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Distribution of the ΔG210 Protoporphyrinogen Oxidase Mutation in Illinois Waterhemp (*Amaranthus tuberculatus*) and an Improved Molecular Method for Detection

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Molecular assays are often implemented by weed scientists for detection of herbicide-resistant individuals; however, the utility of these assays can be limited if multiple mechanisms of evolved resistance exist. Waterhemp resistant to protoporphyrinogen oxidase (PPO)– inhibiting herbicides is conferred by a target-site mutation in *PPX2L* (a gene coding for PPO), resulting in the loss of a glycine at position 210 (ΔG210). This ΔG210 mutation of *PPX2L* is the only known mechanism responsible for PPO-inhibitor resistance (PPO-R) in waterhemp from five states (Illinois, Indiana, Iowa, Kansas, and Missouri); however, a limited number of populations have been tested, especially in Illinois. To verify the ubiquity of the ΔG210 in PPO-R waterhemp populations in Illinois, a previously published allele-specific PCR (asPCR) was used for the detection of the ΔG210 mutation to associate this mutation with phenotypic resistance in 94 Illinois waterhemp populations. The ΔG210 mutation was detected in all populations displaying phenotypic resistance to lactofen (220 g ai ha^{-1}), indicating the deletion is likely the only mechanism of resistance. With evidence that the ΔG210 mutation dominates PPO-R waterhemp biotypes, molecular detection techniques have considerable utility. Unfortunately, the previously published asPCR is time consuming, very sensitive to PCR conditions, and requires additional steps to eliminate the possibility of false negatives. To overcome these limitations, a streamlined molecular method using the TaqMan® technique was developed, utilizing allele-specific, fluorescent probes for high-throughput, robust discrimination of each allele (resistant and susceptible) at the 210th amino acid position of *PPX2L*.
Nomenclature: Lactofen; waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer (syn. *rudis*) AMATA.
Key words: PPO-inhibiting herbicides, herbicide resistance, genotyping, polymorphism, qPCR.

Weed scientists have made concerted research efforts to understand herbicide resistance mechanisms in light of the increasing occurrences of herbicide resistance (Neve 2007). Improving methods for detecting herbicide-resistant biotypes is a fundamental step toward those efforts. Molecular techniques allow for the rapid detection of herbicide resistance (Burgos et al. 2013; Délye et al. 2002); however, the usefulness of molecular techniques are limited when multiple mechanisms of resistance exist for a single herbicide site of action, especially if one or more mechanisms are not detectable via molecular techniques. Often these resistance mechanisms are due to non–target-site mechanisms, such as metabolism or exclusion-based mechanisms (Burgos et al. 2013).

Waterhemp has been a recurrent subject for weed science research because of its evolved resistance to many commercially available herbicides from various sites of action, including protoporphyrinogen oxidase (PPO)– inhibiting herbicides (Legleiter and Bradley 2008; Shoup et al. 2003). A codon deletion at the 210th amino acid position in the gene coding for the PPO enzyme (*PPX2L*) is currently the only known mechanism conferring resistance to PPO-inhibiting herbicides in waterhemp. This deletion results in a loss of a glycine residue at the 210th amino acid position (ΔG210), greatly reducing the ability of the herbicide to inhibit the PPO enzyme (Dayan et al. 2010). This polymorphism has been highly associated with phenotypic resistance to PPO-inhibiting herbicides in isolated waterhemp populations in Illinois, Iowa, Indiana, Kansas, and Missouri (Heap 2014; Lee et al. 2008; Schultz et al. 2015; Thinglum et al. 2011). The ΔG210 mutation has been identified in many Illinois waterhemp populations with suspected phenotypic resistance to PPO inhibitors; however, the presence of the mutation has not been directly associated with

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phenotypic resistance verified under controlled conditions (i.e., greenhouse). A systematic screening of multiple Illinois waterhemp populations for the ΔG210 mutation, corroborated with a phenotypic screen for resistance to PPO-inhibiting herbicides in the greenhouse, would provide considerable evidence that the ΔG210 mutation is likely the only mechanism present in Illinois. Therefore, the first objective of this research was to screen 94 Illinois waterhemp populations to confirm the association between the phenotypic resistance to PPO-inhibiting herbicides and the presence of the ΔG210 mutation of *PPX2L*.

Two methods have been developed for the detection of ΔG210 in waterhemp: asPCR and DNA sequencing (Lee et al. 2008; Patzoldt et al. 2006). The asPCR method is inexpensive, but requires visualization of the PCR product on agarose gel (Lee et al. 2008). Furthermore, in the method described by Lee et al. (2008), amplification only occurs in the presence of the mutation, necessitating an additional step to verify the presence of amplifiable DNA to eliminate false-negative results. This is often accomplished via spectroscopy or an additional PCR with the use of primers that flank the mutation of interest. The repeat nature of the sequence within and adjacent to the deleted codon compromises the discriminatory ability of the primers that can be designed; therefore, the asPCR method for the ΔG210 PPO mutation is very sensitive to PCR conditions and DNA quality and quantity. DNA sequencing provides an alternative to the asPCR; however, with current technology DNA sequencing is cost and time prohibitive for large genotyping experiments and requires the use of highly purified DNA extracts. Several genotyping methods (including TaqMan® assays) have an advantage over sequencing because they can utilize crude DNA extracts, allowing for a more rapid DNA extraction, thus providing greater efficiencies in time and expense (Giancola et al. 2006). Allowing for the use of crude DNA extracts is critical, given that DNA extraction is often listed as the limiting factor for high-throughput genotyping (Stein et al. 2001).

The TaqMan technique is a derivative of quantitative PCR (qPCR) applied often by plant breeders who require high-throughput capabilities, robust identification of molecular markers, and rapid turnover of results (Meksem et al. 2001). Primer and probe oligomers are designed like a standard qPCR, flanking and overlapping the region of interest, respectively; the TaqMan technique, however, uses two internal allele-specific probes. This allelic specificity allows for identification of the genotypes at the gene of interest (Giancola et al. 2006). Délye et al. (2002) utilized this technique to detect resistance to herbicides that inhibit acetyl coenzyme A carboxylase in blackgrass [*Alopecurus myosuroides* Huds.] and rigid ryegrass [*Lolium rigidum* Gaudin] with great success. For the purpose of genotyping waterhemp at the *PPX2L* locus, the TaqMan technique has a clear theoretical advantage: (1) results are delivered in real time, eliminating the need for gel electrophoresis and secondary verification of amplifiable DNA; (2) it is currently more cost effective than DNA sequencing; and (3) the permissible use of crude DNA extracts. Therefore, the second objective was conditional: if the ΔG210 mutation dominated Illinois waterhemp populations displaying phenotypic resistance to PPO inhibitors, a TaqMan-based technique was to be developed to enhance detection of the ΔG210 mutation in waterhemp.

Materials and Methods

Screening Illinois Waterhemp Populations for the ΔG210 Mutation of *PPX2L*. Waterhemp seed accessions were collected in 41 counties in Illinois (Figure 1) from 94 discrete waterhemp populations. Each population consisted of seeds combined from female waterhemp plants growing within a single field. Figure 1 highlights the Illinois counties that were sampled in gray, with the number of populations sampled within each county indicated on the map. Two sampling strategies for collecting PPO-inhibitor–resistant waterhemp populations were implemented: random and directed. Fields selected for random sampling were chosen based on the presence of waterhemp late in the growing season, likely because of the failure of glyphosate to control emerged plants in a glyphosate-based soybean weed management system. Fields targeted for directed sampling had a history of either conventional soybeans or PPO-inhibiting herbicide failure applied postemergence for control of waterhemp. As indicated on Figure 1, sampling primarily took place in the southern half of the state because of a greater frequency of waterhemp escapes from soybean weed management tactics in that geography. Among the 94 populations sampled, three positive and negative control populations were included for reference. The positive control populations were known to contain waterhemp with reduced sensitivity to PPO-inhibiting herbicides and were previously confirmed to express the ΔG210 mutation of *PPX2L*. The negative control populations have historically been controlled with the 1× field use rate of lactofen in field and greenhouse conditions.

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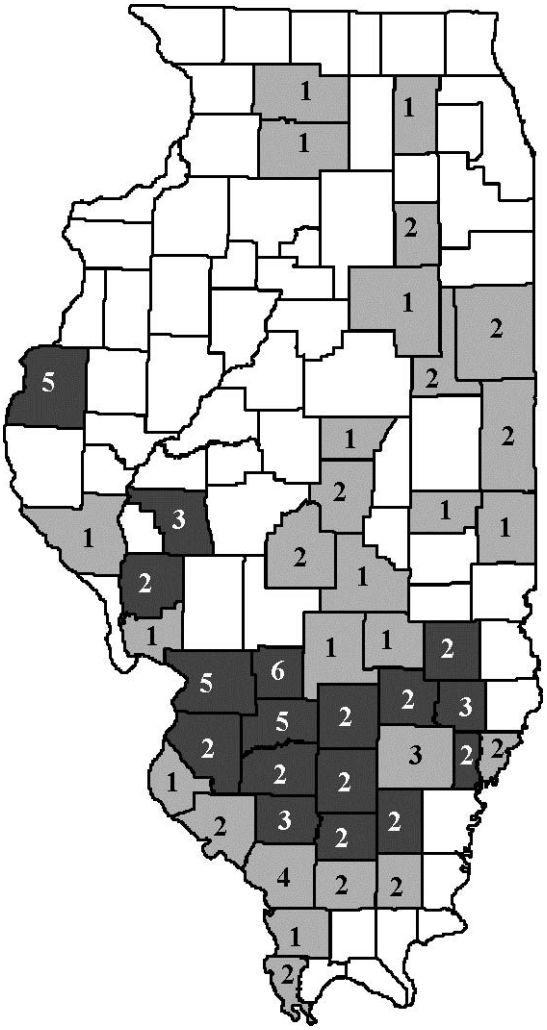

Figure 1. Survey geography and confirmation of waterhemp populations resistant to PPO inhibitors in Illinois by county, collected in fall 2006. Seed from waterhemp populations that had escaped management during the growing season, predominately soybean fields, were collected from all counties shaded in gray (light and dark). The number in each county indicates the number of waterhemp populations that were tested for resistance to PPO-inhibiting herbicides in a phenotypic greenhouse screen. Counties highlighted in dark gray contained waterhemp populations that were confirmed to be phenotypically resistant to PPO-inhibiting herbicides based on a greenhouse bioassay. All waterhemp populations confirmed to display phenotypic-resistance to PPO-inhibiting herbicides from the greenhouse bioassay contained the ΔG210 mutation of *PPX2L*.

Sixteen waterhemp plants were grown from each of the 94 populations in 10-cm^2 pots filled with growing media. Throughout the course of the experiment the greenhouse conditions were set to maintain an air temperature near 32 ± 5 C and a 16-h photoperiod with 430-W sodium lighting providing 250 μmol m^{-2} s^{-1} of supplemental photosynthetically active radiation. At a height of 8 to 12 cm, eight plants from each population were treated with 220 or 660 g ai ha^{-1} (1× and 3× field use rates, respectively) of lactofen (Cobra®, Valent USA, Walnut Creek, CA) plus 1% (v/v) crop oil concentrate (Prime Oil®, Winfield Solutions, LLC) with the use of a single-nozzle, CO_2 spray chamber equipped with an EVS8002 nozzle (TeeJet Technologies, Wheaton, IL) set to deliver 187 L ha^{-1} of carrier at 207 kPa of pressure. Visual herbicide efficacy ratings (0% = no efficacy; 100% = plant death) of individual plants were recorded 21 d after treatment (DAT). Special consideration was taken to make note of individuals with the least % of lactofen efficacy at 21 DAT. This process was necessary to identify individual plants within a population that may be resistant to PPO-inhibiting herbicides instead of using the pooled average of all plants within a population. Tissue was collected from surviving individuals to be genotyped with the use of the asPCR described by Lee et al. (2008) for the detection of ΔG210. The Lee et al. (2008) PCR was the only method available at the time the samples were genotyped and is an adequate method for the detection of ΔG210. During the course of the experiment plants were arranged in a randomized complete block design in the greenhouses at the Horticultural Research Center (HRC) of Southern Illinois University–Carbondale and the experiment was performed twice.

Method for the Robust Genotypic Determination of Waterhemp Resistant to PPO-Inhibiting Herbicides. The asPCR method described by Lee et al. (2008) has the limitation of only amplifying the allele responsible for resistance, Δ*PPX2L*, requiring additional effort to limit false negative samples through spectroscopy or additional PCR reactions. Lee et al. (2008) suggested that modifications to the asPCR could enhance its sensitivity and allow for quantitative measures. Therefore, the second objective was to develop a novel assay with the use of the TaqMan technique for the detection of waterhemp expressing the ΔG210 mutation. The experiments follow the precedent of Lee et al. (2008) by testing artificially bred samples with known genotypes, as well as several waterhemp populations known to be either resistant or susceptible to PPO inhibitors based on the phenotypic response to PPO-inhibiting herbicides.

Sequence data for waterhemp *PPX2L* was accessed through GenBank (http://www.ncbi.nlm.nih.gov/genbank/). TaqMan probes and primers were developed by ABI (Applied BioSystems Inc., Grand Island, NY) after submitting a portion of the *PPX2L* gene to the ABI on-line submission tool. Genomic DNA was extracted with the use of a modified cetyl

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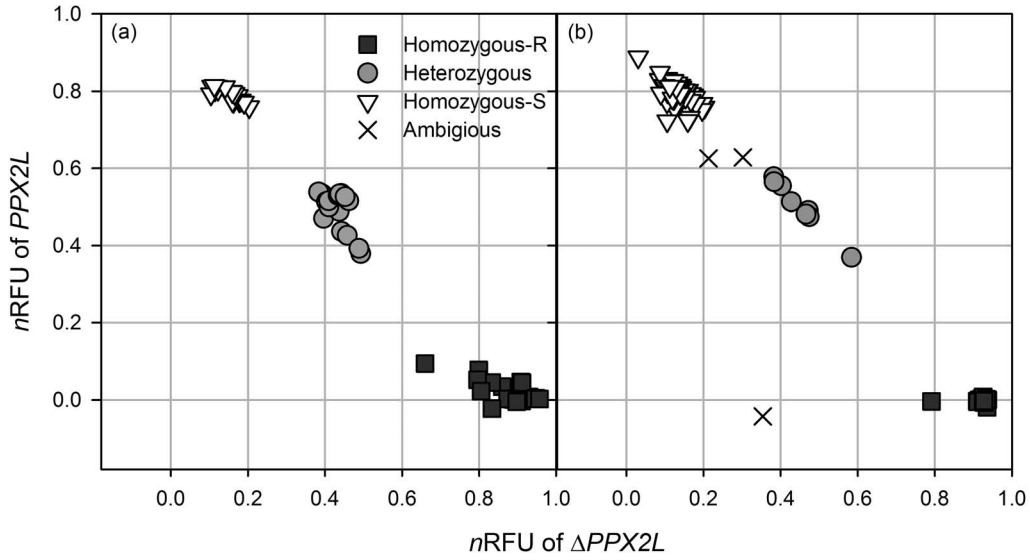

Figure 2. TaqMan® assay *n*RFU from populations with (a) known and (b) unknown genotypes. (a) Waterhemp was artificially crossed in the greenhouse to produce known homozygous-resistant (ACR), heterozygous (HET), and homozygous-susceptible (BRC) genotypes. These artificially bred individuals were then used to verify the accuracy of the TaqMan (10 biological replicates, two technical replicates for each genotype). (b) Waterhemp samples were grown from two PPO-inhibitor–resistant (Murphysboro and Carbondale) and two PPO-inhibitor–susceptible (Belleville and De Soto) populations for subsequent genotypic screening using the TaqMan assay (20 biological replicates).

trimethylammonium bromide (CTAB) method originally developed by Saghai-Maroof et al. (1984). A 20-µl reaction was prepared as per the recommendations of the manufacturer, with 4 µl of 5× GoTaq buffer, 2.4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of 20× TaqMan probes and primers, and 0.2 µl of GoFlexi Taq Polymerase (5 U μl^{-1}). The following primers were designed to flank the ΔG210 deletion of *PPX2L*: forward primer 5′-GCAGTTTGTTGATTATGTTATTGACCCTTTA-3′ (AHGJXAV_F) and reverse primer 5′-CAAGCACAGTATTTAACTCACGGAAA-3′ (AHGJXAV_R). The following probes were designed to overlap the ΔG210 deletion of *PPX2L*: 5′-TTGAGGATCTCCACCACATG-3′ (AHGJXAV_VIC) to detect the wild-type (*PPX2L)* and 5′-CGATTGAGGATCTCCACATG-3′ (AHGJXAV_FAM) to detect Δ*PPX2L*. Cycling conditions were as follows: 2 min at 95 C; 39 cycles of 95 C for 15 s, and 60 C for 1 min; followed by a plate read on every cycle. All reactions included three nontemplate controls (NTCs) and three controls from each genotype: homozygous-resistant (ACR), homozygous-susceptible (BRC), and heterozygous (HET). Sources for the controls are described below in the "Genotypic Confirmation of Waterhemp Samples with Known and Unknown Genotypes" section. Reactions were amplified with the use of a CFX384 RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA), which does not require the use of a passive reference dye (i.e., ROX). The Bio-Rad CFX Manager software (Bio-Rad Laboratories) was used for data analysis, which automatically reports the relative fluorescence units (RFU) of each allele. Data can also be expressed as normalized relative fluorescence units (*n*RFU) according to the equation developed by Livak et al. (1995):

$$n\mathrm{RFU}A1 = \mathrm{RFU}A1/[\mathrm{RFU}A1 + \mathrm{RFU}A2 + \mu(NTC)].$$

Genotypic Confirmation of Waterhemp Samples with Known and Unknown Genotypes. To test waterhemp with known genotypes, homozygous-resistant and heterozygous waterhemp plants were obtained by crossing ACR (Adams County Resistant) × ACR and WCS (Wayne County Sensitive) × ACR, respectively. Both the ACR and WCS populations were described by Patzoldt et al. (2006) in the original characterization of the ΔG210 mutation. The homozygous-susceptible accession came from a seed lot collected in 2002 from the Belleville Research Center (BRC), where PPO-inhibiting herbicides have remained highly efficacious. Sanger sequencing was performed (Genewiz Inc., North Plainfield, NJ) on three samples from the three seed accessions to confirm genotypes (Table 2). Prior to sequencing, genomic DNA was amplified with primers that flank the ΔG210 mutation (AmPPX2LwalkDR, 5′-ACAGCCTCCAGAAAATGTTG-3′ and AmPPX2LwalkDF, 5′-GAGAAAACACAATGC TACTGAA-3′) under

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Table 1. Number of waterhemp populations from directed and random sampling with confirmed presence of the ΔG210 mutation.

Sampling method	Waterhemp populations			
	Confirmed resistant[a]	Total sampled	Confirmed resistant[a]	FOR[b,c]
	No.		%	
Random	12	70	17	15 (4.1)
Directed	23	24	96	29 (3.3)
Total	35	94	37	–

[a] The % confirmed resistant was derived from the number of confirmed resistant populations divided by the total number of populations sampled, within each sampling method.

[b] Numbers within parentheses represent the ± standard error of the FOR. The FOR was calculated from populations with confirmed presence of the ΔG210 only. Populations considered phenotypically susceptible were excluded from the mean FOR.

[c] Abbreviation: FOR, frequency of resistance.

the reaction conditions described by Thinglum et al. (2011). Approximately 400 seeds of each genotype were planted into separate 25-cm^2 flats filled with growing media (Fafard Growing Mix 2, Conrad Fafard Inc., Agawam, MA) and plants were maintained at the HRC. Ten plants from each accession were sampled at the six to eight–leaf stage for DNA extraction, then genotyped with the use of the TaqMan assay previously described, with three controls of each genotype and three NTCs included in every plate.

Waterhemp is a genetically diverse species; therefore, confirmation of the assay's allelic discriminatory ability on several waterhemp populations was essential, as was done for the Lee et al. PCR (2008). Soil samples were collected from four sites: two fields containing waterhemp with phenotypic resistance to PPO-inhibiting herbicides, near Murphysboro and Carbondale, IL, and two sites containing waterhemp with phenotypic susceptibility to the latter herbicides, near Belleville and De Soto, IL. Soil samples were dried, crushed, and placed in a thin layer on top of planting media in a 25-cm^2 flat. Flats were maintained at HRC under the same greenhouse conditions listed above. Twenty samples from each population were collected and genotyped via the TaqMan assay with the use of previously described methods. To complement the TaqMan assay data of unknown samples, Sanger sequencing was performed on 12 randomly selected individuals from the two phenotypically resistant populations with the intent of capturing homozygous-resistant, heterozygous, and homozygous susceptible individuals from each population for comparison with TaqMan assay data.

Results and Discussion

Prevalence of the ΔG210 Mutation in Illinois Waterhemp Phenotypically Resistant to PPO-Inhibiting Herbicides. Of the 94 populations screened from collections made in fall 2006, 35 populations from 17 counties contained waterhemp with the ΔG210 mutation of *PPX2L* (Table 1). Among the populations sampled with the directed sampling strategy, 96% (23 of 24) contained the ΔG210 mutation (Table 1). Although populations screened with the directed strategy were specifically selected because PPO-inhibitor resistance was suspected, it is still remarkable that the ΔG210 mutation was identified in 96% of the suspected populations. Herbicides that inhibit the PPO enzyme may provide variable foliar herbicide efficacy when applied to large weeds (Wilson 2005); therefore, it was expected that herbicide failure in several of the sampled fields would be due to poor application techniques, not the presence of the target-site mutation. This finding may indicate that the distribution of waterhemp resistant to PPO-inhibiting herbicides is underestimated in fields where foliar-applied PPO-inhibiting herbicides failed to control waterhemp, given that growers commonly report poor control from herbicides within this site of action due to poor spray coverage, large plants, etc. (personal observation). Additionally, the estimated frequency of resistance (FOR) within each population was calculated based off of visual control ratings, where individuals were considered phenotypically resistant if control was less than or equal to 30 or 70% for the 1× and 3× rates of lactofen, respectively. Populations with random and directed sampling strategies had average FOR values of 15 and 29%, respectively, indicating that populations targeted for the detection of PPO-inhibitor resistance had nearly twice as many resistant individuals in the population on average when compared with waterhemp populations that were randomly sampled (Table 1). It must be noted that only 32 individuals were screened from each population (16 plants for each lactofen rate). Given that this is a relatively small sample size these values may underestimate the prevalence of waterhemp resistant to PPO-inhibiting herbicides, especially in populations with a low frequency of individuals resistant to PPO-inhibiting herbicides.

Herbicide efficacy values were assigned to each population and are presented as the "least % control," which represents the lowest visual control rating given to an individual within a given population, with respect to lactofen rate. This was done to target the least

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Table 2. Partial sequencing reads overlapping the ΔG210 mutation of *PPX2L*/Δ*PPX2L* in waterhemp with known and unknown genotypes versus genotypic determination via the TaqMan® assay.

Waterhemp genotype[a]	*n*RFU[b] R-allele	*n*RFU S-allele	Partial sequence of *PPX2L*/Δ*PPX2L*[c]
	*n*RFU		
Known homozygous resistant	0.997	0.003	5-**GGGTACATGTGGAGATCCTCAATC**-3
Unknown sample	0.977	0.013	5-**GGGTACATGTGGAGATCCTCAATC**-3
Unknown sample	1.000	0.000	5-**GGGTACATGTGGAGATCCTCANTC**-3
Known heterozygous	0.341	0.659	5-**GGGTACATGTGGAGAACATCCNCC**-3
Unknown sample	0.448	0.547	5-**GGGTACATGTGGAGAACATCNTCC**-3
Unknown sample	0.382	0.618	5-**GGGTACATGTGGAGAACATCNNCN**-3
Known homozygous susceptible	0.040	0.959	5-**GGGTACATGTGGTGGAGATCCTCA**-3
Unknown sample	0.208	0.786	5-**GGGTACATGTGGTGGAGATCCTCA**-3
Unknown sample	0.055	0.945	5-**GGGTACATGTGGTGGAGATCCTCA**-3

[a] Known homozygous-resistant, heterozygous, and homozygous-susceptible samples were taken from ACR, HET, and BRC populations, respectively. Unknown samples displayed were taken from the PPO-inhibitor-resistant waterhemp sites near Murphysboro and Carbondale, IL.

[b] Abbreviations: *n*RFU, normalized relative fluorescent units; R, resistant; S, susceptible.

[c] The "TGG" repeats within the polymorphic region are underlined when present. Nucleotides are in bold type when nucleotides from the heterozygous genotype match nucleotides in either homozygous genotype.

sensitive individuals in each population so those individuals could subsequently be screened for the presence of the ΔG210 mutation. The ΔG210 mutation of *PPX2L* causes resistance to PPO inhibitors in waterhemp (Patzoldt et al. 2006); therefore, screening susceptible individuals for the presence of ΔG210 prior to lactofen application was deemed unnecessary. The whole-plant bioassay of the three positive-control populations yielded least % control values of 5, 10, and 10%, which is similar to the previously reported control values for waterhemp expressing the ΔG210 mutation following foliar applications of lactofen at a 1× rate (220 g ai ha^{-1}) (Lee et al. 2008). Conversely, the three negative-control populations yielded least % control values of 98, 99, and 99% as expected. Populations with a least % control below 30 and 70% for 1× and 3× rates of lactofen, respectively, were evaluated for the presence of the ΔG210 in surviving plants using the Lee et al. (2008) asPCR. Results indicated that all populations containing individuals that were considered phenotypically resistant tested positive for the presence of the ΔG210, demonstrating the presence of the ΔG210 is highly associated with phenotypic resistance to PPO-inhibiting herbicides among the Illinois waterhemp populations tested.

TaqMan Assay Accuracy for the Detection of ΔG210. Given the strong association between the ΔG210 and PPO-inhibitor resistance in Illinois waterhemp populations, a TaqMan assay was developed to allow for rapid and robust detection of the causal mutation. Sanger sequencing data of waterhemp with known genotypes yielded results as expected: homozygous-susceptible individuals contained the 5′-TGGTGG-3′ repeat at the 209th and 210th amino acid position, whereas homozygous-resistant individuals lacked the repeat in the 210th amino acid position (Table 2). The sequence of heterozygous individuals had continuity with homozygous sequences until the 210th amino acid position, at which point the sequencing results either alternated nucleotides from either homozygous condition due to the presence of both alleles, or became ambiguous (Table 2). Furthermore, the Sanger sequencing trace files exhibited double peaks in the mismatched nucleotide positions downstream of ΔG210, a clear indication of the heterozygous condition (Supplemental Figure 1; http:dx.doi.org/10.1614/WS-D-15-00037.S1). The *n*RFU values derived from the TaqMan assay of samples with known genotypes were in their expected relative proportions based on the Sanger sequencing data (Table 2). The *n*RFU values of either allele, *PPX2L* or Δ*PPX2L*, was greater than 0.70 when detected in the homozygous conditions and between 0.35 and 0.60 when in the heterozygous condition (Table 2). Technical replicates returned values similar to the original values. As with the samples with known genotypes, TaqMan assay results for samples with unknown genotypes were in accordance with Sanger sequencing data. The TaqMan assay did not detect the Δ*PPX2L* allele in either of the waterhemp populations that were phenotypically susceptible to

PPO-inhibiting herbicides (Figure 2). Conversely, Δ*PPX2L* was encountered in the homozygous-resistant and heterozygous genotypes in populations with suspected PPO-inhibitor resistance. From a total of 80 unknown samples tested, less than 5% of the samples were ambiguous (Figure 2). Similarly, low numbers of ambiguous tests have been reported by others using the TaqMan assay for genotyping (Giancola et al. 2006).

In conclusion, these data support the assertion that the ΔG210 is currently the predominant, and likely the only, mechanism of resistance to PPO-inhibiting herbicides in waterhemp to date (Dayan et al. 2014, Schultz et al. 2015, Thinglum et al. 2011). With just one predominant resistance mechanism, utilization of the TaqMan assay described will provide robust and rapid detection of PPO-inhibitor resistance in waterhemp with high-throughput screening capabilities. Furthermore, the assay described has the sensitivity to detect the resistant and susceptible allele which will allow for future investigations of waterhemp heterozygous for PPO-inhibitor resistance. Given the increasing distribution of waterhemp resistant to PPO-inhibiting herbicides, weed scientists could adopt the methods described in this article for detection of resistance in their respective states to assist growers in making sound weed management decisions. This is especially the case in soybeans and cotton (*Gossypium hirsutum* L.), where herbicide options are limited and PPO-inhibiting herbicides are essential for management of *Amaranthus* species (Shoup et al. 2003; Sosnoskie and Culpepper 2014). With increasing use of PPO-inhibiting herbicides one cannot rule out the possibility that alternative resistance mechanisms have evolved or will evolve; therefore, continued caution must be taken when interpreting data generated from molecular assays. Although the assay described in this article is specific to the detection of waterhemp resistant to PPO-inhibiting herbicides, the TaqMan technique could expedite the detection of many target-site herbicide resistance mechanisms. With the ability to multiplex several sets of probes and primers into one reaction well, current and future genotyping efforts have the potential to be streamlined with the use of the technique described.

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