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Research Article

Will the *Amaranthus tuberculatus* Resistance Mechanism to PPO-Inhibiting Herbicides Evolve in Other *Amaranthus* Species?

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Resistance to herbicides that inhibit protoporphyrinogen oxidase (PPO) has been slow to evolve and, to date, is confirmed for only four weed species. Two of these species are members of the genus *Amaranthus* L. Previous research has demonstrated that PPO-inhibitor resistance in *A. tuberculatus* (Moq.) Sauer, the first weed to have evolved this type of resistance, involves a unique codon deletion in the *PPX2* gene. Our hypothesis is that *A. tuberculatus* may have been predisposed to evolving this resistance mechanism due to the presence of a repetitive motif at the mutation site and that lack of this motif in other amaranth species is why PPO-inhibitor resistance has not become more common despite strong herbicide selection pressure. Here we investigate inter- and intraspecific variability of the *PPX2* gene—specifically exon 9, which includes the mutation site—in ten amaranth species via sequencing and a PCR-RFLP assay. Few polymorphisms were observed in this region of the gene, and intraspecific variation was observed only in *A. quitensis*. However, sequencing revealed two distinct repeat patterns encompassing the mutation site. Most notably, *A. palmeri* S. Watson possesses the same repetitive motif found in *A. tuberculatus*. We thus predict that *A. palmeri* will evolve resistance to PPO inhibitors via the same *PPX2* codon deletion that evolved in *A. tuberculatus*.

1. Introduction

Herbicides that inhibit protoporphyrinogen oxidase (PPO) have been used for many years for control of broadleaf weeds in large-scale crop production systems in the United States. Their use began to slowly decline during the late 1990s due to the introduction and subsequent widespread adoption of glyphosate-resistant crop varieties, such as Roundup Ready soybean, corn, and cotton. Glyphosate-resistant crops currently dominate throughout much of the United States and elsewhere in the world, and these systems rely almost exclusively on glyphosate as the sole means for weed management [1]. Unfortunately, the continuous broad-scale use of glyphosate over time has triggered the evolution of glyphosate-resistant biotypes among an increasing diversity of weed species [2]. In many cases, these glyphosate-resistant biotypes have been selected from weed populations that already had resistance to one or more other herbicide families, such as ALS inhibitors, triazines, and, less frequently, PPO inhibitors. As glyphosate resistance continues to increase in frequency, distribution, and the number of species [2], growers are once again relying on PPO-inhibiting herbicides as an alternative approach to control weeds.

The enzyme protoporphyrinogen oxidase (EC 1.3.3.4) is one of the most important targets for herbicide development [3, 4]. PPO is the last common enzyme in the tetrapyrrole biosynthesis pathway and is responsible for converting protoporphyrinogen IX (Protogen) to protoporphyrin IX (Proto). In plants, two isoforms of the PPO enzyme are encoded by two different nuclear genes, *PPX1* and *PPX2*. These two enzymes share little sequence identity and are functionally compartmentalized, with *PPX1* being targeted to plastids and *PPX2* targeted to the mitochondria. Inhibition of PPO disrupts the synthesis of chlorophylls and hemes, which results in the damaging photodynamic effect characteristic of PPO inhibiting herbicides [3].

Natural resistance to PPO-inhibitors has been slow to evolve [3, 5], yet it has been confirmed in four weed species [2]. The first weed to evolve resistance to PPO herbicides was *Amaranthus tuberculatus* (Moq.) Sauer (waterhemp) in 2000

[6]. PPO resistance has subsequently been confirmed in *Euphorbia heterophylla* L. (wild poinsettia) and *Amaranthus quitensis* Kunth, both from South America, and *Ambrosia artemisiifolia* L. (common ragweed). The mechanism of PPO-inhibitor resistance, a unique target-site amino acid deletion, was first elucidated in a biotype of *A. tuberculatus* from Illinois [7]. This mechanism involves the loss of a glycine at position 210 in the mitochondrial isoform of the PPO enzyme. Loss of this amino acid is considered to have occurred via a slippage-like mechanism within a trinucleotide repeat of the *PPX2* gene [7, 8]. Specifically, the sequence motif spanning position 210 (i.e., . . .TGTGGTGGA. . .) contains both a GTG and a TGG bi-repeat. Loss of either one of these repeat elements results in a loss of a glycine codon (GGT) without affecting the reading frame. This glycine deletion alters the binding domain of the enzyme without negatively affecting substrate affinity, and thus overall sensitivity to PPO-inhibiting herbicides is reduced by at least 100-fold [3]. The presence of a short repetitive motif at the mutation site, together with the favorable biochemical consequences of the deletion, seems to have predisposed *A. tuberculatus* to evolving this unique resistance mechanism, which thus far is the only identified mechanism of evolved PPO-inhibitor resistance. Despite being an unusual mutation, it is found commonly in *A. tuberculatus* populations across the Midwestern United States [9–11]. Assuming a slippage mechanism is responsible for evolved PPO resistance in *A. tuberculatus*, the question arises whether other weedy amaranth species possess the same sequence repeat and thus are also predisposed to acquiring this mutation.

It is unknown how conserved the *PPX2* gene is among weed species or whether other weedy members of the genus *Amaranthus*, such as *A. hybridus* L., *A. retroflexus* L., *A. powellii* S. Watson, and most notably Palmer amaranth (*A. palmeri* S. Watson), share the same repeat motif as in *A. tuberculatus*. These species are aggressive and pernicious weeds in their own right and have evolved resistances to multiple herbicides [2], though as of yet not to PPO inhibitors. However, there is growing concern that over-reliance on the PPO herbicides for controlling glyphosate-resistant *A. palmeri* in Roundup Ready crops (soybean, cotton, and corn) in the Southeastern United States will promote PPO resistance in this species [12]. In addition to glyphosate, populations of *A. palmeri* have evolved resistance to ALS inhibitors, dinitroanilines, and triazines [2, 13, 14], so the threat of a multiple-resistant individual or population is real and will be much more difficult to control once PPO resistance occurs in this species.

In this paper, we investigate inter- and intraspecific variability of the *PPX2* gene among weedy amaranths with conventional PCR and sequencing methods in conjunction with a newly developed PCR-RFLP (restriction fragment length polymorphism) assay. Our primary objective was to determine if the repeat motif encompassing Gly210 of *PPX2* from *A. tuberculatus* is shared among other weedy amaranth species. A second objective was to obtain baseline sequence information for future genetic and molecular studies of evolved PPO-inhibitor resistance in weedy amaranths.

2. Materials and Methods

Plant species and populations sampled for this study are listed in Table 1. Genomic DNA of some accessions utilized in this study was previously isolated for analysis of genetic similarity [15] and herbicide target-site genes [16, 17]. Source material for additional *Amaranthus* accessions was obtained either from herbarium specimens (for *A. acanthochiton* Sauer and *A. caudatus* L.) or from germplasm collections of the authors. In the case of *A. quitensis*, seed was obtained from the North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, and represented semi domesticated types from the Pacific side of South America and field weeds collected from Brazil. Seeds were initially sown in containers with 3 : 1 : 1 : 1 mixture of commercial potting mix to soil to peat to sand. When seedlings exhibited true leaves, the plants were thinned and transplanted into new containers of the same size to ensure ample material for DNA extractions. Plants were fertilized as necessary with slow-release fertilizer and grown under mercury halide and sodium vapor lamps along with incident sunlight. Lamps were programmed for a 16-hour photoperiod and the temperature maintained at 22 C at night and 28 C daytime. Leaf material for DNA extraction was harvested from one individual mature plant per accession and flash frozen in liquid nitrogen prior to DNA extraction using the modified CTAB method [18].

PCR amplifications were performed using MJ Research thermal cyclers in 25 μL volumes with 1x buffer (GoTaq Flexi Buffer, Promega Corp., Madison, WI, USA), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer, 1.0 μL total genomic DNA (10–50 ng), and 1.25 units of GoTaq polymerase. Parameters for PCR featured an initial denaturation of 95 C for 2 min, followed by 37 cycles (95 C for 45 sec, 55 C for 30 sec, and 72 C for 90 sec) and a final extension of 72 C for 5 min. Three primer sets were used for amplifications of the exon 9 region of *PPX2*. Since genomic *PPX2* information with intron sizes and sequence identity was limited, we initially used the primer set ARMS7-F (5′-TCTGATGAGCATGTTCAGGAAAGGCAAG) and *PPX2*ex10-R (5′-CTGGAAATGTATGGTGCATC) to amplify a large fragment (~1200 bp) between exons 7 and 10 for all species. Following sequence comparisons to identify conserved motifs in the introns adjacent to exon 9, the primer sets *PPX2*int8-F1 (5′-CAACTTGCCATGCTCTATTCC) and *PPX2*int9-R1 (5′-ATGGCGAAATGAGTTAAGGTTC), or *PPX2*int8-F2 (5′-ATTGCCATGCTCTATTCATTCC) and *PPX2*int9-R2 (5′-CGCCTATTCAAATCAAATGTCC), were used to amplify smaller fragments of ~500 bp and ~400 bp, respectively. PCR products were visualized on a 1% agarose gel containing 0.5 μg mL^{-1} ethidium bromide and cleaned using an E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturer's instructions. Purified PCR products were directly sequenced using an ABI Prism BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA, USA), and run on an ABI 3730XL capillary sequencer at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois. Sequencing reactions were prepared in 13–16 μL volumes and

Table 1: *Amaranthus* accessions tested for *PPX2* sequence variation.

Species	Accession no.	Location information	PCR-RFLP result
A. acanthochiton Sauer	398443	New Mexico, USA	Uncleaved
A. albus L.	MH36	Washington, USA	Uncleaved
(Tumble pigweed)	MH38	Mississippi, USA	Uncleaved
	PT70	Illinois, USA	Uncleaved
A. caudatus L.	CAU	Bolivia	Cleaved
A. hybridus L.	MH154	North Carolina, USA	Cleaved
(Smooth pigweed)	PT12	Illinois, USA	Cleaved
	MH165	Ohio, USA	Cleaved
A. palmeri S. Watson	Alex 1	Illinois, USA	Uncleaved
(Palmer amaranth)	Alex 2	Illinois, USA	Uncleaved
	S1 union	Illinois, USA	Uncleaved
	S1 pul	Illinois, USA	Uncleaved
	Mass 1	Illinois, USA	Uncleaved
	GA-5	Georgia, USA	Uncleaved
	GA-7	Georgia, USA	Uncleaved
	GA-8	Georgia, USA	Uncleaved
	GA-10	Georgia, USA	Uncleaved
	MH253	New Mexico, USA	Uncleaved
	MH247	California, USA	Uncleaved
	MH254	Texas, USA	Uncleaved
A. powellii S. Watson	MH234	Washington, USA	Cleaved
(Powell's amaranth)	MH237	New York, USA	Cleaved
	MH242	Ontario, Canada	Cleaved
A. quitensis Kunth	511736	Bolivia	Cleaved
	511738	Ecuador	Cleaved
	511745	Ecuador	Cleaved
	511751	Peru	Cleaved
	568154	Tarija, Bolivia	Cleaved
	652421	Goias, Brazil	Cleaved
	652423	Goias, Brazil	Cleaved
	652426	Federal District, Brazil	Cleaved
	652429	Federal District, Brazil	Cleaved
	652430	Federal District, Brazil	Cleaved
A. retroflexus L.	MH84	New Mexico, USA	Cleaved
(Redroot pigweed)	PT25	Illinois, USA	Cleaved
	PT67	Illinois, USA	Cleaved
A. spinosus L.	MH267	Puerto Rico	Uncleaved
(Spiny amaranth)	MH203	Texas, USA	Uncleaved
	MH205	Louisiana, USA	Uncleaved
A. tuberculatus (Moq.) Sauer	PT43	Illinois, USA	Uncleaved
(Waterhemp)	ACR	Adams Co., IL, USA	Uncleaved
	WCS	Wayne Co., IL, USA	Uncleaved
	MH320	Ohio, USA	Uncleaved
	MO-a	Missouri, USA	Uncleaved
	MO-b	Missouri, USA	Uncleaved
	MO-c	Missouri, USA	Uncleaved
	MO-1	Missouri, USA	Uncleaved

contained 1.8 μL ddH_2O, 5.2 μL 12.5% glycerol, 2.0 μL 5x sequencing buffer, 2.0 μL 10 μM primer, 1.0 μL BigDye Terminator v3.1, and 1.0–4.0 μL PCR product. Cycle sequencing conditions started at 96 C for 1 min, followed by 30 cycles of (96 C for 30 sec, 50 C for 15 sec, 60 C for 4 min) and final extension of 60 C for 4 min. Forward and reverse sequences were manually edited and assembled into contiguous sequences (contigs) using the Alignment Explorer in MEGA5 [19]. Alignment of coding regions and exon-intron boundaries were determined by comparison with published genomic and cDNA *PPX2* sequences of *A. tuberculatus* (DQ394875, DQ394876, DQ386113, DQ386114, DQ386116, DQ386117, DQ386118) and *A. hypochondriacus* L. (EU024569) from GenBank.

Comparative sequence alignments of exon 9 of the *PPX2* gene showed nucleotide variations at the Gly210 mutation site. To facilitate the screening of additional accessions of each species for variation at this site, a PCR-RFLP assay was developed using the restriction enzyme EciI (New England Biolabs Inc., USA). Amplified products from all primers sets were digested and analyzed for fragment patterns. Following PCR, 10 μL of each reaction was added to 10 μL of a digestion mixture and incubated at 37 C for 1.5 hrs. The digestion mixture contained EciI (1 unit μL^{-1}) and 1x concentrations of the supplied buffer (NEB2) and BSA. After digestion, the fragments were separated on a 1% agarose gel and visualized under UV with ethidium bromide staining. PCR amplifications and digestions for each sample were replicated at least twice to validate the assay.

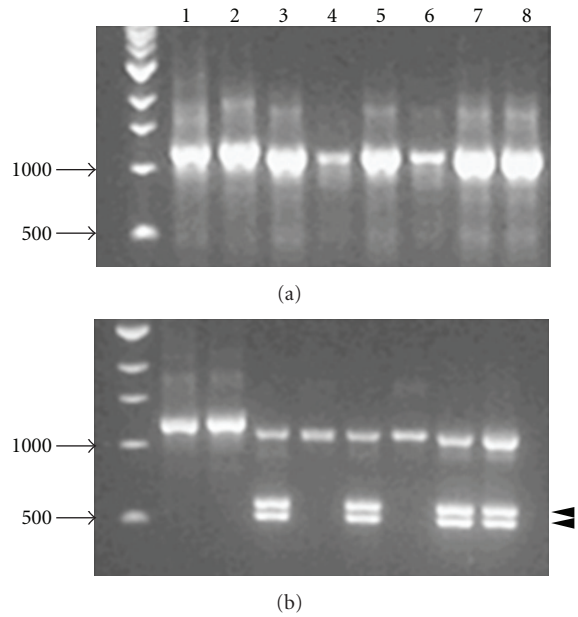

Figure 1: (a) Amplification of a segment of the *PPX2* gene with the primers ARMS7-F and *PPX2*ex10-R. (b) Digested PCR products with the enzyme EciI. Separation of fragments on a 1% w/v agarose gel with a 1-kb DNA ladder (NEB). Lanes 1–8 represent the following accessions: (1) *A. tuberculatus* PT43, (2) *A. tuberculatus* MH320, (3) *A. retroflexus* PT25, (4) *A. palmeri* MH253, (5) *A. hybridus* MH154, (6) *A. palmeri* MH247, (7) *A. powellii* MH242, and (8) *A. powellii* MH237.

3. Results and Discussion

Sequence comparisons between a PPO-inhibitor-sensitive biotype (i.e., wild-type) of *A. tuberculatus* (GenBank accession DQ394875) and *A. hypochondriacus* (GenBank accession EU024569) revealed the presence of a single-nucleotide polymorphism (SNP) in the third position of the Gly210 codon in exon 9 of the genomic *PPX2* gene. Although this SNP is a synonymous substitution, its position is significant in being part of a codon that, when deleted, confers resistance to PPO-inhibiting herbicides in *A. tuberculatus* [7]. In biotypes of *A. tuberculatus,* the thymine present at this site creates a bi-GTG or bi-TGG nucleotide repeat that spans the Gly210 codon. In the nucleotide sequence of *A. hypochondriacus*, a cytosine rather than a thymine is present, so no repeat motifs are formed. Repetitive sequences are thought to be more prone to slippage during replication, and it would not matter in this case if the deleted triplet was a TGG or a GTG since the reading frame is maintained in both instances [8]. Moreover, the resultant loss of a glycine at this position imparts herbicide resistance without adversely affecting the normal functions of the PPO enzyme [3].

The initial PCR experiments tested for cross-species amplification of the *PPX2* gene using primers based on sequence data from *A. tuberculatus*. The primers ARMS7-F and *PPX2*ex10-R produced a single product of approximately 1200 bp in the five amaranth species tested (Figure 1(a)). The amplified products for each species were fairly uniform in size with only minor differences in length being attributable to short indel (insertion/deletion) mutations located in the introns. Digestion of these PCR products with the restriction enzyme EciI produced two smaller fragments of approximately 500 and 580 bp in *A. hybridus*, *A. retroflexus*, and *A. powellii*, but no fragments in the multiple accessions of *A. tuberculatus* and *A. palmeri* (Figure 1(b)). Sequencing these PCR products confirmed the digestion patterns by showing that both accessions of *A. palmeri* had the same repeat motif as *A. tuberculatus* and thus lacked the restriction site. Conversely, a single restriction site was present in the sequences of the three other species since they possessed a cytosine substitution similar to *A. hypochondriacus* rather than a thymine as in *A. tuberculatus* and *A. palmeri*. The uncut bands observed for *A. hybridus*, *A. retroflexus*, and *A. powellii* in Figure 1(b) were determined to be the result of incomplete digestion rather than heterozygosity as their intensity decreased, but was not completely eliminated, through additional digestion experiments with different enzyme and template concentrations and longer incubation times. Sequencing evidence also confirmed that none of these accessions were heterozygous for the C↔T polymorphism. Subsequent PCR experiments using the primers ARMS7-F and *PPX2*ex10-R produced single bands of ~1200 bp in *A. albus* L., *A. quitensis*, and *A. spinosus* L., which again suggested that this portion of the gene is relatively conserved across amaranth species. Digestion with EciI produced cleaved products only for the accessions of *A. quitensis* (data not shown).

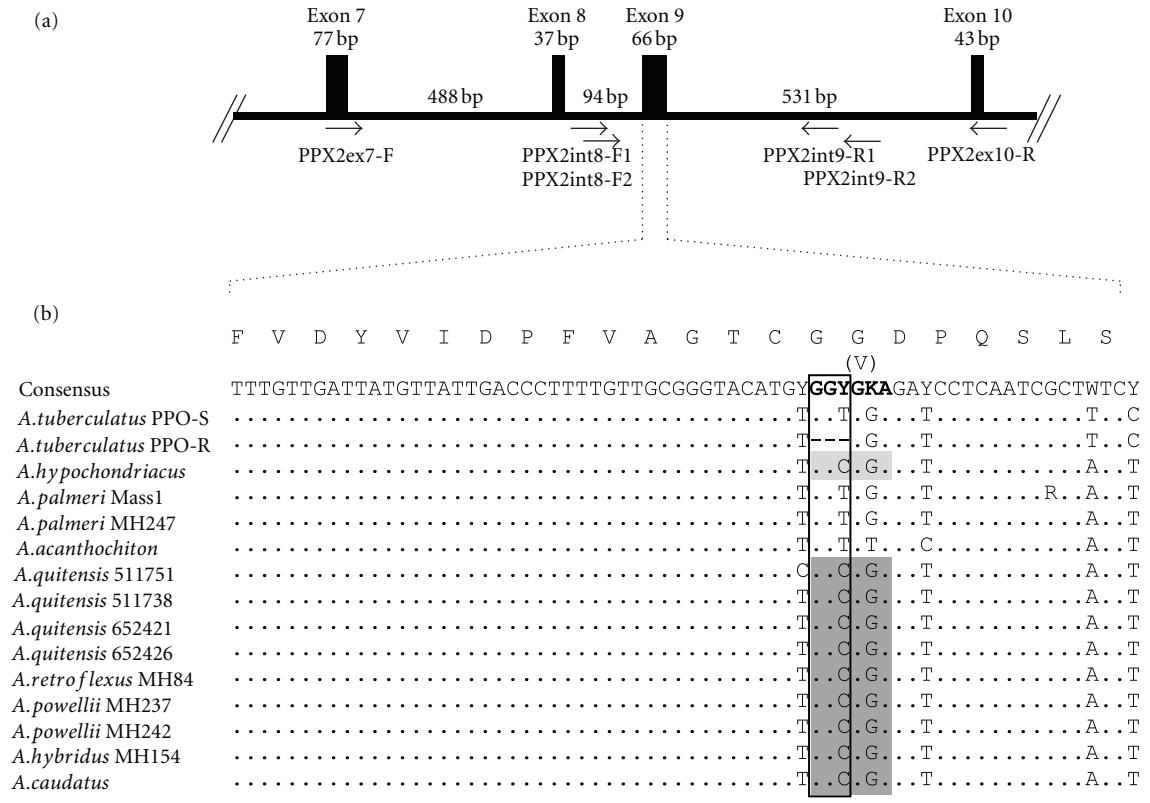

Figure 2: (a) Partial *PPX2* gene structure showing exon/intron positions and sizes. (b) Alignment of exon 9 from different *Amaranthus* species. The consensus nucleotide sequence and translated amino acid sequence are at the top. Conserved nucleotides are represented by dots. The boxed codon indicates the site of the Gly210 deletion mutation that is present in the PPO-resistant biotype of *A. tuberculatus* (GenBank DQ394876) and not in the wild-type (GenBank DQ394875) or any of the other species. Sequences that are cleaved in the PCR-RFLP assay are indicated by the shaded box which depicts the EciI recognition site. An inferred amino acid change of Gly211 to Val211 is also shown that results from a G to T nucleotide polymorphism in the Gly211 codon in the sequence of *A. acanthochiton*. The *PPX2* sequence of *A. hypochondriacus* is from GenBank (EU024569).

Based on sequence data from the first series of experiments, two additional primer sets were designed that are positioned in relatively conserved regions of the introns immediately flanking exon 9 (Figure 2(a)). The first primer set, *PPX2*int8-F1 and *PPX2*int9-R1, worked well for amplifying and sequencing exon 9 in all species except some accessions of *A. tuberculatus*. In this case, the modified primers *PPX2*int8-F2 and *PPX2*int9-R2 were successfully used. PCR experiments with both primer sets resulted in single amplified products of the expected size (~500 bp) in all accessions tested (data not shown). Results of the digestion analyses conducted with the different primer sets were in complete agreement with one another and with the sequencing evidence for every accession tested (Table 1). No infraspecific variation was observed in the PCR-RFLP fragment patterns for any species, although infraspecific variation was detected in *A. quitensis* after sequencing four randomly chosen accessions. In accession 511751 of this species, sampled from a Peruvian population, a silent T → C nucleotide mutation was observed in the codon immediately preceding Gly210, which in effect produced a bi-CGG sequence repeat spanning the resistance mutation site (Figure 2(b)). The position of this polymorphism has not been directly implicated in resistance to PPO herbicides, but it does open the possibility for an alternative repeat motif that could lead to the same Gly210 mutation.

Sequence comparisons among the remaining accessions, including wild-type and confirmed PPO-resistant *A. tuberculatus* (i.e., biotypes without and with the deletion mutation), revealed six additional SNPs in exon 9, but only one resulted in a nonsynonymous substitution (Figure 2(b)). This substitution occurred at position 47 in the sequence of *A. acanthochiton* (a dioecious species) and resulted in an inferred change of glycine to valine (amino acid position 211). Only one accession sequenced appeared heterozygous, and this too was from a sample of a dioecious species (i.e., *A. palmeri* Mass1). This polymorphism was in the third position of codon 215 (serine) and thus did not result in an amino acid change. These observations are not surprising as dioecious species are obligately outcrossing and thus expected to have higher levels of sequence polymorphisms compared to the primarily self-pollinated monoecious species [20, 21].

Nonetheless, additional sampling of these widespread and ecologically diverse species is needed to determine the extent of sequence variability present in natural populations.

Although the experimental results were not in conflict with one another, it is important to point out some limitations of the PCR-RFLP assay. This test cannot be used to screen plants for the same target-site deletion mutation responsible for PPO-inhibitor resistance in *A. tuberculatus*. For example, PCR-amplified products for both the wild-type and PPO-resistant biotypes of *A. tuberculatus* were uncut by EciI. However, mutated alleles can be detected using an allele-specific PCR method [9]. This allele-specific marker should theoretically work in other amaranth species (e.g., Palmer amaranth) that have the same repeat motif and, of course, the same deletion mechanism as operative in *A. tuberculatus*. On the other hand, supposing a similar codon deletion mutation occurred at the Gly210 site in an aberrant biotype of *Amaranthus* that normally possesses a cytosine SNP in the wild-type (*A. quitensis*, for example), then the PCR-RFLP test would be able to distinguish between the mutated and non-mutated alleles. There is a risk, however, of generating false positives or negatives due to other mutations within the EciI recognition site. Furthermore, the likelihood of mutations occurring outside the recognition site and endowing resistance must also be considered [22]. Even with these caveats, the PCR-RFLP assay is one more molecular tool that can be used in conjunction with other molecular markers to monitor the evolution of PPO-inhibitor resistance in weedy amaranths.

4. Conclusions

Even though resistance to PPO-inhibiting herbicides has been slow to evolve, it may be expected to occur in weedy species with large populations that are under strong and continuous selection pressure [23]. Inherently high levels of genetic variation also help to increase the likelihood that mutated alleles conferring resistance will be selected under strong herbicide pressure. Although *PPX2* variability was relatively low among the ten species investigated in this study, the results do show that two groups can be recognized: species with and species without a repeat motif. It is noteworthy that the *PPX2* gene of Palmer amaranth shared the same repeat motif as waterhemp, which suggests that Palmer amaranth will evolve resistance to PPO inhibitors via the Gly210 deletion mutation. Of course, the possibility of Palmer amaranth evolving another resistance mechanism (e.g., a different target-site mutation or a non-target-site mechanism) cannot be ruled out. In fact, preliminary evidence for a different target-site mutation in the *PPX2* gene of common ragweed was recently reported [24]. The occurrence of a second repeat pattern in *A. quitensis* is also noteworthy, especially since this species is one of the four known with PPO-inhibitor resistance. Further studies with resistant and sensitive biotypes of this species will be useful in linking sequence patterns with the likelihood of evolving PPO-inhibitor resistance. Finally, the information provided in this study will facilitate the use and further development of molecular markers for screening amaranth populations suspected of PPO-inhibitor resistance for target-site-based mechanisms.

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