemented with metal halide and sodium vapor lights. During cross-pollination, plants were placed in a growth chamber[3] at the University of Illinois in Urbana, Illinois. Conditions were set to 29/22 C day/night temperature with 16-h photoperiod and natural light was imitated with fluorescent and incandescent light bulbs.

2.3.1.3 Herbicide Applications and Evaluations:

All applications were applied on common ragweed plants at the 6 leaf stage using a research track sprayer[4] fitted with a TeeJet[5] even flat-fan nozzle (80015EVS), delivering 187 L ha^{-1} at 207 kPa in a compressed air chamber. The nozzle was maintained approximately 45 cm above the canopy. Immediately after herbicide treatment, the plants were returned to the greenhouse. Herbicide efficacy was based on visual estimates of percent injury including treated plants compared with untreated controls on a scale of 0 to 100, where 0 represented no damage and 100 represented plant death, 18 days after treatment (DAT). Growth reduction, foliar chlorosis, and overall necrosis were considered when making the visual estimates of injury. Prior to herbicide application, tissue samples were taken from each plant for DNA isolation. Total DNA

was extracted from young leaf tissue using a modified CTAB protocol (Doyle and Doyle 1990).

2.3.1.4 Generation of F_1 Line:

A cross was set up between one Del-R plant with two DV1-S plants that were isolated and grown in a growth chamber at the University of Illinois in Urbana, Illinois. To ensure that progeny resulted from an R × S cross, seeds were collected from the DV1-S plants, under the assumption that resistance was dominant. To increase germination, the F_1 seeds were immersed into a solution of ethephon (10% (v/v) Florel[6]), which is converted to ethylene in the plant (Brennan et al. 1978; Patzoldt et al. 2001), and incubated in a cold chamber maintained at 4 C for twenty-four hours. Seeds were then removed from the solution and promptly planted in a greenhouse at the University of Illinois.

2.3.1.5 Evaluation of the F_1 Line:

Once the F_1 progeny and DV1-S common ragweed plants reached the 6 leaf stage, they were sprayed with fomesafen[7] at 82 g ai ha^{-1}, 164 g ai ha^{-1}, or 246 g ai ha^{-1} plus 1% (vol/vol) crop oil concentrate[8] (COC) treatment. Plants were assessed to determine if they were resistant (R) or sensitive (S). Two resistant plants were selected from plants sprayed with 246 g ai ha^{-1} plus COC and DV1-S plants from untreated sensitive controls for generating the F_2 line.

2.3.1.6 Generation of F_2 Line:

Two different methods were utilized to obtain enough progeny to analyze the F_2 population. First, a single F_1 plant was isolated in a growth chamber and allowed to self-pollinate. Second, two F_1 plants were isolated in a growth chamber and allowed to cross-pollinate to create full-sib F_2 progeny. Seeds from both breeding methods were treated with Florel as described previously for increased germination and grown in a greenhouse at the University of Illinois in Urbana, Illinois.

2.3.1.7 Evaluation of F_2 Line:

Inheritance of PPO inhibitor resistance was determined by evaluating R or S responses of common ragweed plants from the F_2 population after they reached the 6 leaf stage with fomesafen at 164 g ai ha^{-1} plus 1% (v/v) COC. While screening the F_1 population, this intermediate rate of fomesafen was determined to discriminate between resistant and sensitive plants. Progeny from each method to create the F_2 population were assessed: 17 plants from the full-sib F_2 method and 9 plants from the traditional F_2 method for a total of 26 plants. Responses were subjected to a chi-square goodness-of-fit test to determine whether they fit a single dominant gene model of inheritance.

2.3.2 cDNA Library Screening

2.3.2.1 cDNA Library Construction:

cDNA was synthesized from poly(A)+ RNA derived from newly emerging leaves in the apical meristem of a PPO-inhibiting herbicide sensitive common ragweed line. The cDNAs were size-selected for insert lengths greater than 500 bp and cloned

directionally into pre-digested Lambda Zap II arms (*EcoR*I/*Xho*I) according to the Lambda ZAP® II Undigested Vector Kit[9] manufacturer's instructions. The library was amplified by infecting 600 µl of cells (at an OD_{600} of 0.5) with $2x10^4$ pfu of unamplified primary library, followed by plating in top agar onto 150 mm agar plates. Five such aliquots were plated yielding a total of 10^5 pfu of the primary library that was amplified.

2.3.2.2 Plating of the cDNA Library:

Titering of the cDNA library was performed per Lambda ZAP® II Undigested Vector Kit manufacturer's instructions and determined to be less plaques than expected by a factor of 10. Primary plating of the cDNA library was performed per Lambda ZAP® II Undigested Vector Kit manufacturer's instructions by mixing 0.39 µl of the amplified cDNA library with 600 µl of XL1-Blue cells per plate, incubating at 37 C for 15 min, and then adding to 7 ml of NZY top agar, which was in a 48 C water bath. Test tubes were then vortexed, poured over fresh 150-mm Luria-Bertani (LB) agar plates (Maniatis et al. 1989) and swirled to ensure an even coating. Putative positive plaques were subjected to secondary plating which started from a vortexed 1.5 ml microcentrifuge tube containing a plug from a potentially positive plaque. A 1:100 dilution of phage in SM buffer (Maniatis et al. 1989) was made and 1 µl was added to 200 µl of XL1-blue cells, incubated at 37 C for 15 min, and added to 4 ml of NZY top agar, which was in a 48 C water bath. Test tubes were then vortexed, poured over fresh 100-mm LB agar plates and swirled to ensure an even coating. Plaque lifts, denaturing, neutralizing, and rinsing of positively charged nylon membranes were performed using the Lambda ZAP® II Undigested Vector Kit instructions per manufacturer.

2.3.2.3 Creation of *PPX1* and *PPX2* Probe Templates:

A *PPX1* probe template was created by amplifying cDNA from waterhemp with primers AmPPX1southernF 5'-AGCTAAACATCGTGTTGGAGGTA, and AmPPX1aspR 5'-AGATTTGTAGCACCTCCAATG. Amplification of the product was prepared from polymerase chain reaction (PCR) containing 1 µl of cDNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, water, and 1.5 units of Mango Taq DNA Polymerase[10] in 1X PCR buffer. PCR reactions were made in a final volume of 20 µl and PCR amplification was performed using a PTC-100 thermocycler[11]. An initial denaturation at 94 C for 2 min was followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 2 min. PCR products were separated in a 0.75% (w/v) Certified™ Molecular Biology Agarose[12] gel containing 0.1 µg ml^{-1} ethidium bromide and visualized with ultraviolet light. The amplified fragment of the expected size was purified using the QIAquick® PCR Purification Kit[13] and selected for DNA sequencing.

Template for the *PPX2* probe was obtained from the common ragweed cDNA library using primers designed for waterhemp *PPX2L*: (Gen2PPX2-F 5'-GAGGGGGCAAATACTATGAC, Gen2PPX2-R 5'-GCACGATCGGGAAACATCA). Amplification of the *PPX2* template occurred from PCR containing 2 µl of the amplified cDNA library, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 1.5 units of Mango Taq DNA Polymerase in 1X PCR buffer. PCR reactions were made in a final volume of 20 µl and PCR amplification was performed using a PTC-100 thermocycler. Reactions were incubated for 2 min at 94 C, 35 s at 63 C and 2 min at 72 C then 21 cycles of 30 s at 94 C, 30 s at 65 C increasing by 1 C each cycle, 2 min at

72 C, 2 min at 94 C then 21 cycles of 30 s at 55 C, 2 min at 72 C. PCR products were separated in a 0.75% (w/v) Certified™ Molecular Biology Agarose gel containing 0.1 μg ml^{-1} ethidium bromide and visualized with ultraviolet light. The amplified fragment of the expected size was selected for DNA sequencing.

2.3.2.4 Heterologous *PPX1* and Homologous *PPX2* Probe Creation:

A heterologous *PPX1* probe was made by amplifying a 1:100 dilution of the purified product from the previously described *PPX1* template in section 2.3.2.3 and a homologous *PPX2* probe was created by amplifying the previously described *PPX2* template in section 2.3.2.3 with the following primers: *PPX1*, AmPPX1southernF 5'-AGCTAAACATCGTGTTGGAGGTA, and AmPPX1aspR 5'-AGATTTGTAGCACCTCCAATG; *PPX2*, CRagPPX2-F 5'-CACAGCACAAGCGGTACATT and CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT. Two separate 50 μl reactions were made of the labeled probe and one 50 μl reaction of the unlabeled probe via the PCR DIG Probe Synthesis Kit[14] using a PTC-100 thermocycler. An initial denaturation at 94 C for 2 min was followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 2 min or 1.30 min for *PPX1* and *PPX2*, respectively. PCR products were separated by gel electrophoresis as previously described.

2.3.2.5 Pre-hybridization and Hybridization:

Pre-hybridization and hybridization was performed using the DIG Easy Hyb[15]products and procedures with a couple modifications. First, the denatured probe

was added to previously used pre-warmed DIG Easy Hyb from earlier attempts to troubleshoot the protocol for *PPX1* and *PPX2*. For *PPX1*, the DIG Easy Hyb was pre-heated to 37 C for 20 minutes and 42 C for 20 minutes for *PPX2*. Hybridization occurred overnight at 37 C and 42 C for *PPX1* and *PPX2*, respectively.

2.3.2.6 Post-Hybridization Washes:

Post-hybridization washes followed the DIG Easy Hyb protocol except for a couple modifications. The second wash step used 0.2xSSC, 0.1% SDS at 39 C for *PPX1* and 68 C for *PPX2*. Since the *PPX1* probe was heterologous, the second wash temperature was reduced to decrease stringency. Subsequent washes of the positively charged nylon membranes were performed per manufacturer's instructions with the DIG Wash and Block Buffer Set[16]. Addition of the Anti-Digoxigenin-AP antibody and CSPD were also performed per manufacturer's instructions using the DIG Luminescent Detection Kit[17].

2.3.2.7 Exposure of Membranes to Film and Isolation:

Membranes were sealed between two plastic sheets using an Impulse Sealer[18]. Sealed sheets containing the membranes were placed first in a Kodak X-ray Exposure Holder[19] and then a sheet of Lumi-Film Chemiluminescent Detection Film[20] was placed on top in the dark room. The folder was closed and allowed to expose for 2-24 hours depending on the probe. Lumi-Film Chemiluminescent Detection Film was then developed with a Futura 2000K Automatic X-Ray Film Processor[21].

Plates were compared to the exposed films to identify positive plaques. Positive plaques were isolated with a 1000 µl pipette tip with the tip end cut off for a larger plug or a Disposable Pasteur Pipet Flint Glass[22] for a smaller plug. Plugs were placed in a 1.5 ml microcentrifuge tube containing 500 µl SM buffer with approximately 7 µl chloroform then vortexed and stored in 4 C.

2.3.2.8 Positive Plaque Amplification:

PCR was used to amplify *PPX1* or *PPX2* from a positive cDNA library plaque with the following primers: *PPX1*, [M13 Universal Primer (-20) Reverse 5'-CAGGAAACAGCTATGAC, M13 Universal Primer (-20) Forward 5'-GTAAAACGACGGCCAGT]; *PPX2*, [M13 Universal Primer (-20) Reverse 5'-CAGGAAACAGCTATGAC, CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT] and [M13 Universal Primer (-20) Forward 5'-GTAAAACGACGGCCAGT, CRagPPX2-F 5'-CACAGCACAAGCGGTACATT]. Amplification of the product was prepared from PCR containing 1 µl of the isolated positive plaque solution, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, water, and 1.5 units of Mango Taq DNA Polymerase in 1X PCR buffer. PCR reactions were made in a final volume of 20 µl and PCR amplification was performed using a PTC-100 thermocycler. An initial denaturation at 94 C for 2 min was followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 2 min or 45 s for *PPX1* and *PPX2*, respectively. PCR products were separated by gel electrophoresis, purified, and selected for DNA sequencing as previously described.

2.3.2.9 Sequencing Positive Plaques:

Purified DNA fragments were sequenced using an ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit[23]. Forward and reverse primers were used in separate reactions to sequence both strands of *PPX1* and *PPX2*: *PPX1*, [M13 Universal Primer (-20) Reverse 5'-CAGGAAACAGCTATGAC], (CRag-PPX1-F1 5'-TGGATATTTGTGGGAAGAAGGT), (CRag-PPX1-F2 5'-CGATGCTCACTATGGTGGTG), (CRag-PPX1-F3 5'-CAAATGGATTTGCAAGTCTGC); *PPX2*, [M13 Universal Primer (-20) Reverse 5'-CAGGAAACAGCTATGAC], (CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT), CRagPPX2-F 5'-CACAGCACAAGCGGTACATT). Each reaction contained 5.75 µl of purified template, 4 µl of BigDye® Terminator v3.1 Cycle Sequencing RR-100, 500 nM of primer in 10 µl total reaction volume, and was performed using a PTC-100 thermocycler. Reactions were subjected to 1 min at 96 C then 38 cycles of 30 s at 96, 15 s at 50 C, 4 min at 60 then 4 min at 60 C. Samples were sent to the W.M. Keck Center for Comparative and Functional Genomics High Throughput Sequencing Lab[24] and analyzed using an ABI 3730xl DNA Analyzer. Sequence data were aligned and compared using Sequencher™ 4.9 software[25] and ClustalW (Corpet 1988). ExPASy translate tool (Gasteiger 2003) was used to translate nucleotide sequences into the inferred amino acids. Gene identification was verified for the edited sequences with the nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) against non-redundant nucleotide database optimized for somewhat similar sequences (blastn) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

2.3.3 Rapid Amplification of cDNA Ends (RACE)

2.3.3.1 5'-RACE of *PPX2*:

Total RNA was isolated by using young leaf tissue from a single resistant common ragweed plant with Total RNA Isolation from Plant Tissue Kit[26]. 5'-RACE was performed with the SMARTer™ RACE cDNA Amplification Kit[27] and Advantage® 2 PCR Kit[28] per manufacturer's instructions with several modifications. While generating first-strand cDNA synthesis, 2.75 µl total RNA from common ragweed was used. Since there was 200 ng of total RNA and the gene of interest has low abundance, first-strand reaction product was diluted with 20 µl Tricine-EDTA buffer. Control reactions were performed per manufacturer's instructions and were successful.

During RACE, nested primers for PCR were utilized performing a primary and then a secondary, or inner, reaction. PCR reactions were made according to the manufacturer's instructions but during the primary PCR reaction primers UPM and (CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT) were used. The secondary PCR reaction was made according to the manufacturer's instructions with modification: 2.5 µl of the diluted 1:1000 primary PCR product as a template with primers NUP and (CRag-PPX2-123R 5'-AGGATCTCCACCGCTTGTT). Both products used Program 2 from the manufacturer's instructions with 25 cycles. PCR products were separated by gel electrophoresis as previously described. The amplified fragment of the expected size, about 500-600 bp, was gel isolated and purified. Another secondary PCR reaction was performed using 2.5 µl of the purified product as a template to purify the product even further following the previously described conditions.

2.3.3.2 Cloning, Digestion, and Sequencing of 5'-RACE Product:

5'-RACE PCR product was cloned using the TOPO TA Cloning® Kit for Sequencing[29] per manufacturer's instructions for chemically competent *Escherichia coli* (*E. coli*) using 4 µl of fresh PCR product. Cells were plated on LB agar plates supplemented with 50 mg ml^{-1} kanamycin and placed overnight in an incubator at 37 C. Ten colonies were picked with autoclaved toothpicks and placed in a 3 ml culture of LB supplemented with 50 mg ml^{-1} kanamycin and placed overnight in an incubator at 37 C with agitation at 200 RPM. Saturated cultures were prepped with the QIAprep® Spin Miniprep Kit[30].

Each digestion reaction contained 5 µl of miniprep DNA, 1X NEB EcoRI reaction buffer, 6 units of *EcoR*I restriction enzyme[31], and water in 20 µl total reaction volume. Digestions were performed for 2 hrs at 37 C. The digested DNA products were separated by gel electrophoresis as previously described.

Fragments were sequenced using an ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit. Forward and reverse primers were used in separate reactions to sequence both strands of *PPX2*: [M13 Universal Primer (-20) Forward 5'-GTAAAACGACGGCCAGT] and [M13 Universal Primer (-20) Reverse 5'-CAGGAAACAGCTATGAC]. Sequencing procedures were performed the same as previously described in section 2.3.2.9 except for the sequencing reaction mix. Each reaction contained 1.5 µl of miniprep DNA at 100-200 ng/µl, 2 µl of 5X Buffer, 1 µl of BigDye® Terminator v3.1 Cycle Sequencing RR-100, 5.2 µl of 12.5% (v/v) glycerol, 2 µl of 20 µM primer, and 1.8 µl water in 13.5 µl total reaction volume. Sequence data were aligned and compared using Sequencher™ 4.9 software and ClustalW (Corpet 1988).

ExPASy translate tool (Gasteiger 2003) was used to translate nucleotide sequences into the inferred amino acids.

2.3.4 Amplification and Sequencing of *PPX1* and *PPX2* from a F_1 Plant:

2.3.4.1 Plant Material:

Total RNA was isolated from young leaf tissue of a single resistant F_1 plant, named B_2, with the TRIzol® Reagent[32] method. cDNA was prepared from total RNA using the iScript™ cDNA Synthesis Kit[33] per manufacturer's instructions.

2.3.4.2 *PPX1* and *PPX2* F_1 Template for Sequencing:

PCR was used to amplify *PPX1* from a resistant F_1 plant to identify any polymorphisms between the parental alleles with the following primers: (CRag-PPX1-F 5'- ACCAACGACTCCACATCACA, CRag-PPX1-R 5'- GACAAAGCCTCTGCTGCTTC) and (CRag-PPX1-F5 5'- AACCTTCCTTCCCTGAACCA, CRag-PPX1-R3 5'- CTTATACACGCCTCGTGACAAA). Amplification of the product was prepared from PCR containing 1 µl of cDNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, water, and 1.5 units of *Taq* DNA Polymerase[34] in 1X PCR buffer. PCR reactions were made in a final volume of 20 µl and amplification was performed using a PTC-100 thermocycler. An initial denaturation at 94 C for 2 min was followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 2.00 min. PCR products were separated by gel electrophoresis, purified, and selected for DNA sequencing as previously described.

PCR was used to amplify *PPX2* from a resistant F_1 plant to identify any polymorphisms between the parental alleles with the following primers: (CRag-PPX2-F3 5'-AGTGGTTGTGCTGCTGCTTA, CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT) and (CRag-PPX2-F2 5'-CTCCAGATCAAAACAAACAATTTC, CRag-PPX2-R6 5'-TGGTTCGAATAAGAATCCAAGT). Amplification was performed using PCRs containing 1 µl of cDNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, water, and 1.5 units of Mango Taq DNA Polymerase in 1X PCR buffer. PCR reactions were made in a total reaction volume of 20 µl and amplification was performed using a PTC-100 thermocycler. Reactions including primers CRag-PPX2-F3 and CRag-PPX2-R were subjected to touchdown PCR and were incubated for 21 cycles at 2 min at 94 C, 30 s at 63 C and 2 min at 72 C, 30 s at 94 C, 30 s at 61 C increasing by 1 C each cycle, 2 min at 72 C now followed by 21 cycles of 3 min at 94 C, 30 s at 54 C, 2 min at 72 C. Reactions including primers CRag-PPX2-F2 and CRag-PPX2-R6 had an initial denaturation at 94 C for 2 min was followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 2.00 min. PCR products were separated by gel electrophoresis, purified, and selected for DNA sequencing as previously described.

2.3.4.3 Sequencing:

Purified DNA fragments were sequenced using an ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit following the manufacturer's recommendations. Forward and reverse primers were used in separate reactions to sequence both strands of

PPX1 and *PPX2*: *PPX1*, (CRag-PPX1-F 5'-ACCAACGACTCCACATCACA), (CRag-PPX1-R 5'-GACAAAGCCTCTGCTGCTTC), (CRag-PPX1-F5 5'-AACCTTCCTTCCCTGAACCA), (CRag-PPX1-R3 5'-CTTATACACGCCTCGTGACAAA); *PPX2*, (CRag-PPX2-F3 5'-AGTGGTTGTGCTGCTGCTTA), (CRagPPX2-R 5'-GGCAGTCATGATCACAGCAT), (CRag-PPX2-F2 5'-CTCCAGATCAAAACAAACAATTTC), CRag-PPX2-R6 5'-TGGTTCGAATAAGAATCCAAGT). Sequencing procedures were performed the same as previously described in section 2.3.2.9. Sequence data were aligned and compared using Sequencher^{TM} 4.9 software and ClustalW (Corpet 1988). ExPASy translate tool (Gasteiger 2003) was used to translate nucleotide sequences into the inferred amino acids.

2.4 Results and Discussion

2.4.1 Analysis of the F_2 population:

Creation of the F_1 population operated under the assumption that the herbicide resistance trait in common ragweed was dominant. Seeds collected from the S plant resulted in resistant progeny, thus indicating that our assumption was correct. Analyzing an F_2 population would allow us to further confirm dominance. Common ragweed is a highly outcrossing plant and it has been demonstrated that plants exposed to only self-pollen set very few seeds relative to those that were cross-pollinated (Friedman and Barrett 2008). Two different methods were utilized in order to obtain enough progeny to analyze for the F_2 population: traditional F_2 and full-sib F_2 (Figure 2.1). Progeny was collected from both breeding methods to create the F_2 population and screened with

fomesafen to determine if plants were R or S. From the full-sib F_2 method, 14 plants were resistant and 3 plants were susceptible, and the traditional F_2 method yielded 7 resistant plants and 2 susceptible plants. Herbicide efficacy responses were subjected to a chi-square goodness-of-fit test to determine genetic inheritance.

The null hypothesis is that the observed and expected values are not significantly different and a 3:1 (R:S) segregation ratio exists in the F_2 population between the two phenotypes. The alternative hypothesis is that there is a significant difference between the observed and expected values and there is not a 3:1 (R:S) segregation ratio in the F_2 population between the phenotypes. The result of the chi-square=0.46, 1 degree of freedom, P-value=0.50, indicated no significant difference between the observed and expected frequencies (Table 2.1). We thus concluded that the data are consistent with the inheritance of PPO-resistance in common ragweed conferred by a single, dominant nuclear gene. Given the relatively small number of plants utilized as the F_2 population we cannot rule out a more complex form of genetic control, for example, a second genetic factor involved in resistance. Under the assumption of resistance due to a single, dominant gene, a logical candidate gene was one of the target-site genes, and attention was therefore focused on obtaining these genes from common ragweed.

2.4.2 cDNA Library Screening:

Target-site genes for PPO-inhibiting herbicides have been studied in several plant species including waterhemp, spinach, maize, soybean, and *Arabidopsis*. As mentioned earlier, research uncovered the resistance mechanism in waterhemp to PPO-inhibiting herbicides and it was due to a single codon deletion in *PPX2L* (Patzoldt et al. 2006).

Knowing that resistance to PPO-inhibitors can be accomplished synthetically or naturally via manipulating the wild-type sequence of *PPX1* or *PPX2* in various ways, it proved to be important to have the sequence of these target-site genes for common ragweed.

In order to obtain the sequence of the target-site genes in common ragweed we screened a cDNA library with gene-specific non-radioactive probes. The advantage to using a cDNA library is that it contains only the coding regions of a genome. There were a total of about 819,000 and 234,000 plaques screened for *PPX1* and *PPX2*, respectively. From the first round of screening 22 and 14 potentially positive clones for *PPX1* and *PPX2*, respectively, were isolated and the second round yielded 7 and 1 for *PPX1* and *PPX2*, respectively. The third round of screening of the potentially positive plaques utilized PCR with gene-specific and vector-specific primers, sequencing, and using blastn from NCBI (http://www.ncbi.nlm.nih.gov) with the sequence data to verify their identity. Sequence from the clones $4\text{-}4_5$ and $1\text{-}2_5$ for *PPX1* and *PPX2*, respectively, resulted in the confirmation of putative positive clones. Clone $4\text{-}4_5$ shared 83% identity with *Cichorium intybus* protoporphyrinogen oxidase *PPX1* and clone $1\text{-}2_5$ shared 76% identity with *Arabidopsis thaliana* protoporphyrinogen oxidase *PPX2* (Table 2.2).

Screening of the common ragweed cDNA library resulted in the recovery of 1,653 bp and 1,406 bp of nucleotide sequence from *PPX1* and *PPX2*, respectively. Translation of the nucleotide sequences and subsequent alignments revealed approximately how much of the gene sequences were missing. Both target-site gene clones contained an in-frame stop codon but neither contained the likely start codon. It was determined from the alignment that *PPX*1 was missing from 1 (referencing waterhemp) to 5 (referencing chicory and tomato) amino acids (Figure 2.2) depending on to which species it was

compared. Observations of the 5' end of *PPX1* reveal an N-terminal extension known as the plastid-targeting transit peptide. Typically, transit peptides do not exhibit a high level of amino acid or length conservation (Ralph et al 2004), which we also observed with common ragweed compared to other plant species. For *PPX2*, it was potentially missing 24 (referencing tobacco, soybean, and potato *PPX2*), 27 (referencing *Arabidopsis PPX2*), or 54 (referencing waterhemp *PPX2L*) amino acids (Figure 2.3) from the 5' end of the gene. In the case of *PPX2*, it is of particular interest to have the 5' end of the gene in order to determine if it contains a dual-targeting transit peptide as seen in waterhemp.

2.4.3 Rapid Amplification of cDNA Ends (RACE):

RACE is a procedure used to obtain sequence from an RNA transcript in a cell. Typically when a cDNA library, which is a collection of cloned cDNA in host cells, is screened a full length clone may not be isolated. Therefore, RACE is utilized to acquire the missing sequence which typically is the 5'- or 3'-end of the gene. After screening the common ragweed cDNA library it was determined that we did not have full length sequences for *PPX1* and *PPX2* and were missing the 5'-end of both genes. Since resistance in waterhemp was due to a deletion in the *PPX2L* gene (Patzoldt et al. 2006), we decided to perform 5'-RACE for only the *PPX2* gene in common ragweed in attempts to determine if this gene actually contains a dual-targeting transit peptide. In regards to *PPX1*, it was not necessary to perform 5'-RACE since we were only missing the plastid-targeted transit peptide sequence meaning we had obtained the full coding sequence of the functional enzyme, which would most likely contain any resistance-conferring polymorphisms if present.

Nested primers for PCR were utilized during 5'-RACE in attempts to reduce contamination due to the possible amplification from unexpected primer binding sites. This methodology proved to be effective since we obtained sequence from the 5'-end of *PPX2*. From the 5'-RACE sequencing results of *PPX2*, 38 codons of additional sequence was obtained and an in-frame methionine codon was identified 23 amino acids upstream from the beginning sequence of the cDNA library clone, $1\text{-}2_5$ (Figure 2.4 and 2.5). This in-frame methionine is the beginning of the putative mitochondria-targeted PPO gene isoform (Watanabe et al. 2001). Many continued attempts to acquire more upstream sequence were performed with numerous primers and protocol modifications but proved to be unsuccessful. At this point we cannot definitively answer if an in-frame methionine exists upstream of the already obtained methionine in *PPX2* for common ragweed.

2.4.4 Amplification and Sequencing of *PPX1* and *PPX2* from a F_1 Plant:

In order to definitively conclude if PPO-inhibiting herbicide resistance is target-site based and co-segregates with *PPX1* or *PPX2*, we needed to first identify any polymorphisms between R and S plants. Sequence from the target-site genes, *PPX1* and *PPX2*, was obtained from cDNA of an F_1 plant so only the coding regions were analyzed. When analyzing the sequence data of the target-site genes in Sequencher^{TM} 4.9 software several polymorphisms between the parental alleles of *PPX1* and *PPX2* were identified. Polymorphisms were observed when two peaks were present representing two different bases at the same nucleotide suggesting heterozygosity (Figure 2.6).

Analysis of the nucleotide sequences between the F_1 (R) and the cDNA library clone (S) revealed 6 and 1 different nucleotide(s) for *PPX1* and *PPX2*, respectively.

Inspection of the inferred amino acid sequence of the F_1 (R) *PPX1* revealed 17 heterozygous polymorphisms; 10 silent amino acid mutations and 7 missense amino acid mutations. Analysis of the inferred amino acid sequence of the F_1 (R) *PPX2* revealed 14 heterozygous polymorphisms: 5 silent amino acid mutations and 9 missense amino acid mutations. When considering a mutation which could be responsible for resistance, one which confers a change in R group property is of interest and it was found that the *PPX1* sequence contained two (64 and 370) and *PPX2* possessed four (46, 98, 248, and 452) such mutations (Table 2.3; Table 2.4). Amino acid numbering for polymorphisms in *PPX1* is based on the homologous positions in chicory (AF160961) and amino acid numbering for *PPX2* is based on the common ragweed sequence starting with methionine at the beginning of the mitochondrial isoform. The polymorphisms found in *PPX1* and *PPX2* provide promising leads for the identification of a candidate responsible for conferring PPO-inhibiting herbicide resistance in common ragweed.

2.4.5 Acknowledgements

I thank Dr. Ryan M. Lee for performing the genetic crosses, assistance in evaluating the crosses, creation of the *PPX2* template used for screening the cDNA library, and overall guidance. I would also like to thank Dr. Mark VanGessel of the University of Delaware for providing the PPO- and ALS-resistant common ragweed seed, Dr. Dean Volenberg for providing the PPO-sensitive common ragweed seed, and the staff at the W.M. Keck Center for Comparative and Functional Genomics, High-Throughput Sequencing and Genotyping Unit for their creation of the common ragweed cDNA

library. I thank Dr. Stephen P. Moose for allowing me to utilize equipment in his lab to aid in the process of screening the cDNA library.

2.4.6 Sources of Material

[1] LC1 Professional Growing Mix, Sun Gro Horticulture, Inc., 110 110th Ave. NE, Suite 490, Bellevue, WA 98004.

[2] Osmocote 13:13:13 (N:P:K), The Scotts Company, 14111 Scottslawn Road, Marysville, OH 43041.

[3] Environmental Growth Chambers, 510 E. Washington St., Chagrin Falls, OH 44022.

[4] Track Sprayer: Allen Machine Works, 607 E. Miller Rd., Midland, MI 48640.

[5] TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60189-7900.

[6] Florel®, Southern Agricultural Insecticides, Inc., P.O. Box 429, Hendersonville, NC 28793.

[7] Fomesafen: Flexstar®, Syngenta Crop Protection, P.O. Box 18300, Greensboro, NC 27419.

[8] COC: Herbimax®, Loveland Products, Inc., P.O. Box 1286, Greeley, CO 80632.

[9] Lambda ZAP® II Undigested Vector Kit, Stratagene, 11011 N. Torrey Pines Road, La Jolla, CA 92037.

[10] Mango Taq™ DNA Polymerase, Bioline, 305 Constitution Drive, Taunton, MA 02780.

[11] PTC-100 thermocycler, MJ Research, Inc., 590 Lincoln Street, Waltham, MA, 02451.

[12] Certified™ Molecular Biology Agarose, Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547.

[13] QIAquick® PCR Purification Kit, Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355.

[14] PCR DIG Probe Synthesis Kit, Roche Diagnostics GmbH, Sandhoferstrasse 116, DE-68305 Mannheim, Germany.

[15] DIG Easy Hyb, Roche Diagnostics GmbH, Nonnenwald 2, DE-82372 Penzberg, Germany.

[16] DIG Wash and Block Buffer Set, Roche Diagnostics GmbH, Sandhoferstrasse 116, DE-68305 Mannheim, Germany.

[17] DIG Luminescent Detection Kit, Roche Diagnostics GmbH, Sandhoferstrasse 116, DE-68305 Mannheim, Germany.

[18] Impulse Sealer, American International Electric, 2835 Pellissier Place, Whittier, CA 90601.

[19] Kodak X-ray Exposure Holder, Eastman Kodak Company, 1669 Lake Avenue, Rochester, NY 14652.

[20] Lumi-Film Chemiluminescent Detection Film, Roche Diagnostics Corp., P.O. Box 50457, Indianapolis, IN 46256.

[21] Futura 2000K Automatic X-Ray Film Processor, Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275.

[22] Disposable Pasteur Pipets Flint Glass, Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275.

[23] ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., 850 Lincoln Centre Drive, Foster City, CA 94404.

[24] W.M. Keck Center for Comparative and Functional Genomics, University of Illinois Biotechnology Center, 340 Edward R. Madigan Laboratory, 1201 W. Gregory Drive, Urbana, IL 61801.

[25] Sequencher™ 4.9 software, Gene Codes Corp., 640 Avis Drive, Ann Arbor, MI 48108.

[26] RNA Isolation from Plant Tissue Kit, Macherey-Nagel Inc., 2850 Emrick Boulevard, Bethlehem, PA 18020.

[27] SMARTer™ RACE cDNA Amplification Kit, Clontech Laboratories Inc., 1290 Terra Bella Avenue, Mountain View, CA, 94043.

[28] Advantage® 2 PCR Kit, Clontech Laboratories Inc., 1290 Terra Bella Avenue, Mountain View, CA, 94043.

[29] TOPO TA Cloning® Kit for Sequencing, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[30] QIAprep® Spin Miniprep Kit, Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355.

[31] *EcoR*I restriction enzyme, New England Biolabs, 240 Country Road, Ipswich, MA 01938.

[32] TRIzol® Reagent, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[33] iScript™ cDNA Synthesis Kit, Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547.

[34] *Taq* DNA Polymerase, Bioline, 305 Constitution Drive, Taunton, MA 02780.

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2.6 Tables and Figures

Table 2.1. Chi-square analysis results from the common ragweed F_2 population.

Response to Herbicide	Observed	Expected
Alive	21	19.5
Dead	5	6.5
	Chi-Square	0.46
	P-Value	0.50

Table 2.2. Top hits after a BLASTn search in the NCBI database of the edited sequences of the potentially positive plaques from the common ragweed cDNA library.

Common Ragweed Clone	BLASTn Results	Accession	Identity
PPX1	*Cichorium intybus* protoporphyrinogen oxidase (*PPX1*)	AF160961	83%
PPX2	*Arabidopsis thaliana* protoporphyrinogen oxidase (*PPX2*)	NM_121426	76%

Table 2.3. All differences present between the S plant (library) and R plant (F_1) sequences for *PPX1*. Amino acid numbering is based on the homologous positions in Chicory (AF160961).

Codon No.	S Plant	R Plant	Amino Acid Polymorphism	Side Chain Polarity	Side Chain Acidity or Basicity
30	ACA	WCA	T/S	-	-
31	AAC	CCA	N/Q	-	-
32	CCA	MCM	P/T-P	Non-polar/Polar	-
50	ACG	ACS	T/T	-	-
52	TGT	CGT	C/R	Non-polar/Polar	-
54	TCA	TCT	S/S	-	-
59	ACT	ACY	T/T	-	-
64	TCA	YCA	S/P	Polar/Non-polar	-
73	CCA	CCG	P/P	-	-
94	GCG	GCS	A/A	-	-
115	GGG	GGT	G/G	-	-
287	CCG	CCR	P/P	-	-
290	AAG	MAG	K/Q	-	Basic/Neutral
370	GAA	RAA	E/K	Non-polar/Polar	Acidic/Basic
376	TCA	TCM	S/S	-	-
389	TCC	TCS	S/S	-	-

Table 2.3. (cont.)

Codon No.	S Plant	R Plant	Amino Acid Polymorphism	Side Chain Polarity	Side Chain Acidity or Basicity
394	GCA	GCR	A/A	-	-
408	TTC	TTY	F/F	-	-
424	ATC	ATM	I/I	-	-
475	AAC	AMC	N/T	-	-
515	GGA	GGW	G/G	-	-
531	CTA	YTA	L/L	-	-

Table 2.4. All differences present between the S plant (library) and R plant (F_1) sequences for *PPX2*. Amino acid numbering is based on the full-length common ragweed sequence starting with methionine.

Codon No.	S Plant	R Plant	Amino Acid Polymorphism	R Group Property	Side Chain Acidity or Basicity
46	GAG	GRG	E/G	Polar/Non-polar	Acidic/Neutral
90	GTC	GTT	V/V	-	-
98	CGG	CKG	R/L	Polar/Non-polar	Basic/Neutral
129	CAA	CAR	Q/Q	-	-
141	AAG	AAS	E/K	-	Acidic/Basic
143	TCT	TCY	S/S	-	-
170	GTT	GTY	V/V	-	-
195	AGA	RAA	R/K	-	-
248	TCT	YCC	P/S	Non-polar/Polar	-
249	CAT	MAT	H/N	-	Basic/Neutral
323	TTG	TTS	L/F	-	-
327	GTA	GTW	V/V	-	-
359	ACG	ACR	T/T	-	-
445	GAG	GAK	E/D	-	-
452	CCC	YCC	P/S	Non-polar/Polar	-

Figure 2.1. Schematic of the two breeding methods utilized to obtain F_2 population.

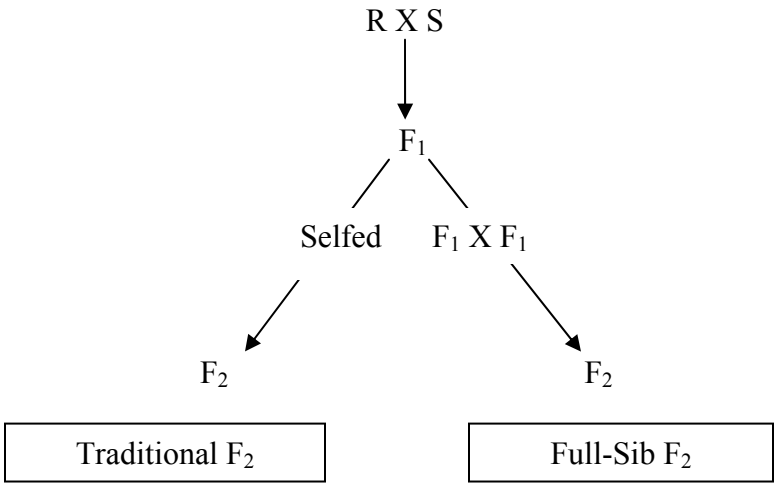
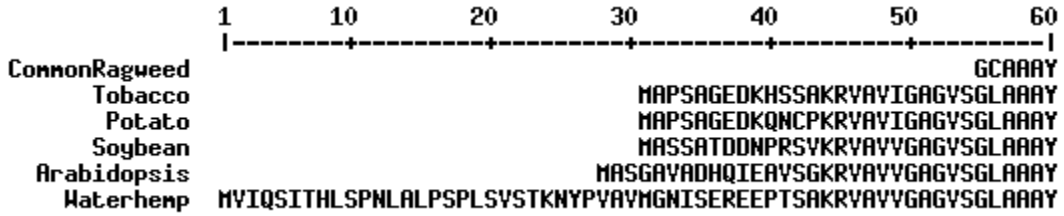


Figure 2.2. Partial alignment of *PPX1* protein from various plant species which shows the amount of the 5' end of the gene is potentially missing from common ragweed.

```
                1       10        20        30        40        50        60        70        80        90      97
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+------|
CommonRagweed       NLPSLNHYRTWSS--PPISHRTRST----NPPNHLTTYGPTNNNH-NRRTTTFCCSIANDTPISPSITNDSTSQPLLDCVIIGAGISGLCIA
Chicory   MTSLTDVCSLNCCRSWSSLPPPVSGGSLTS----KNPRYLITYSPAHRKC-NR--WRFRCSIAKDSPITPPISNEFNSQPLLDCVIVGAGISGLCIA
Tomato    MTTTAVANHPSIFTHRSPLPSPSSSSSSPSFLFLNRTNFIPYFSTSKRNSVNCNGWRTRCSVAKDYTV-PPSEVDGNQFPELDCVVVGAGISGLCIA
Waterhemp           MSAMALSSSILQCPPHSDISFRFFAHTRTPSPIFFGRTRKLSYIHC-----STSSSSTANYQNTITSQGEGDKVLDCVIVGAGISGLCIA
```

Sequences are from chicory (AF160961), tomato (CAA12400), and waterhemp (ABD52327).

Figure 2.3. Partial alignment of *PPX2L* and *PPX2* proteins from various plant species that shows the amount of the 5' end of the gene potentially missing from common ragweed.



Sequences are from tobacco (Y13466), potato (CAA12401), soybean (BAA76348), *Arabidopsis* (NM_121426), and waterhemp (DQ386117).

Figure 2.4. Partial alignment of deduced sequence between common ragweed PPX2 obtained from cDNA library screening and 5'-RACE. From the 5'-RACE, 114 bp of new sequence was obtained and the in frame methionine codon was 23 amino acids upstream from the beginning of the library sequence.

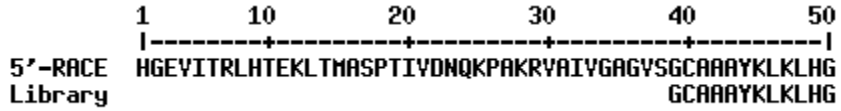
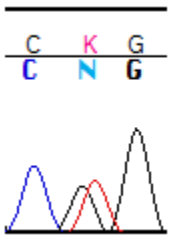


Figure 2.5. Alignment of the available *PPX2* amino acid sequence of common ragweed after 5'-RACE with other various species *PPX2(L)* amino acid sequences.

```
                1        10        20        30        40        50        60
                |--------+---------+---------+---------+---------+---------|
ComonRagweed                                HGEVITRLHTEKLTMASPTIVDNQKPAKRVAIVGAGVSGCAAAY
Soybean                                     SLSRTPQPPRMASSATDDNPRSVKRVAVVGAGVSGLAAAY
Potato                                  LAGHFTFPQILHFWVPMAPSAGEDKQNCPKRVAVIGAGVSGLAAAY
Waterhemp   MVIQSITHLSPNLALPSPLSVSTKNYPVAVMGNISEREEPTSAKRVAVVGAGVSGLAAAY
Tobacco                                         MAPSAGEDKHSSAKRVAVIGAGVSGLAAAY
Arabidopsis                                   MASGAVADHQIEAVSGKRVAVVGAGVSGLAAAY
```

Sequences are from tobacco (Y13466), potato (CAA12401), soybean (BAA76348), *Arabidopsis* (NM_121426), and waterhemp (DQ386117).

Figure 2.6. Example of chromatogram at a polymorphic region, this particular sample is common ragweed *PPX2* at position 98.



CHAPTER 3

INVOLVEMENT OF COMMON RAGWEED'S *PPX* GENES IN HERBICIDE RESISTANCE

3.1 Abstract

There are hundreds of documented weed biotypes resistant to one or more of almost all known herbicidal modes of action. Resistance to one particular herbicidal class, protoporphyrinogen oxidase (PPO, Protox) inhibitors, seems to have evolved at a relatively slow rate, with only four resistant weed species to date world-wide. Common ragweed, an already troublesome weed to control, was the third weed with documented resistance to PPO-inhibiting herbicides. Previous research with waterhemp linked resistance of this same herbicidal class to a 3-bp deletion in the *PPX2L* gene, which encodes proteins targeted to both plastids and mitochondria. We used the information from waterhemp as a model to elucidate resistance in common ragweed. Molecular marker analysis of an F_2 common ragweed population sprayed with a PPO-inhibitor, fomesafen, revealed that the *PPX2* gene co-segregated with PPO-inhibiting herbicide resistance. A particular polymorphism, R98L, was suspected of being responsible for conferring herbicide resistance due to its functionality within the active site and conservation across multiple species. Complementation of a *hemG* mutant strain of *Escherichia coli* with a construct containing the R98L mutation identified qualitatively confirmed the role of the mutation in resistance. Growth curves were performed with the same complemented strain of the *hemG* mutant *Escherichia coli* to quantitatively confirm the role of the mutation in resistance.

3.2 Introduction

Many challenges exist in modern agriculture systems for growers to produce a healthy and profitable crop. Herbicides have globally revolutionized weed control due to the fact that they are often the most reliable and least expensive methods of control available (Heap 1997). However, evolution of herbicide-resistant weeds has threatened the efficacy of herbicides. Strong selection pressures exerted from herbicide applications on a plant population can result in resistance evolution. Widespread adoption of repetitively applying similar herbicidal modes of action and reduced discovery of new herbicides have resulted in difficult to control herbicide-resistant weeds (Cole et al. 2000). There has been a global evolution of 345 resistant weeds biotypes in 194 weed species and over 340,000 fields (Heap 2010). Multiple mechanisms of resistance exist in weeds to herbicides: insensitive site of action, enhanced metabolism, compartmentalize/sequestration, or decreased uptake or translocation.

Few weeds have evolved documented cases of resistance to the protoporphyrinogen oxidase (PPO, Protox) inhibiting herbicides. Protox is an enzyme which oxidizes protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX) and is involved in the tetrapyrrole biosynthetic pathway with heme and chlorophyll biosynthesis (Beale and Weinstein 1990). There are three main herbicidal chemical classes which share Protox as a site of action: diphenylethers, N-phenylpthalimides, and aryl triazinones. These herbicides are known as contact burning due to their rapid ability to cause tissue damage and necrosis when applied to susceptible plants. These herbicides compete for the substrate binding site, and as a result, block Protox. Protoporphyrinogen IX accumulates in the plastid and leaks into the cytoplasm and surrounding cellular

membranes, where an enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX rapidly occurs (Jacobs and Jacobs 1993; Jacobs et al. 1991; Lehnen et al. 1990). Since this is occurring outside of the plastid, where protective antioxidant mechanisms are present, there is nothing to prevent cellular damage from reactions with the accumulating protoporphyrin IX. Ultimate necrosis of a susceptible plant occurs from the interaction of protoporphyrin IX with oxygen and light causing the production of reactive oxygen species, which trigger lipid peroxidation and cell membrane damage.

Various methods have been utilized in order to obtain synthetic PPO-inhibiting herbicide resistance. Usually, this involves altering the target-site genes *PPX1* or *PPX2*, which encode plastid- and mitochondrial-targeted PPO isoforms, respectively. Synthetic resistance was achieved in tobacco (*Nicotiana tabacum* L.) and soybean [*Glycine max* (L.) Merr.] from the over expression of *PPX2* (Ichinose et al. 1995; Warabi et al. 2001). Resistance was also created by introducing a single mutation, double mutation, or over expression of *PPX1* in an alga species, maize (*Zea mays* L.), *Arabidopsis thaliana* L, or rice (*Oryza sative* L.) (Randolph-Anderson et al. 1998; Holmberg 2000, Lermontova and Grimm 2000, Hanin et al. 2001; Jung et al. 2010).

Natural resistance to PPO-inhibiting herbicides has been identified in four weed species to date: waterhemp (*Amaranthus rudis* Sauer), common ragweed (*Ambrosia artemisiifolia* L.), wild poinsettia (*Euphorbia heterophylla* L.), and pigweed (*Amaranthus quitensis* Kunth) (Heap 2010). Previously, it has been determined that resistance in waterhemp is the result of a unique mechanism: a single codon deletion, designated as ΔG210, in the *PPX2L* gene, which encodes both plastid- and mitochondrial-targeted PPO isoforms (Patzoldt et al. 2006). Multiple PPO-resistant waterhemp populations were

screened and it was determined that ΔG210 was the only mechanism of resistance present in all of these populations (Lee et al. 2008; Thinglum et al. 2009).

A particular biotype of common ragweed, which is researched in this thesis, represents the third weed to evolve resistance to PPO-inhibiting herbicides (Moreira et al. 2006). This biotype of common ragweed has resistance to various PPO- and ALS-inhibiting herbicides and cross resistance to multiple herbicides within these two sites of action (Moreira et al. 2006). Common ragweed has currently evolved resistance to five different herbicidal modes of action world-wide (Heap 2010).

Several important findings were determined from the previous chapter in this thesis about PPO-inhibiting herbicide resistance in common ragweed. Chi-square analysis of the F_2 population, after an application of fomesafen, revealed a 3:1 (R:S) segregation ratio. Thus it was concluded that data were consistent with resistance conferred by a single, dominant, nuclear gene. A common ragweed cDNA library was screened and the candidate genes, *PPX1* and *PPX2*, for PPO-inhibiting herbicide resistance were isolated. After comparing amino acid sequence alignments from various plant species, it was determined that the cDNA clones for *PPX1* and *PPX2* were not full-length. 5'-RACE was performed to obtain more *PPX2* gene sequence which led to the identification of the putative start codon encoding the mitochondrial transit peptide. Currently, we cannot definitely answer if there is a *PPX2L* gene present in common ragweed. Sequence analysis of *PPX1* and *PPX2* from an F_1 plant identified 2 and 4 missense mutations, respectively, as possible candidates for PPO-inhibiting herbicide resistance in common ragweed.

Several questions about resistance in this common ragweed biotype were solved but some still remain which will be addressed in this chapter. Co-segregation analysis, functional complementation assays, and growth curve experiments identified an R98L *PPX2* mutation as the likely basis of resistance to PPO-inhibiting herbicides in this common ragweed biotype. Last, a DNA based assay was developed to identify the R98L mutation in common ragweed samples.

3.3 Materials and Methods

3.3.1 *PPX2* Co-segregation with *Fok*I

3.3.1.1 PCR Reaction:

Partial amplification of the *PPX2* gene, containing a silent mutation at position 143, was performed with genomic DNA extracted from plants in the F_2 population by utilizing the forward 5'-GGTTCAAATTCTTTTAGAGCCATT -3' (CRag-PPX2-123F) and reverse 5'-AGGATCTCCACCGCTTGTT-3' (CRag-PPX2-123R) primers. Each 20 µl PCR reaction mix consisted of 1 µl of 50 ng μl^{-1} genomic DNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 1.5 units of *Taq* DNA Polymerase[1] in 1X PCR buffer, and water. PCR amplification was performed in a PTC-100 thermocycler[2] with an amplification protocol of an initial denaturation at 94 C for 2 min followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 55 C for 30 s, and extension at 72 C for 60 s. PCR products were separated in a 0.75% (w/v) Certified™ Molecular Biology Agarose[3] gel containing 0.1 µg ml^{-1} ethidium bromide and visualized with ultraviolet light.

3.3.1.2 Digestion:

Each digestion reaction contained 10 µl of PCR product, 1X NEB 4 reaction buffer, 1.2 units of *Fok*I restriction enzyme[4], and water in 20 µl total reaction volume. Digestions were performed for 3 hrs at 37 C. Digested DNA products were separated in a 3.0% (w/v) Certified™ Molecular Biology Agarose gel containing 0.1 µg ml^{-1} ethidium bromide and visualized with ultraviolet light. Analysis consisted of visual ratings of herbicide injury, as described in chapter two of this thesis, plotted against the result of digestion. Plants were grouped together by *Fok*I digestion pattern and mean visual ratings determined for each of these groups. Standard deviations of the groups were calculated to find the standard error of the mean for each data point:

$$SE_{\bar{X}} \equiv \frac{s}{\sqrt{n}}$$

$SE_{\bar{X}}$ is the standard error of the mean, s is the sample standard deviation and n is the size of the sample.

3.3.2 Functional Complementation Assay

3.3.2.1 Preparation of Waterhemp *PPX2* R98L Mutants:

Waterhemp *PPX2L* has two in-frame start codons, one for the plastid- and one for the mitochondrial-targeted proteins. A shortened version of the *PPX2L* sequence beginning with the mitochondrial methionine, from the sensitive waterhemp biotype, was cloned into pET28a(+) DNA[7] expression vector at *Eco*RI/*Hind*III. This plasmid was used as a template to substitute the wild-type Arg98 to Leu98 using a QuickChange® Site-directed Mutagenesis Kit[8]. Mutagenic primers were designed using Stratagene's web-based QuikChange® Primer Design Program available online: forward 5'-

AGAAGCAACAGTTGCCAATTTCACAAAATAAACTATACATAGCTAGAGCCGG -3' (a292c_g293t_F) and reverse 5'-CCGGCTCTAGCTATGTATAGTTTATTTTGTGAAATTGGCAACTGTTGCTTCT-3' (a292c_g293t_R). Protocols followed the QuickChange® Site-directed Mutagenesis Kit per manufacturer's instructions using 0.5 µl of S-*PPX2* waterhemp construct used as template and 18 cycles of the thermocycler program. Cells were plated on Luria-Bertani (LB) agar plates (Maniatis et al. 1989) supplemented with 50 mg ml^{-1} kanamycin and placed overnight in an incubator at 37 C. Eight colonies (R_1-R_8) were picked with autoclaved toothpicks and placed in 3 ml cultures of LB medium supplemented with 50 mg ml^{-1} kanamycin overnight in an incubator at 37 C with agitation at 200 RPM. Saturated cultures were prepped with the QIAprep® Spin Miniprep Kit[9].

Each digestion reaction contained 5 µl of miniprep DNA, 1X REact® 2 reaction buffer, 3 units of *EcoR*I restriction enzyme[10], 3 units of *Hind*III restriction enzyme[11], and water in 20 µl total reaction volume. Digestions were performed for 2½ hrs at 37 C. Digested DNA products were separated by gel electrophoresis as previously described to check that the insert was present and of the proper size.

3.3.2.2 Sequencing of Waterhemp *PPX2* R98L Mutants:

PCR was used to amplify a fragment of the insert from the mutant colonies R_1-R_5 with the following primers: (T7promoterprimer 5'-TAATACGACTCACTATAGG) and (PPXvector2-R 5'-ACCAACGCTTTCCTGAACAT). Amplification of the product was prepared from PCR containing 1 µl of 1:50 diluted miniprep DNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, water, and 1.5 units of *Taq*

DNA Polymerase in 1X PCR buffer. PCR reactions were made in a final volume of 20 µl and PCR amplification was performed using a PTC-100 thermocycler. An initial denaturation at 95 C for 5 min was followed by incubation at 38 cycles of: denaturation at 95 C for 1 min, annealing at 56 C for 45 s, and extension at 72 C for 60 s and then a final extension at 72 C for 5 min. PCR products were separated by gel electrophoresis as previously described, purified using the QIAquick® PCR Purification Kit[12], and selected for DNA sequencing.

Plasmids R_1-R_5 were determined to contain an insert of the expected size and so they were sequenced to ascertain if the introduced Leu98 mutation was present. Purified DNA fragments were sequenced using an ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit[13]. A reverse primer was used in each reaction to sequence the *PPX2* mutants: (PPXvector2-R 5'-ACCAACGCTTTCCTGAACAT). Each reaction contained 5.75 µl of purified template, 4 µl of BigDye® Terminator v3.1 Cycle Sequencing RR-100, 500 nM of primer in 10 µl total reaction volume, and was performed using a PTC-100 thermocycler. Reactions were subjected to 1 min at 96 C then 38 cycles of 30 s at 96, 15 s at 50 C, 4 min at 60 then 4 min at 60 C. Samples were sent to the W.M. Keck Center for Comparative and Functional Genomics High Throughput Sequencing Lab[14] and analyzed using an ABI 3730xl DNA Analyzer. Sequence data were analyzed and edited using Sequencher™ 4.9 software[15]. All sequencing reactions yielded usable chromatograms except colony R_4. Colonies R_1, R_2, R_3, and R_5 contained the introduced Leu mutation at position 98.

3.3.2.3 Transformation of Chemically Competent Cells for Sensitive Isolates:

As previously described in section 3.3.2.1, the *PPX2* construct from a sensitive waterhemp biotype was cloned into the pET28a(+) DNA expression vector and transformed into Subcloning Efficiency™ DH5α™ Competent Cells[16] according to manufacturer's protocol with a few exceptions: 100 µl of cells were initially used, 7 µl of miniprep DNA was added to thawed cells, cells were incubated in ice with cold water for 15 min instead of 30 min, cells were heat shocked for 45 s instead of 20 s at 45 C instead of 42 C, and 900 µl instead of 950 µl of LB media was added to the cells. Cells were then incubated at 37 C for 1 hr and then spun in a table-top microcentrifuge for 30 s at 15,000 RPM. Most of the liquid was removed, saving about 100-200 µl. Cells were re-suspended by pipetting gently, and about 50 µl of the suspension were plated on each of two LB agar plates supplemented with 50 mg ml^{-1} kanamycin.

Four colonies (S_1, S_2, S_3, and S_4) were picked with autoclaved toothpicks and placed in 3 ml cultures of LB media supplemented with 50 mg ml^{-1} kanamycin and placed overnight in an incubator at 37 C while shaking with agitation at 200 RPM. Saturated cultures were prepped with the QIAprep® Spin Miniprep Kit. Each digestion reaction contained 5 µl of miniprep DNA, 1X REact® 2 reaction buffer, 3 units of *Eco*RI restriction enzyme, 3 units of *Hind*III restriction enzyme, and water in 20 µl total reaction volume. Digestions were performed for 2½ hrs at 37 C. Digested DNA products were separated by gel electrophoresis as previously described to check that the insert was present and of the proper size. All four colonies contained the expected insert and S_1 and S_4 were selected to make glycerol stocks. Glycerol stocks contained 500 µl of 50% (v/v)

sterile glycerol and 500 µl of cells which were inverted several times and then immediately stored at -80 C.

3.3.2.4 Transformation of BT_3 Electrocompetent *hem*G Mutant of *Escherichia coli* (*E. coli*):

The *hem*G mutant of *E. coli* was transformed with plasmid constructs encoding *PPX2L* which only differed at position R98L. The immediate precursor of protoporphyrin IX is protoporphyrinogen IX; this conversion is catalyzed by protoporphyrinogen oxidase, encoded by the *hem*G gene (Yang et al. 1996). This mutant *E. coli* is deficient in the *hem*G gene and, therefore, cannot make its own PPO and grows very slow. In order to rescue the growth of the mutant, hematin, the hydroxide of heme, can be exogenously supplied. Transformation competent BT_3 cells were thawed in ice water while covered with aluminum foil due to their photo-sensitive nature. Once thawed, 50 µl of cells were added to a pre-chilled electroporation cuvette[17] with 1 µl of miniprep DNA (R_1 or S_4) and shielded from the light. The cuvette was placed in the cuvette chamber of an electroporation system and the appropriate pulse was applied. Following the pulse, the cells were immediately rescued with 750 µl of LB media supplemented with 20 µg ml^{-1} hematin and transferred to a 1.5 ml microcentrifuge tube. Samples were incubated at 37 C for 1 hr while in the dark to allow expression of the antibiotic-resistance gene. At the end of this expression period cells were spun in a table-top microcentrifuge for 30 s at 15,000 RPM and most of the liquid was removed except approximately 100-200 µl. Cells were re-suspended by gently pipetting and approximately 50 µl was split between two agar plates of LB media supplemented with

50 mg ml^{-1} kanamyacin and 20 μg ml^{-1} hematin. Plates were inverted and incubated overnight in the dark at 37 C. Two colonies were picked from the (R_1, R_2) and (S_1, S_2) plates each.

3.3.2.5 Petri Plate Assay:

Transformed isolates of the mutant BT_3 *E. coli* (R_1, R_2, S_1, and S_2) and untransformed controls (C_1 and C_2) were tested for their ability to grow on LB media alone or supplemented with 20 μg ml^{-1} hematin or with technical grade lactofen[18] ranging from 0.001 to 100 μM. All plates were supplemented with 50 mg ml^{-1} kanamyacin and incubated at 37 C in the dark for 14 hrs (Patzoldt et al. 2006).

3.3.3 Growth Curve Experiment

3.3.3.1 Culture Preparation:

Glycerol stocks of isolates C_1, C_2, S_1, S_2, R_1, and R_2 were scraped with autoclaved toothpicks and individually placed in 3 ml cultures of LB supplemented with 20 μg ml^{-1} hematin and 50 mg ml^{-1} kanamycin. They were incubated in the dark overnight at 37 C with agitation at 200 RPM. Isolates were tested for their ability to grow on LB medium alone or supplemented with 20 μg ml^{-1} hematin or with technical grade lactofen. During the first replication, 100 μM and 1 μM lactofen were used whereas 10 μM was used for the second replication. All cultures were supplemented with 50 mg ml^{-1} kanamyacin in 50 ml plastic screw-cap tubes. Each tube contained 10 ml of one of the various mediums and was inoculated with 100 μl of a saturated culture from one of the six isolates.

Cultures were placed in an incubator for 7-10 hrs at 37 C with agitation at 200 RPM in the dark.

3.3.3.2 Analyses:

Samples were taken from each culture at 0, 3, 4, 5, 5½, 6, 6½, and 7 hrs. During the second replication, samples were taken from each culture at 0, 3, 4, 5, 6, 7, 8, 9, and 10 hrs. Samples consisted of 100 µl of each culture and were placed in a cuvette and analyzed for measurement of the optical density at 600 nm (OD_{600}) with a DU® Series 500 Spectrophotometer[19]. After each sampling, the cultures were immediately placed back in the incubator to continue growth for the next sampling until ideally reaching the stationary phase. The two isolates of the same strain, e.g. C_1 and C_2, were averaged at each time point and plotted on the graph as a single data point. Standard deviations of the samples were calculated to find the standard error of the mean for each data point as previously described in section 3.3.1.2.

3.3.4 R98L Mutation Specific DNA Based Assay

3.3.4.1 PCR Reaction:

Partial amplification of the *PPX2* gene, containing the R98L polymorphism, was performed with genomic DNA extracted from common ragweed plants by utilizing the forward 5'-AGCAGTTTGATTGATGATCTTGG-3' (PPX2-98-F3) and reverse 5'-CAGTGCAATTGGGTTAGAAGG-3' (PPX2-98-R1) primers. Each 20 µl PCR reaction mix consisted of 1 µl of 50 ng μl^{-1} genomic DNA, 300 nM each of forward and reverse primers, 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 1.5 units of Bullseye Taq DNA Polymerase[5]

in 1X PCR buffer, and water. PCR amplification was performed in a PTC-100 thermocycler with an amplification protocol of an initial denaturation at 94 C for 2 min followed by incubation at 36 cycles of: denaturation at 94 C for 20 s, annealing at 56.5 C for 30 s, and extension at 72 C for 1.00 min. PCR products were separated by gel electrophoresis as previously described.

3.3.4.2 Digestion:

Each digestion reaction contained 5 µl of PCR product, 1X NEB 2 reaction buffer, 10 units of *BsrG*I restriction enzyme[6], water, and supplemented with 0.02 µg μl^{-1} bovine serum albumin (BSA) in 20 µl total reaction volume. Digestions were performed for 6 hrs at 60 C to result in a 2-fold increase in activity. Digested DNA products were separated by gel electrophoresis as previously described.

3.4 Results and Discussion

3.4.1 *PPX2* Co-segregation Analysis with *Fok*I:

In order to ascertain if the target-site genes confer PPO-inhibiting herbicide resistance, PCR products digested with a restriction enzyme, *Fok*I, were used as molecular markers to follow the inheritance of *PPX2* alleles in a F_2 common ragweed population. In the previous chapter, this same population was found to segregate 3:1 for R or S responses to fomesafen and resistance was thus due to a single-dominant gene. The recognition site of *Fok*I contains a polymorphism conferring a silent mutation and cuts when TCC is present at amino acid position 143 but will not with TCT (Figure 3.1). Representative samples of genomic DNA from plants of the F_2 population after digestion

illustrate the inheritance of the parental alleles amongst the F_2 population (Figure 3.2). Grouping of plants by presence/absence of the *Fok*I recognition site showed an association with herbicide responses. Specifically, plants digested with *Fok*I were sensitive, whereas undigested and heterozygous plants were resistant (Figure 3.3). One exception existed with a plant scored heterozygous which died after herbicide application but this phenomenon could be due to some other factor besides herbicide sensitivity. Visual inspection of the data clearly shows co-segregation of this molecular marker, *Fok*I, with the presence of the allele containing TCT at position 143 in the F_2 population. Therefore, this strongly indicated that *PPX2* is likely to confer PPO-inhibiting herbicide resistance in common ragweed. The association of the *Fok*I site with resistance does not implicate the polymorphism with resistance. Rather, it simply shows that the allele of *PPX2* inherited from the R parent is responsible for PPO-inhibiting herbicide resistance.

3.4.2 Functional Complementation Assay:

Based on the results from the co-segregation analysis in the F_2 population with the molecular marker, it was determined that resistance to PPO-inhibiting herbicide resistance in common ragweed co-segregates with *PPX2*. In the previous chapter we also learned that there are four polymorphisms that confer missense mutations in *PPX2*, which would more likely be responsible for resistance than a silent mutation. When the amino acid sequences of various species are aligned around these four polymorphic regions, only Arg98 is highly conserved (Figure 3.4). It has been previously documented that Arg98 is a highly conserved residue throughout the entire PPO family and functionally involved in substrate coordination within the active site (Heinemann et al. 2007). Since

this residue, which is polymorphic in common ragweed, is important in functionality and highly conserved, we decided to target it as a potential mechanism for resistance.

In order to determine if the R98L mutation in *PPX2* was able to confer resistance to PPO-inhibiting herbicides, a functional complementation assay was performed. A very similar assay was previously performed in waterhemp to prove the resistance mechanism to PPO-inhibiting herbicides (Patzoldt et al 2006) and was used as a model for common ragweed. Plasmid constructs encoding *PPX2* from waterhemp were transformed into a mutant BT_3 *E. coli* strain. Constructs differed only at position Arg128 in waterhemp, which is homologous to Arg98 in both tobacco and common ragweed. The *PPX2* gene from waterhemp was utilized instead of common ragweed since when this experiment was performed the 5'-RACE data was not available and we were still missing sequence from the 5' end of *PPX2*. Initially, the shortened available sequence from *PPX2* of an R common ragweed plant was ligated into a plasmid construct and transformed into the mutant BT_3 *E. coli* strain, which cannot produce heme natively unless exogenously supplied. We found that the construct was not able to complement the mutant. This phenomenon is probably due to fact that the mitochondrial-targeted transit peptide of *PPX2* is important for the functionality of the protein (Lermontova et al. 1997). The isolates labeled S_1 and S_2 contained *PPX2* with Arg98 and isolates R_1 and R_2 contained *PPX2* with Leu98. Both the S and R constructs were able to grow on the LB growth medium, indicating they rescued the growth of the mutant BT_3 *E. coli* strain and thus encoded functional proteins (Figure 3.5). Growth medium supplemented with lactofen considerably hindered the growth of *E. coli* transformed with the S construct but not *E. coli* transformed with the R construct (Figure 3.5). Therefore, this assay

qualitatively proved that the single point mutation in *PPX2* producing a leucine at position 98, instead of an arginine, was capable of conferring resistance to lactofen.

3.4.3 Growth Curve Experiment:

Qualification of the R98L polymorphism in *PPX2* as the resistance mechanism of PPO-inhibiting herbicides in common ragweed was accomplished via a functional complementation assay with various constructs transformed into a mutant *E. coli* strain. Conducting growth curves on the strains previously created from the functional complementation assay would give further confirmation of the resistance mechanism in a more quantitative fashion. Two replications were performed in order to determine the proper concentrations of technical grade lactofen in the media and the length of time to monitor the strains.

During the first replication, LB medium supplemented with 20 μg ml^{-1} hematin, LB media alone, or lactofen at 100 μM and 1 μM were used to test the various strains' abilities to grow. As shown in Panel A of Figure 3.6, the control strain grew in LB supplemented with hematin but at a much slower rate than the resistant and sensitive, which grew at about the same rate. As shown in Panel B of Figure 3.6, the control strain did not grow in LB media alone, but the resistant and sensitive grew vigorously at about the same rate proving that they complemented the mutant. Panel C in Figure 3.6 showed no growth in LB supplemented with 100 μM lactofen from the control strain and very little from the sensitive as well leaving both perpetually in lag phase. The resistant strain did not only grow but also started log phase, which confirmed resistance to lactofen. As displayed in Panel D of Figure 3.6, the control strain had no growth in LB supplemented

with 1 μM lactofen leaving it in lag phase. The resistant strain vigorously grew and started stationary phase which once again confirmed resistance and the sensitive strain also grew but at a much slower rate leaving it in only early log phase at the time the experiment was terminated. After analysis of these growth curves it can be determined that resistance due to the R98L mutation is further confirmed. Based on the results, it was decided for the second replication to use a concentration between 100 μM and 1 μM lactofen and to extend the sampling time to better differentiate between the resistant and sensitive strains.

In general, the results from the second replication confirmed the conclusions from the first replication. Mediums used in the second replication were LB medium supplemented with 20 μg ml^{-1} hematin, LB media alone, or LB plus lactofen at 10 μM. At 10 μM lactofen, the growth of the sensitive strain lagged behind the resistant strain by greater than 2 hrs, clearly distinguishing the different sensitivities to lactofen (Figure 3.7). After analysis of these growth curves it can be concluded that resistance in common ragweed to PPO-inhibiting herbicides is due to the R98L mutation in *PPX2*. Also, based on the data from the growth curves there does not appear to be a fitness penalty associated with the R98L mutation in the bacterial system but that does not mean this would also be true at the whole plant level.

Data from the growth curve experiments was utilized to estimate the resistance level of the enzyme. Evaluations of the resistant and sensitive strains' relative rates of growth were pooled from the two replications comparing data from the LB media supplemented with hematin with the three lactofen rates. The relative growth rate of the bacteria was calculated at the time point that yielded the maximum growth rate rather

than arbitrarily picking a time point due to the fact that the bacterial strains reached the stationary phase at different time points in each of the three lactofen rates. Total amount of growth was calculated by figuring the difference between the OD_{600} value yielding the maximum growth rate and the initial OD_{600} value. Growth rate was then calculated by dividing the total amount of growth by the time yielding the maximum growth rate. Relative growth rate of the bacteria in media containing the herbicide was then calculated by dividing this number by the growth rate of bacteria in media containing no herbicide (LB supplemented with hematin). As displayed in Figure 3.8, the R and S lines are roughly parallel. The R line is shifted to the right of the S line, for example, the 1 μM sensitive data point is directly across from the 10 μM resistant data point showing about a 10 fold resistance level (Figure 3.8). According to the whole plant data, this common ragweed biotype was 14 fold resistant to lactofen (Moreira et al. 2006) and similarly, the resistant enzyme from the microbial growth assay is 10 fold resistant to lactofen.

3.4.4 R98L Mutation Specific DNA Based Assay:

PCR products were digested with a restriction enzyme, *BrsG*I, used as a DNA based assay to follow the presence/absence of the R98L mutation in *PPX2*. The recognition site of *BrsG*I contains the R98L polymorphism and cuts with a leucine codon present at amino acid position 98 but will not if there is an arginine codon (Figure 3.9). This assay was applied to the entire F_2 population, where each sample was previously documented to be resistant or sensitive, to illustrate the inheritance of the R98L allele of *PPX2* and test the accuracy of the marker (Figure 3.10). As expected, visual inspection of the data showed co-segregation with the presence of this marker, *BrsG*I, and resistance

to PPO-inhibiting herbicides (data not shown). A perfect association existed with one exception, which was the same plant from the *Fok*I co-segregation analysis. Application of this DNA based assay would be to test suspect resistant common ragweed plants to PPO-inhibiting herbicides sent in from growers. Results from the assay could used to make the proper management decisions in order to manage the common ragweed population. However, further research is needed to determine if other mechanisms of resistance to PPO-inhibiting herbicides exist in common ragweed.

3.4.5 Acknowledgements

I thank Dr. Ryan M. Lee for the creation of the *PPX2* sensitive waterhemp template and overall guidance. I would also like to thank the staff at the W.M. Keck Center for Comparative and Functional Genomics High for their high throughput sequencing. I give thanks to Dr. Jack M. Widholm for allowing me to use his electroporator in order to transform the mutant *E. coli*. I would also like to thank Dr. Ryouichi Tanaka from Hokkaido University for supplying our laboratory with the BT_3 *hem*G mutant of *E. coli* used in these experiments. Last, I thank Valent for supplying us with technical grade lactofen. Funding for this project was provided by the Illinois Soybean Association.

3.4.7 Sources of Material

[1] *Taq* DNA Polymerase, Bioline, 305 Constitution Drive, Taunton, MA 02780.

[2] PTC-100 thermocycler, MJ Research, Inc., 590 Lincoln Street, Waltham, MA, 02451.

[3] Certified™ Molecular Biology Agarose, Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547.

[4] *Fok*I restriction enzyme, New England Biolabs, 240 County Road, Ipswich, MA 01938.

[5] Bullseye Taq DNA Polymerase, MIDSCI, 280 Vance Road, St. Louis, MO 63088.

[6] *BrsG*I enzyme, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[7] pET28a(+) DNA, Novagen®, EMD Chmicals, Inc., 480 South Democratic Road, Gibbstown, NJ 08027.

[8] QuickChange® Site-directed Mutagenesis Kit, Stratagene, 11011 N. Torrey Pines Road, La Jolla, CA 92037.

[9] QIAprep® Spin Miniprep Kit, Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355.

[10] *Eco*RI restriction enzyme, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[11] *Hind*III restriction enzyme, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[12] QIAquick® PCR Purification Kit, Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355.

[13] ABI Prism BigDye® Terminator™ v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., 850 Lincoln Centre Drive, Foster City, CA 94404.

[14] W.M. Keck Center for Comparative and Functional Genomics, University of Illinois Biotechnology Center, 340 Edward R. Madigan Laboratory, 1201 W. Gregory Drive, Urbana, IL 61801.

[15] Sequencher™ 4.9 software, Gene Codes Corp., 640 Avis Drive, Ann Arbor, MI 48108.

[16] Subcloning Efficiency™ DH5α™ Competent Cells, Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008.

[17] Electroporation cuvette, 0.2 cm gap, USA Scientific, Inc., P.O. Box 3565, Ocala, FL 34478.

[18] Lactofen Technical Grade, Valent®, 6560 Trinity Court, Dublin, CA 94568.

[19] DU® Series 500 Spectrophotometer, Beckman Coulter, Inc., 250 South Kraemer Boulevard, Brea, CA 92821.

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3.6 Figures

Figure 3.1. Common ragweed nucleotide sequence surrounding position 143. Underlined nucleotides indicated codon 143 and the polymorphic residue is outlined. Brackets indicate the *Fok*I recognition site and the arrows represent where the restriction enzyme cuts.

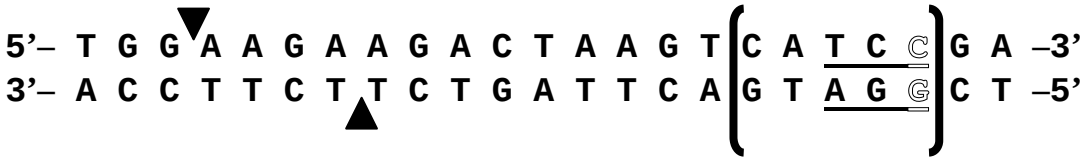


Figure 3.2. Segregation of common ragweed samples from the F_2 population after digestion with the *Fok*I restriction enzyme. N indicates a homozygous no cut, H indicates a heterozygote, and C indicates a homozygous cut result after digestion.

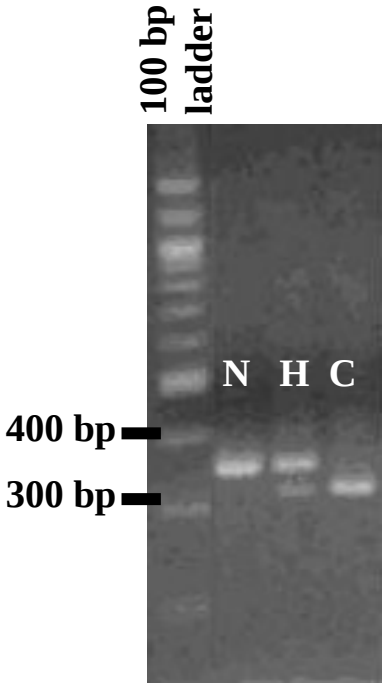


Figure 3.3. Co-segregation analysis of the *PPX2* allele from genomic DNA digested with *Fok*I of an F_2 population treated with fomesafen at 164 g ai ha^{-1} plus 1% (v/v) COC. Herbicide efficacy was based on visual estimates of percent injury including treated plants compared with untreated controls on a scale of 0 to 100, where 0 represented no damage and 100 represented plant death, 18 days after treatment (DAT). Vertical bars represent +/- the standard error of the mean.

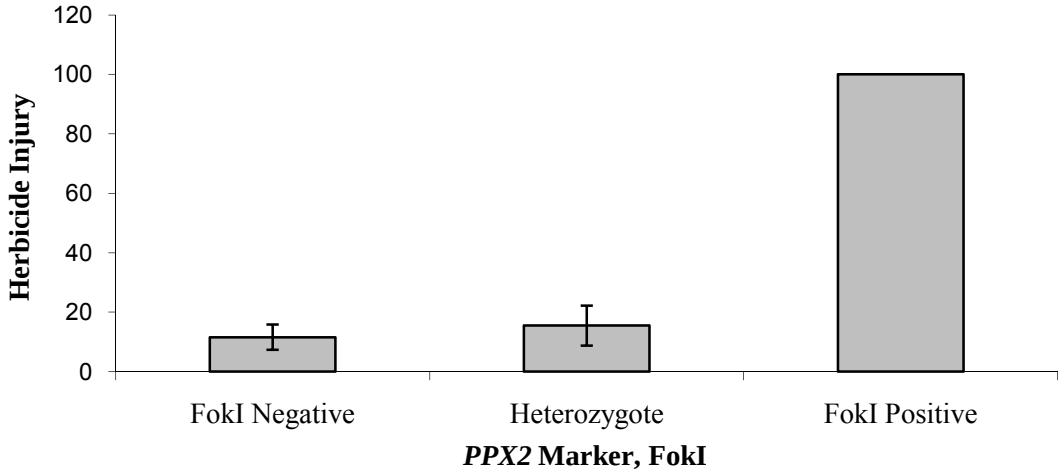


Figure 3.4. Alignment of amino acid sequences of *PPX2* from various plant species at the heterozygous positions of the common ragweed F_1 plant. Amongst all four residues only position 98 is highly conserved throughout all the species. Note: amino acid numbering is based on the full-length common ragweed sequence starting at methionine.

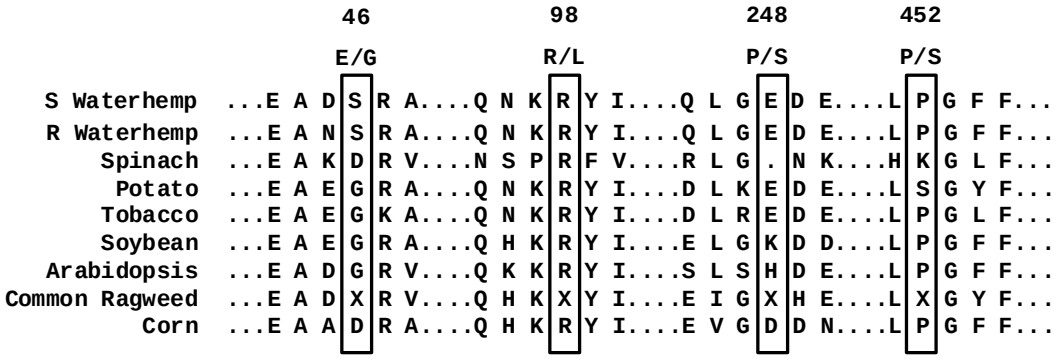


Figure 3.5. Functional complementation assay of the BT_3 *hem*G mutant of *Escherichia coli* grown on agar plates containing LB medium alone, 100 μM lactofen, or 20 μg ml^{-1} hematin. Isolates were as follows: C_1 and C_2, untransformed controls; S_1 and S_2, transformed with a vector encoding waterhemp-derived *PPX2* with Arg98; and R_1 and R_2, transformed with a vector encoding identical *PPX2* with the exception of Leu98.

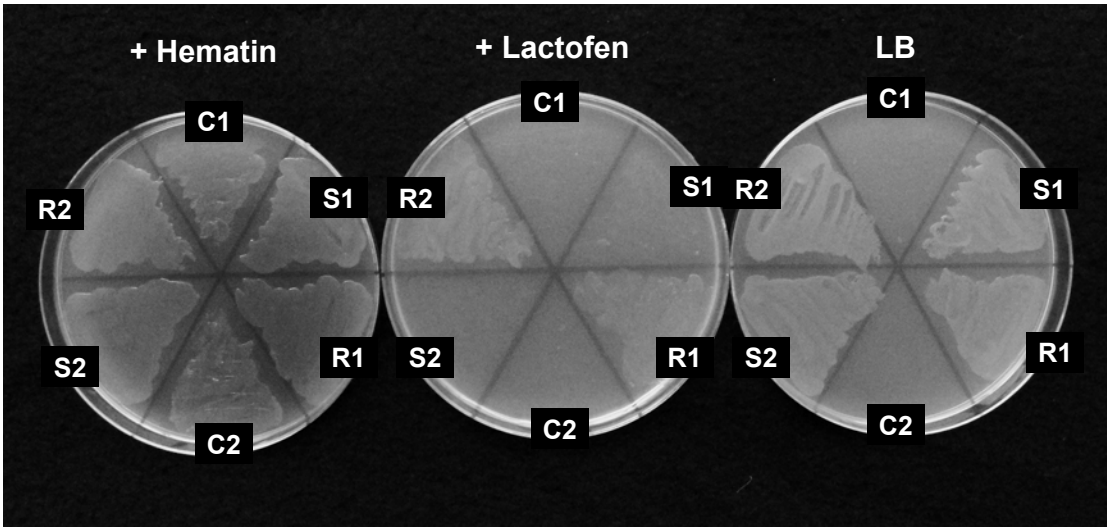
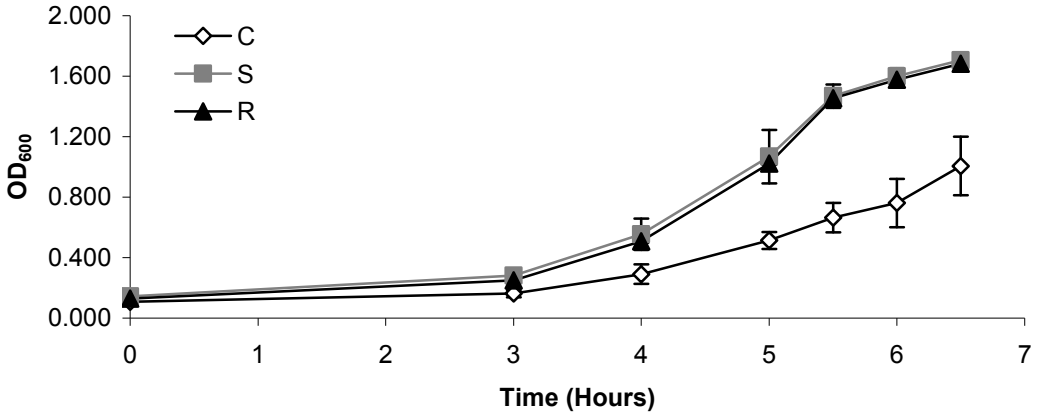


Figure 3.6. Growth curves of the BT_3 *hem*G mutant of *Escherichia coli* grown in LB medium supplemented with (A) 20 μg ml^{-1} hematin, (B) LB medium alone, (C) 100 μM of lactofen, or (D) 1 μM of lactofen (D). Isolates were as follows: C, untransformed control; S, transformed with a vector encoding waterhemp-derived *PPX2* with Arg98; and R, transformed with a vector encoding identical *PPX2* with the exception of Leu98. Vertical bars represent +/- the standard error of the mean (n=2).

A.



B.

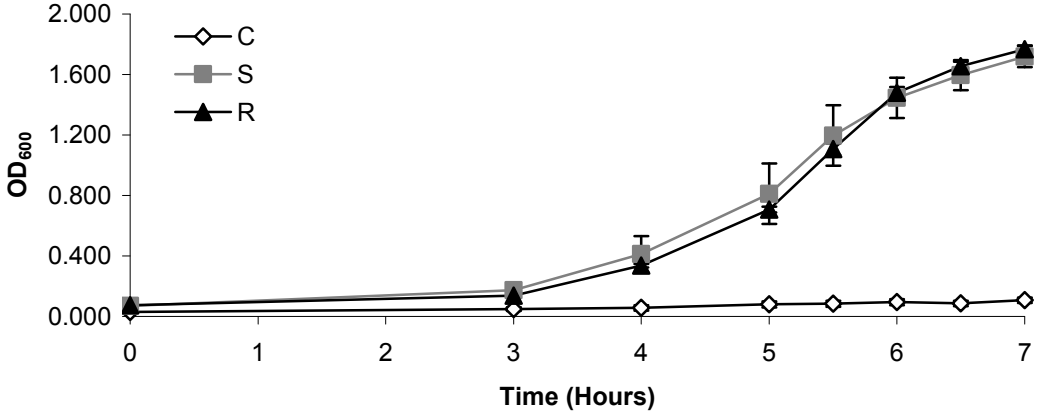
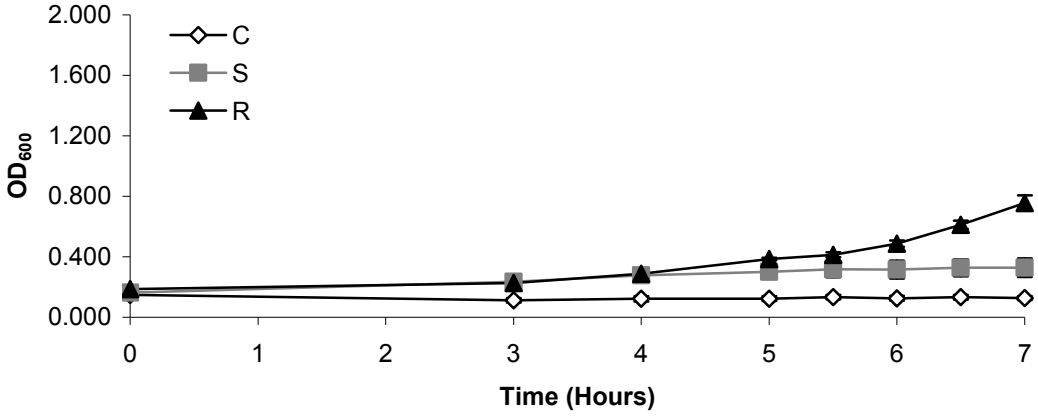


Figure 3.6. (cont.)

C.



D.

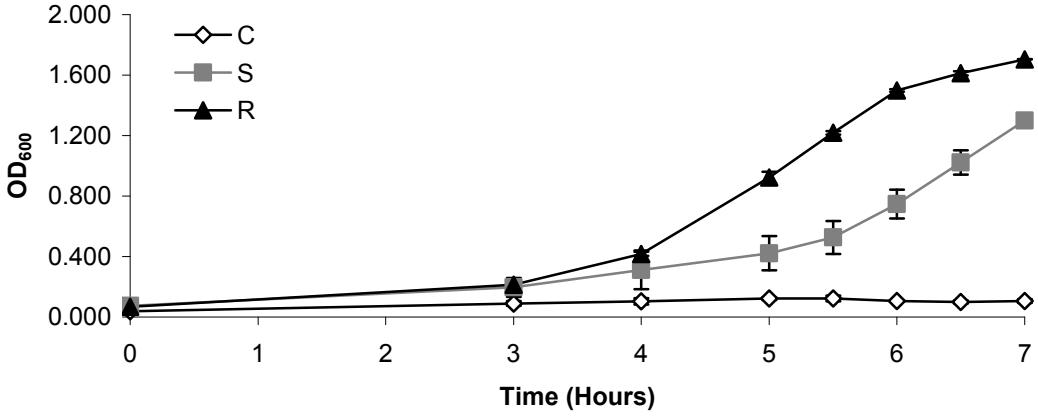
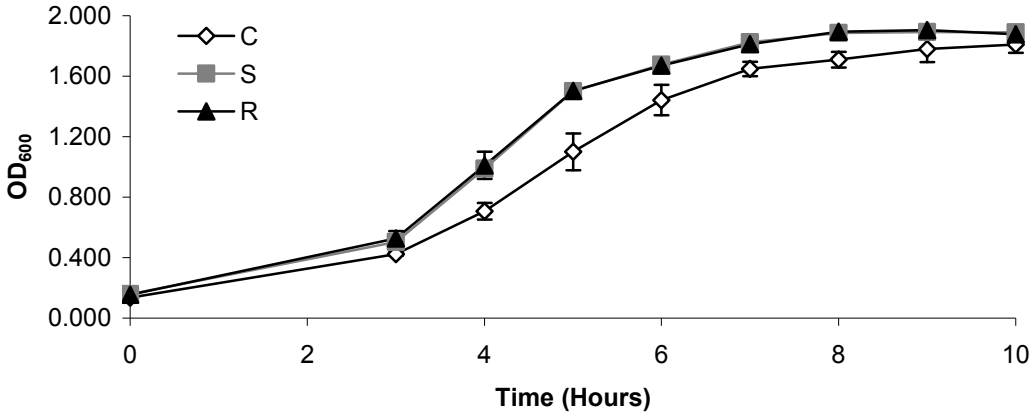


Figure 3.7. Growth curves of the BT_3 *hem*G mutant of *Escherichia coli* grown in LB medium supplemented with (A) 20 μg ml^{-1} hematin, (B) LB medium alone, or (C) 10 μM of lactofen. Isolates were as follows: C, untransformed control; S, transformed with a vector encoding waterhemp-derived *PPX2* with Arg98; and R, transformed with a vector encoding identical *PPX2* with the exception of Leu98. Vertical bars represent +/- the standard error of the mean (n=2).

A.



B.

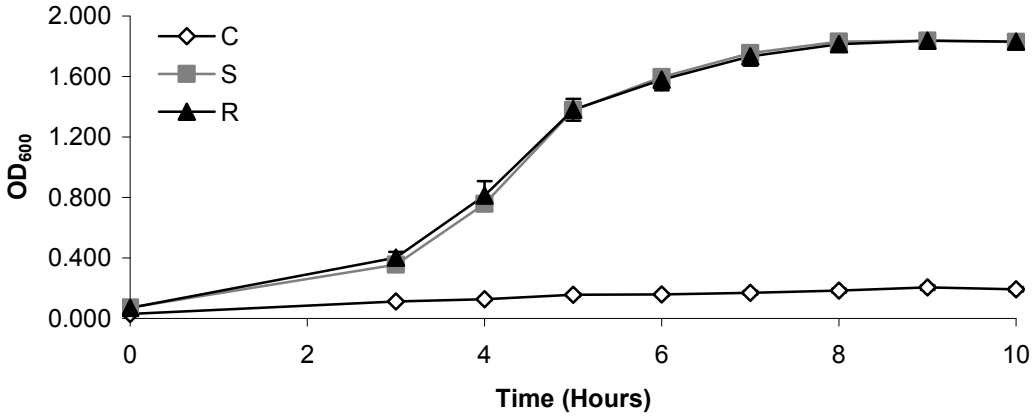


Figure 3.7. (cont.)

C.

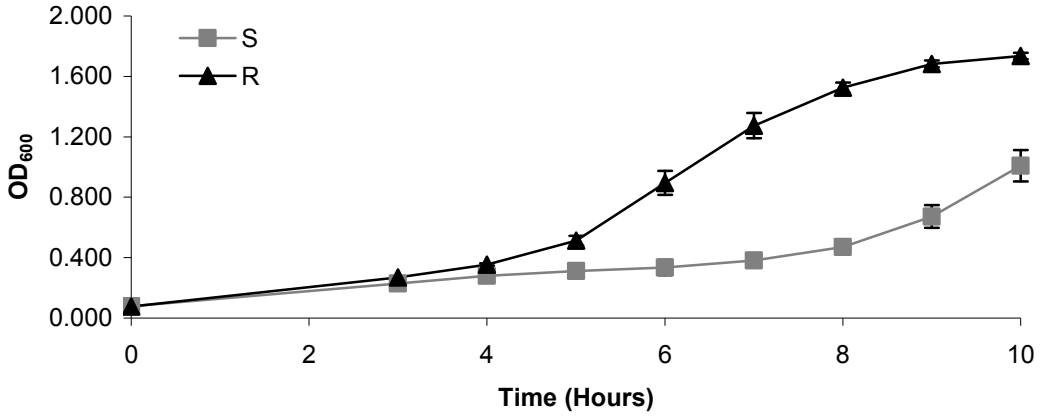


Figure 3.8. Microbial growth data were pooled from the data of the two growth curve replications (Figure 3.6 and 3.7) in order to estimate the resistance level of the enzyme. R represents resistant and S represents sensitive.

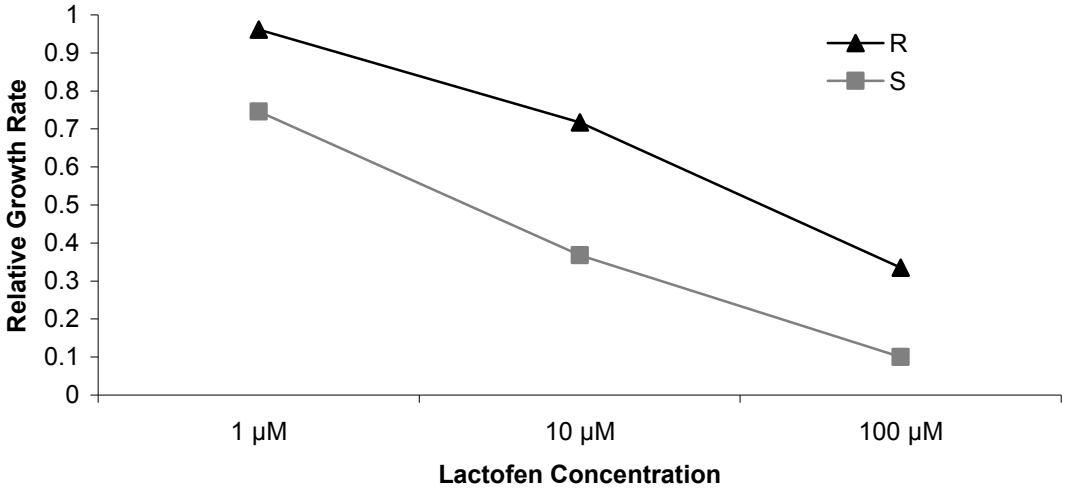


Figure 3.9. Common ragweed nucleotide sequence around the R98L mutation. Underlined nucleotides indicate codon 98 and the polymorphic nucleotide is outlined. Brackets indicate the *BrsG*I recognition site and the arrows represent where the restriction enzyme cuts.

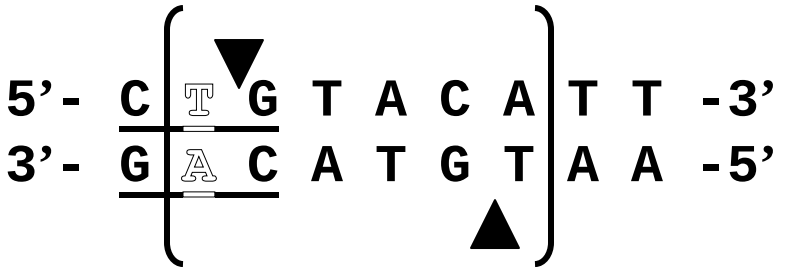
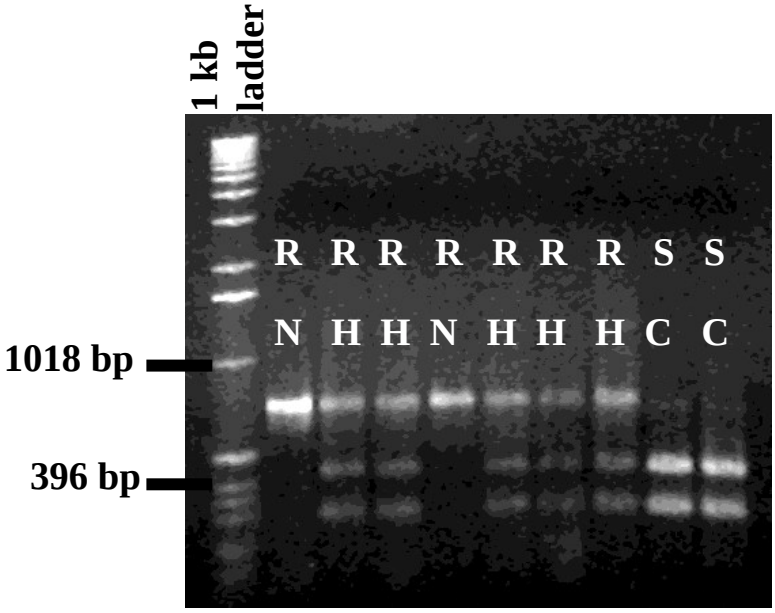


Figure 3.10. Representative sample of common ragweed from the F_2 population after digestion with the *BrsG*I restriction enzyme to test for the presence/absence of the R98L mutation, R indicates resistant and S indicates sensitive. N indicates a homozygous no cut, H indicates a heterozygote, and C indicates a homozygous cut result after digestion.



CHAPTER 4

CONCLUDING REMARKS

4.1 Conclusions, Future Directions, and Speculations

Uses of molecular biology tools have started to expand the field of weed science. These tools can be utilized to improve the study of the mechanisms and evolution of herbicide resistance in weed species, which may eventually lead to the discovery of better management. Herbicide resistance in weed populations endangers the efficacy of herbicides, which is apparent from the global evolution of 345 resistant weeds biotypes in 194 weed species and over 340,000 fields (Heap 2010). Almost all known herbicidal modes of action have documented cases of herbicide-resistant weeds and some have more than others. For example, there are currently over 60 different weed species world-wide resistant to PSII inhibitors versus only four weed species that have evolved resistance to PPO-inhibiting herbicides (Heap 2010). The third weed to evolve resistance to PPO-inhibiting herbicides is a biotype of common ragweed discovered in Delaware during 2005 (Moreira et al. 2006), which was researched in this thesis. The overall purpose of this thesis was to elucidate the resistance mechanism in common ragweed to PPO-inhibiting herbicides. Information from this resistance mechanism could potentially allow for better management strategies, improve our understanding of plant processes, and provide a means to create herbicide resistant crops (Tharayil-Santhakumar 2010).

Previous research led to the identification of the resistance mechanism in waterhemp to PPO-inhibiting herbicides, which provided a model to study resistance in common ragweed. Resistance in waterhemp was found to be the result of a single codon

deletion, ΔG210, in the *PPX2L* gene, which encodes both plastid and mitochondrial transit peptides (Patzoldt et al. 2006). Chapter two revealed the numerous experiments performed to isolate the target-site genes, *PPX1* and *PPX2*, in common ragweed. Chi-square analysis of the F_2 population, after an application of fomesafen at 164 g ai ha^{-1} plus 1% (v/v) COC, revealed a 3:1 (R:S) segregation ratio and data consistent with inheritance of resistance in common ragweed conferred by a single, dominant, nuclear gene. Naturally, the next step to elucidating the resistance mechanism was to obtain the sequences of *PPX1* and *PPX2*. Screening of the common ragweed cDNA library resulted in the recovery of 1,653 bp and 1,406 bp of nucleotide sequence from *PPX1* and *PPX2*, respectively. Translation of the nucleotide sequences and subsequent alignments revealed that the 5' ends of both genes were missing. For *PPX1*, we had obtained the full coding sequence of the functional enzyme but not for *PPX2* so 5'-RACE was performed on this gene only. Nested PCR primers were utilized during 5'-RACE of *PPX2* and resulted in obtaining the in-frame methionine at the beginning of the putative mitochondrial-targeted peptide. At this point we cannot definitively answer if an in-frame methionine exists upstream of the already obtained methionine in *PPX2* for common ragweed. Many continued attempts to acquire more upstream sequence were performed involving multiple RACE procedure modifications and sequencing several cDNA clones but proved to be unsuccessful. Some possible explanations for this is that when the cDNA was made from RNA, during the RACE procedure, only prematurely terminated sequence from the *PPX2* gene was transcribed due to characteristics in gene sequences that can hinder reverse transcriptase; the gene is expressed in very low abundance; or *PPX2L* does not exist. Northern blot analysis could be performed with

PPX2 in common ragweed for detection of RNA size in order to determine if it is instead a *PPX2L* gene. Another method would be to sequence *PPX2* from a genomic DNA library in order to determine if there is an in-frame methionine upstream. Sequence analysis of *PPX1* and *PPX2* from an F_1 plant determined 2 (64 and 370) and 4 (46, 98, 248, and 452) missense mutations, respectively, as possible candidates for PPO-inhibiting herbicide resistance in common ragweed.

Chapter three discussed the involvement of the target-site genes in PPO-inhibiting herbicide resistance in common ragweed. Visual inspection of the molecular marker data clearly shows co-segregation of *Fok*I and *BrsG*I with the presence of the resistant allele in the F_2 population. Therefore, this strongly indicated that *PPX2* is likely to confer PPO-inhibiting herbicide resistance in common ragweed. Having learned that data are consistent with resistance due to a single, dominant gene and co-segregated with *PPX2*, we took a closer look at the four missense mutations that were found. It was determined that the amino acid at position 98 was highly conserved amongst species and functionally important within the active site (Heinemann et al. 2007). It is also important to note that the cause of human variegate porphyria, in South Africa, is mainly due to R59W (Meissner et al. 1996) and this residue is homologous to Asn67 in tobacco and common ragweed, where no mutation exists. In order to test if the R98L mutation was responsible for herbicide resistance, a functional complementation was utilized to prove the resistance mechanism. During the functional complementation assay, growth medium supplemented with lactofen considerably hindered the growth of mutant *E. coli* transformed with the wild-type *PPX2* from waterhemp but not *E. coli* transformed with the R98L construct. Therefore, this assay qualitatively proved that the single point

mutation in *PPX2* producing a leucine at position 98, instead of an arginine, was capable of conferring resistance to PPO-inhibiting herbicides. Resistance was now verified through a qualitative functional complementation assay but we also confirmed it using a more quantitative method with growth curve analysis. Media supplemented with lactofen considerably hindered the growth of the sensitive strain, wild-type *PPX2*, but not the resistant strain, R98L *PPX2*, which grew vigorously. After analysis of the growth curves it could be concluded that resistance in common ragweed to PPO-inhibiting herbicides is further confirmed to result from the R98L mutation in *PPX2*. Based on the data from the growth curves there does not appear to be a fitness penalty associated with the R98L mutation in the bacterial system but that does not mean this would also be true at the whole plant level. We can also conclude from the functional complementation assay and growth curve analysis that resistance is target-site based. Microbial growth assays showed that the resistant enzyme had a 10 fold resistance level to lactofen compared to the sensitive enzyme. When comparing the whole plant and microbial resistance data, it confirms about the same level of resistance in both to lactofen, with 10-14 fold. After this confirmation, we can now use the *BrsG*I marker as a quick and easy DNA based assay to determine if common ragweed samples contain the R98L mutation. Common ragweed suspected to be resistant to PPO-inhibiting herbicides can be sent in from growers and screened with the molecular based marker. Results could then be used to aid in making proper management decisions to manage the common ragweed population.

Naturally, after discovering the R98L resistance mutation in common ragweed, it spawned further questions and future possible experiments. An important experiment would be to obtain all of the 5' end sequence of *PPX2*, whether by means of Northern

blot or genomic DNA library sequencing. During the course of the research in this thesis, another biotype of common ragweed from Ohio was documented with resistance to multiple PPO-inhibiting and ALS-inhibiting herbicides (Heap 2010). The DNA based assay could be used on samples from this new biotype to determine if the resistance mechanism to PPO-inhibiting herbicides is due to the R98L mechanism or something new. Last, biochemical analysis could be performed on the sensitive and resistant enzymes of the biotype from Delaware to determine if there is a change in enzyme kinetics or active site structure.

With knowledge from the R98L resistance mechanism in common ragweed and ΔG210 in waterhemp, some speculations can be made about both of them. In waterhemp, the ΔG210 resistance mechanism is enabled by the surrounding sequence. This mechanism is facilitated by a bi-GTG repeat (or a bi-TGG repeat), which allows for the deletion of either a GTG or TGG resulting in the loss of a glycine codon (Patzoldt et al. 2006). Deletion of ΔG210 would not be probable in common ragweed, soybean, *Arabidopsis*, or corn since the TGG repeat region, which facilitates the mutation, is not present at the homologous positions (Figure 4.1). Although the ΔG210 deletion cannot occur in soybean, it does contain a GCA repeat region around the same location as the glycine, which could allow for the deletion of GCA resulting in the loss of an alanine codon (Figure 4.1). Conversely, the ΔG210 deletion mutation could potentially evolve in spinach, potato, and tobacco due to their sequences containing the TGG repeat region, if intense selection pressure was applied (Figure 4.1). The R98L mutation is achieved by only a single base pair substitution (Table 4.1) in common ragweed and could also be possible in potato, tobacco, *Arabidopsis*, soybean, and corn (Figure 4.1). In order for the

R98L mutation to occur in waterhemp and spinach (Figure 4.1), it would require a substitution in at least two base pairs which is more unlikely (Table 4.2). After these comparisons, it would be interesting to see in plants like potato and tobacco, which are capable of both the R98L and ΔG210 mutation, which mutation would evolve if intense selection pressure was applied. DNA sequence predisposes a plant to which mutation can evolve and, therefore, could allow us to predict which resistance mechanism may be selected.

Previous research involving the biochemical analysis of the ΔG210 deletion in waterhemp brought to light several insights. The rate of the reaction, or V_{max}, is decreased with the presence of the deletion, which is probably not detrimental to the enzyme (Dayan et al. 2010). Conversely, the affinity of the substrate for the enzyme, or K_m, is not changed for the resistant enzyme (Dayan et al. 2010). Since the K_m remained relatively constant between the sensitive and resistant enzymes, little or no fitness penalty likely exists. Based on previous research involving the biochemical analysis of single base pair substitutions at position 98 in tobacco (Heinemann et al. 2007), some speculations can be made about the effects of the R98L mutation in common ragweed. The rate of the reaction, or V_{max}, would probably increase with R98L mutation, which is probably not detrimental to the enzyme and could possibly be advantageous. Similarly, the affinity of the substrate for the enzyme, or K_m, would also probably increase for the resistant enzyme. Since the K_m would probably increase between the sensitive and resistant enzymes a high fitness penalty could be associated with the R98L mutation but this contradicts the results from the bacterial growth curve assays performed in this thesis which may not be represented at the whole plant level. Due to potential fitness penalties,

the ΔG210 deletion may provide a superior level of resistance to PPO-inhibiting herbicides than that obtained via a single amino acid substitution (Dayan et al. 2010).

A plant whose *PPX2* sequence was not predisposed for the glycine deletion would need a different resistance mechanism, therefore the R98L mutation probably evolved. Even though there might be a potential fitness penalty associated with resistance due to the R98L mutation, it is better than the alternative of plant death associated with susceptibility to PPO-inhibiting herbicides. Once biochemical analysis is performed on the R98L resistant enzyme, it may lead to identification of unique changes in the active site as seen in the resistant waterhemp enzyme, which resulted in a significant increase of volume (Dayan et al. 2010). If a significant conformational change due to the R98L mutation exists, it may potentially explain the importance of a single base pair substitution occurring at position 98 on an enzymatic level to confer resistance. Last, maybe common ragweed does contain the *PPX2L* gene but we just have not sequenced enough of the 5' end. In the end, it is possible that multiple factors like ΔG210 or R98L and the dual-targeting *PPX2L* gene all need to be present in order to confer resistance to PPO-inhibiting herbicides, and that may partially explain why resistance was so slow to evolve (Dayan et al. 2010).

4.2 Literature Cited

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4.3 Tables and Figure

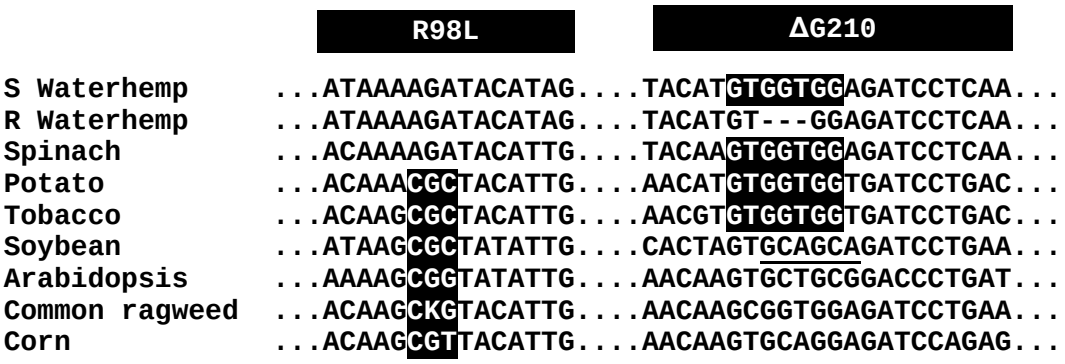
Table 4.1. Codon sequences possible at position 98 in common ragweed, potato, tobacco, *Arabidopsis*, soybean, and corn. The underlined nucleotide represents the nucleotide which would have changed in order to obtain a leucine.

Arginine	Leucine
CGT	C<u>T</u>T
CGC	C<u>T</u>C
CGA	C<u>T</u>A
CGG	C<u>T</u>G

Table 4.2. Codon sequences possible at position 98 in waterhemp and spinach. The underlined nucleotide represents the nucleotide which would have changed in order to obtain a leucine.

Arginine	Leucine
AGA	<u>TT</u>A
	<u>CT</u>A
AGG	<u>TT</u>G
	<u>CT</u>G
AGA/AGG	<u>CTT</u>

Figure 4.1. An alignment of the nucleotide sequences of various species around the two natural resistance mechanisms to PPO-inhibiting herbicides.



Sequences are from S Waterhemp (DQ386117), R Waterhemp (DQ386116), Spinach (AB046993), Potato (AJ225108), Tobacco (AB020500), Soybean (AB025102), Arabidopsis (NM_121426), and Corn (BT033827).