A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase

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Herbicides that act by inhibiting protoporphyrinogen oxidase (PPO) are widely used to control weeds in a variety of crops. The first weed to evolve resistance to PPO-inhibiting herbicides was *Amaranthus tuberculatus*, a problematic weed in the midwestern United States that previously had evolved multiple resistances to herbicides inhibiting two other target sites. Evaluation of a PPO-inhibitor-resistant *A. tuberculatus* biotype revealed that resistance was a (incompletely) dominant trait conferred by a single, nuclear gene. Three genes predicted to encode PPO were identified in *A. tuberculatus*. One gene from the resistant biotype, designated *PPX2L*, contained a codon deletion that was shown to confer resistance by complementation of a *hemG* mutant strain of *Escherichia coli* grown in the presence and absence of the PPO inhibitor lactofen. *PPX2L* is predicted to encode both plastid- and mitochondria-targeted PPO isoforms, allowing a mutation in a single gene to confer resistance to two herbicide target sites. Unique aspects of the resistance mechanism include an amino acid deletion, rather than a substitution, and the dual-targeting nature of the gene, which may explain why resistance to PPO inhibitors has been rare.

Amaranthus | evolution | waterhemp | weed resistance | herbicide resistance

A major concern with the use of herbicides for weed control is the selection of resistant populations. To date, over 300 different herbicide-resistant weed biotypes have been identified worldwide (www.weedscience.com). Numerous factors influence the likelihood of herbicide-resistance evolution in a weed population, and certain herbicides are more prone to resistance evolution than are others. For example, populations of 95 weed species have been reported with resistance to herbicides that inhibit acetolactate synthase, whereas evolved resistance to herbicides that inhibit protoporphyrinogen oxidase (PPO) has been reported for only three weeds (www.weedscience.com), even though these herbicides were first commercialized in the 1960s (1). The first weed to evolve resistance to PPO inhibitors was *Amaranthus tuberculatus* (waterhemp), an increasingly problematic weed of agronomic production systems throughout the midwestern United States (2–4).

PPO is the last common enzyme in the tetrapyrrole biosynthetic pathway that produces heme and chlorophyll (5). In plants, chlorophyll biosynthesis takes place exclusively in plastids, whereas heme is produced in both plastids and mitochondria (6, 7). In both organelles, PPO converts protoporphyrinogen IX (protogen IX) to protoporphyrin IX (proto IX) (8). Two different nuclear genes, *PPX1* and *PPX2*, encode plastid and mitochondrial PPO isozymes, respectively (9, 10). When susceptible plants are treated with PPO inhibitors, the substrate of PPO, protogen IX, accumulates and is exported from the organelles into the cytoplasm (11) where herbicide-insensitive peroxidase-like enzymes in the plasma membrane convert it to proto IX (12). Proto IX accumulates in the cytoplasm and, in the presence of light, induces the formation of singlet oxygen that is damaging to cell membranes (13).

Although PPO-inhibitor-resistant plants have been generated through genetic engineering (14–20), *A. tuberculatus* populations have evolved resistance from the repeated use of these herbicides in agronomic production systems. The consequence of *A. tuberculatus* evolving resistance to PPO inhibitors, combined with its already widespread resistance to acetolactate synthase-inhibiting herbicides, is that the only remaining chemical option for its control following emergence in *Glycine max* (soybean) production systems is glyphosate, which requires the planting of glyphosate-resistant varieties (4). Although the molecular mechanisms of evolved resistance to many herbicides have been identified, such a mechanism has not yet been elucidated for resistance to PPO inhibitors.

Results

Inheritance of PPO Inhibitor Resistance. To characterize the resistance mechanism, plants from a PPO-inhibitor-resistant (R) *A. tuberculatus* biotype were reciprocally crossed with wild-type [herbicide-susceptible (S)] plants to create F_1 lines, followed by subsequent crossing to generate F_2 and backcross (BC) lines. In response to the PPO inhibitor lactofen, the resultant *A. tuberculatus* lines segregated for resistance in ratios similar to those expected for a single genetic unit of inheritance (Table 1). Furthermore, plants from lines that were homozygous or heterozygous for resistance survived 53-fold or 31-fold higher doses of lactofen, respectively, when compared with S plants (Fig. 1 and Table 2 and Fig. 6, which are published as supporting information on the PNAS web site). Thus, resistance to lactofen was inherited as a single, incompletely dominant gene.

Molecular Characterization of *PPX* Genes. cDNA sequences that encode PPO isozymes were obtained from R and S *A. tuberculatus* plants, but with unexpected results. From S plants, cDNA sequences for *PPX1*, *PPX2*, and a longer version of *PPX2*, *PPX2L*, were identified (GenBank accession nos. DQ386112, DQ386113, and DQ386114, respectively) (Fig. 7, which is published as supporting information on the PNAS web site). Comparison of translated sequences of *PPX2* and *PPX2L* indicated that they shared 98% amino acid identity, with the exception of a 30-aa extension in the 5′ end that was unique to *PPX2L*. This extension is predicted to encode a signaling sequence for plastid import (21). Thus, the *PPX2L* gene isolated from *A. tuberculatus* likely encodes both plastid- and mitochondria-targeted PPO isoforms due to the presence of alternate in-frame initiation codons, a phenomenon that was reported previously for *Spinacia*

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Abbreviations: PPO, protoporphyrinogen oxidase; BC, backcross; gDNA, genomic DNA; ai, active ingredient; COC, crop oil concentrate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ386112–DQ386118, DQ394875, and DQ394876).

See Commentary on page 12215.

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Table 1. Inheritance of resistance to the PPO inhibitor lactofen in *A. tuberculatus*

Male parent	Female parent	*N*	No. observed R	No. observed S	Expected ratio (R:S)	χ^2	*P* value
F_1(R)	F_1(R)	400	297	103	3:1	0.120	0.7290
	S	200	98	102	1:1	0.080	0.7772
	R	200	200	0	1:0	0	1
F_1(S)	F_1(S)	400	304	96	3:1	0.213	0.6441
	S	200	109	91	1:1	1.620	0.2030
	R	200	200	0	1:0	0	1

F_1 plants were obtained from reciprocal crosses between a resistant (R) and a sensitive (S) biotype [F_1(R), female parent was R; F_1(S), female parent was S]. Plants from the F_2 and BC lines were treated with lactofen at 110 g ai·ha^{-1} plus 1% (vol/vol) COC and scored as R or S 15 days after treatment. The expected segregation ratio of R to S responses assumes a single genetic unit of inheritance.

oleracea (spinach) *PPX2* (10). In comparison, *PPX1* shared 26% and 25% amino acid identity with *PPX2* and *PPX2L*, respectively, and thus is an evolutionarily distinct isozyme. From R plants, only *PPX1* and *PPX2L* genes (GenBank accession nos. DQ386115 and DQ386116, respectively) were identified on the basis of cDNA sequencing. To confirm the lack of *PPX2* identification in R plants, Southern blot analysis was performed by using genomic DNA (gDNA) probed with a fragment of *PPX2L*. Probing with the fragment of *PPX2L* identified two major bands (presumably *PPX2* and *PPX2L* loci) from S plants but only a single major band (presumably the *PPX2L* locus) from R plants, thus confirming the results obtained from sequencing efforts (Fig. 2).

To determine whether *PPX1* or *PPX2L* mediated PPO inhibitor resistance, PCR-based molecular markers were used to follow the inheritance of alleles of these two genes in *A. tuberculatus* lines segregating 1:1 for R or S responses to lactofen. The molecular marker for *PPX2L* was significantly correlated with lactofen responses ($P < 0.0001$), whereas the marker for *PPX1* was not ($P = 0.4278$) (Fig. 3). In other words, plants were resistant to lactofen only if they inherited the *PPX2L* allele from the R parent. Results of molecular marker studies focused further efforts toward differences among *PPX2L* alleles.

Inspection of the inferred amino acid sequences of *PPX2L* among S and R plants revealed two amino acid polymorphisms

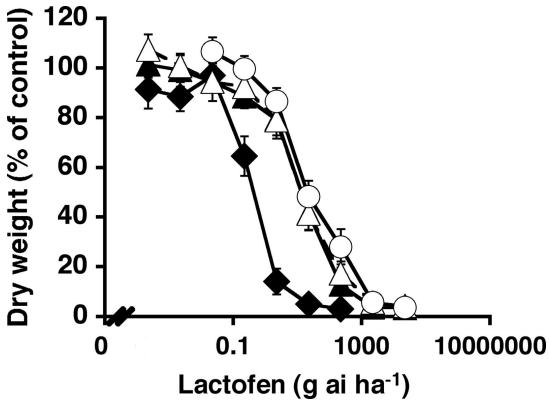

Fig. 1. Dose–response curves of different *A. tuberculatus* lines to the PPO inhibitor lactofen. Lactofen was foliar-applied to greenhouse-grown plants from the S (filled diamonds), R (open circles), F_1(R) (open triangles), or F_1(S) (filled triangles) *A. tuberculatus* lines. Data were collected 15 days after treatment. Vertical bars represent ± SEM ($n = 12$).

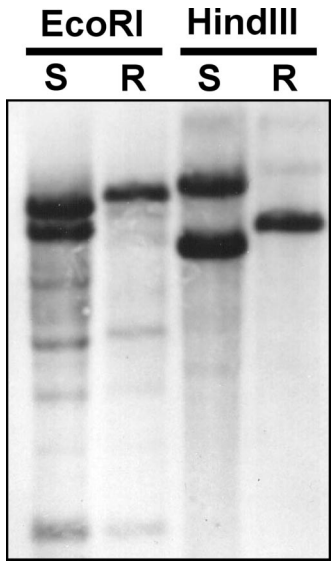

Fig. 2. Southern blot of *A. tuberculatus* gDNA probed with a fragment of *PPX2L*. DNA was isolated from plants that were derived from the S or R biotype and digested with EcoRI or HindIII.

that were correlated with resistance. In an attempt to identify only a single amino acid polymorphism, additional R and S plants were sequenced from independently identified *A. tuberculatus* biotypes (GenBank accession nos. DQ386117 and DQ386118). Sequencing results and subsequent comparisons identified three additional amino acid polymorphisms (five total); however, only one, a glycine deletion at position 210 (ΔG210), was consistently polymorphic between all R and S plants analyzed (Fig. 8, which is published as supporting information on the PNAS web site). *PPX2L* also was sequenced by using gDNA as a template (GenBank accession nos. DQ394875 and DQ394876 for S and R plants, respectively) to further confirm the existence of the 3-bp deletion corresponding to the G210 codon. Alignment of gDNA and cDNA sequences of *PPX2L* identified the codon corresponding to the G210 residue in the ninth exon when starting

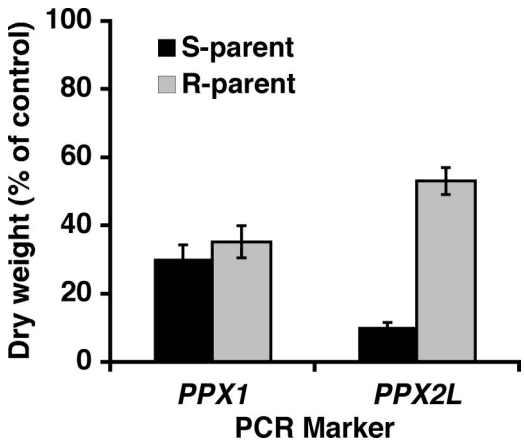

Fig. 3. PCR-based molecular marker analysis of *PPX1* or *PPX2L* alleles. *A. tuberculatus* plants used in the study were derived from F_1 hybrids backcrossed to the S parent (BC_S). Markers were used to determine whether the F_1-derived pollen carried an S or R parental allele. BC_S plants were treated with lactofen at 110 g ai·ha^{-1} plus 1% (vol/vol) COC and harvested 15 days after treatment. Vertical bars represent ± SEM (*PPX1*, $n = 42$ or 40 for S or R parental alleles, respectively; *PPX2L*, $n = 39$ or 49 for S or R parental alleles, respectively).

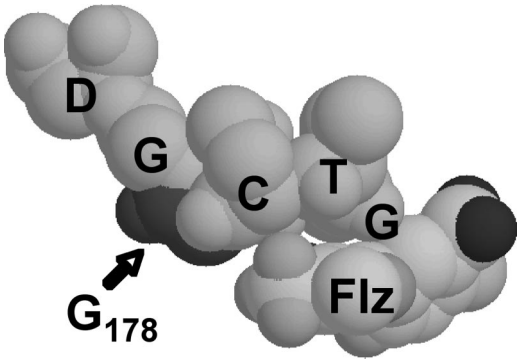

Fig. 4. Selected amino acid residues of *N. tabacum* PPO2 in proximity to the herbicide-binding site. *A. tuberculatus* plants resistant to PPO inhibitors are missing a glycine residue equivalent to G178 of *N. tabacum*. This amino acid deletion is predicted to hinder PPO inhibitor binding. Amino acid residues: D, aspartic acid; G, glycine; C, cysteine; and T, threonine. PPO-inhibiting herbicide: Flz, fluazolate.

from the 5′ end (Fig. 9, which is published as supporting information on the PNAS web site). The 3-bp deletion was also identified in *PPX2L* gDNA sequences of R plants, indicating that the ΔG210 mutation in PPO2L was not the result of an error introduced during mRNA processing.

The ΔG210 mutation was also assessed by using the resolved protein structure of PPO2 from *Nicotiana tabacum* (tobacco) as a reference (22, 23). The equivalent amino acid to G210 of *A. tuberculatus* PPO2L (G178 of *N. tabacum* PPO) was located near the herbicide-binding site, thus supporting the prediction that the G210 deletion was responsible for herbicide resistance (Fig. 4).

Functional Complementation. Complementation assays used a *hemG* (PPO) mutant strain of *Escherichia coli*, SASX38, (24) to assess the effect of the G210 deletion toward herbicide responses. The SASX38 strain grows very slowly unless supplied with exogenous heme or rescued with an alternative source of PPO. Furthermore, because wild-type *E. coli* is naturally tolerant to PPO inhibitors, use of the SASX38 strain enabled a relatively direct assay for herbicide sensitivity of the S and putative R PPO2Ls from *A. tuberculatus* (25, 26). The SASX38 *E. coli* strain was transformed with plasmid constructs encoding PPO2L proteins that differed only in the presence/absence of G210. Both constructs were able to rescue growth of the SASX38 *E. coli* strain, thus indicating that both *PPX2L* genes encoded functional proteins (Fig. 5). However, supplementation of the growth medium with lactofen dramatically inhibited growth of *E. coli* transformed with the wild-type *PPX2L* but not *E. coli* transformed with the ΔG210 *PPX2L* (Fig. 5). Thus, the 3-bp deletion in *PPX2L* resulting in deletion of a glycine at position 210 of PPO2L was sufficient to confer resistance to lactofen.

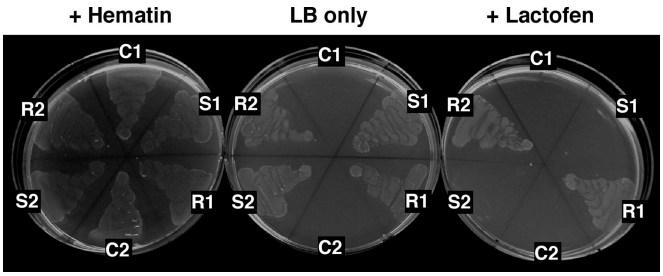

Fig. 5. PPO expression in a *hemG* mutant strain of *E. coli*. *E. coli* were grown on LB medium alone or supplemented with hematin (20 $\mu g \cdot ml^{-1}$) or lactofen (100 nM). *E. coli* isolates were as follows: C1 and C2, nontransformed controls; S1 and S2, transformed with a vector encoding *A. tuberculatus*-derived PPO2L with glycine at position 210; and R1 and R2, transformed with a vector encoding identical PPO2L with the exception of a deletion of glycine at position 210.

Discussion

Seven different mechanisms of PPO inhibitor resistance have been proposed for plants (27). Two of these mechanisms include either enhanced metabolic degradation of the herbicide or an alteration of the herbicide target site, which together constitute the majority of mechanisms for herbicide resistance in weed species. Of these, an altered herbicide target enzyme (PPO) was investigated, based on previous characterization of R *A. tuberculatus* plants (4). It was later determined, in an independently identified PPO-inhibitor-resistant *A. tuberculatus* population, that enhanced metabolism was not responsible for resistance (28).

The mechanism of PPO inhibitor resistance that was selected within natural populations of *A. tuberculatus* was a codon deletion in a gene encoding PPO. Although alterations of herbicide target proteins are common mechanisms for conferring resistance, several characteristics of this specific mechanism merit highlighting. First, PPO inhibitors have two herbicide target sites in plants [i.e., in plastids and in mitochondria (8)]; therefore, in order for target-site resistance to occur, two altered genes would need to be selected. However, *A. tuberculatus* plants have overcome this obstacle by means of mutation in a single gene (*PPX2L*) that is predicted to encode both plastidic and mitochondrial PPO isoforms. Second, the specific alteration of PPO2L that confers resistance to PPO-inhibiting herbicides is an amino acid deletion, rather than a substitution, resulting from a 3-bp deletion in the gDNA. Although intentional selection for resistance to PPO inhibitors identified amino acid substitutions that conferred resistance (20, 29), the codon-deletion approach revealed by *A. tuberculatus* is instructive of an alternative approach to achieve resistance. Third, the R biotype was found to be resistant to multiple chemical families of PPO inhibitors, albeit at different levels (4), indicating that the ΔG210 mutation confers resistance to all PPO inhibitors. Finally, the fact that R *A. tuberculatus* plants lacked one of the PPO genes (*PPX2*) found in plants from the S biotype is curious and requires further research. However, the absence of *PPX2* in the R biotype likely is not related to the resistance phenotype, inasmuch as (*i*) resistance was (incompletely) dominant and exhibited single-locus inheritance, (*ii*) *PPX2L* cosegregated with resistance, and (*iii*) the ΔG210 mutation was sufficient to confer lactofen insensitivity.

Although the origin of the G210 codon deletion of *PPX2L* identified in the R *A. tuberculatus* biotype is uncertain, nucleotide length polymorphisms are not uncommon in this plant species. Codon insertion/deletions (indels) among populations of *A. tuberculatus* were previously identified in other genes encoding herbicide target proteins [e.g., *ALS* (acetolactate synthase) and *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase)] (W.L.P and P.J.T., unpublished results). Furthermore, other indels in addition to the G210 indel were found among *PPX* genes in this study. In *PPX1* (GenBank accession nos. DQ386112 and DQ386115), there were two additional, adjacent proline codons in the nucleotide sequence from R plants relative to S plants. A codon indel was also identified when *PPX2* was compared with *PPX2L* from S plants (GenBank accession nos. DQ386113 and DQ386114). This indel, like the G210 polymorphism between R and S *PPX2L*s, also resulted in a glycine amino acid indel but it was located at a different position (128 nucleotides downstream of the G210 codon). The codon indels observed in *A. tuberculatus* typically are associated with short, simple sequence repeats (SSRs). The G210 indel in *PPX2L* is

part of a bi-GTG repeat (or a bi-TGG repeat); the *PPX2*/*PPX2L* indel is part of a tri-GGA repeat; and the *PPX1* indel is part of a hexa-CCT repeat. Because of their high mutability, SSRs are recognized as a means for providing adaptive genetic variation for evolutionary processes (30). Although the numbers of repeats associated with some of the *PPX* indels are fewer than typically recognized for SSRs, that the indels are found within repeated nucleotides suggests a means for their evolutionary origin.

In regard to PPO-inhibitor-resistant *A. tuberculatus* in agroecosystems, resistance can be transmitted both maternally and paternally and therefore is able to spread through seed dispersal or, more rapidly, by wind dispersal of pollen. Because *A. tuberculatus* is a dioecious plant, it is forced to outcross. This obligate outcrossing, combined with a significant level of resistance that is expressed in the heterozygous state (Fig. 1), will make pollen a very effective means for dissemination of the resistance. In addition to dissemination from a single "source" population, resistance to PPO inhibitors could become more widespread in *A. tuberculatus* populations by independent selection events. In fact, it seems likely that PPO-inhibitor-resistant populations already have evolved independently, given the distinct locations where they have been identified (2–4) and the different *PPX2L* alleles containing the ΔG210 mutation identified in this study (Fig. 8).

A. tuberculatus is one of the most problematic weeds in agronomic fields throughout the midwestern United States. In particular, the propensity of *A. tuberculatus* to rapidly evolve herbicide resistance makes its management difficult (4). The herbicide resistance mechanism reported herein illustrates the sophisticated means by which *A. tuberculatus* can adapt and evolve in response to weed control efforts. With the loss of PPO inhibitors as an effective *A. tuberculatus* management tool in soybean production, farmers will become even more reliant on glyphosate.

Materials and Methods

Detailed procedures for the generation and analysis of *A. tuberculatus* lines and for herbicide dose–response experiments and degree-of-dominance calculations are given in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

***A. tuberculatus* Biotypes.** The R biotype used in this study was derived from an *A. tuberculatus* population originally collected in Adams County, Illinois, and confirmed resistant to PPO-, acetolactate synthase-, and photosystem II-inhibiting herbicides (4). The S biotype was collected in Wayne County, Illinois, and was identified in previous experiments to be susceptible to all herbicides tested (31). *A. tuberculatus* plants derived from the original Adams County population that were PPO-inhibitor-susceptible (S-BioAC), and those from a PPO-inhibitor-resistant biotype collected in Clinton County, Illinois (R-BioCC), were used for sequencing of *PPX2L* alleles only.

Plant Culture. *A. tuberculatus* seeds for each experiment were sown in flats (surface area = 930 cm^2) containing a 1:1:1 mixture of soil:peat:sand. Seedlings for each experiment were transplanted into square plastic pots containing 800 ml of soil plus 0.2% (vol/vol) 14-14-14 Nutricote (Agrivert, New York, NY) when the plants were ≈1 cm in height. Plants were grown in a greenhouse maintained at 28/22°C day/night, with supplemental light (minimum of 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux at the plant canopy) provided by mercury halide and sodium vapor lamps programmed for a 16-hr photoperiod.

Herbicide Applications. Herbicide treatments were applied with a compressed-air, moving-nozzle laboratory sprayer equipped with an 80° flat fan nozzle (Teejet; Spraying Systems Co., Wheaton, IL) delivering 187 liters·ha^{-1} of water at 207 kPa. The nozzle was maintained ≈45 cm above the plant canopy. Plants were returned to the greenhouse immediately after herbicide treatment. All foliar-applied herbicide treatments were made when the *A. tuberculatus* plants were 10–12 cm in height.

Generation of F_1, F_2, and BC Lines. To create F_1 lines, *A. tuberculatus* plants from the R biotype were crossed with plants from the S biotype. F_1 lines were created in which the maternal parent was either S [F_1(S)] or R [F_1(R)]. After maturity, seeds were harvested from each female individually as full-sib lines. F_1 male plants were crossed with female plants from the S biotype, R biotype, or F_1 full-sibs to create BC_S, BC_R, or F_2 lines, respectively. Separate crosses were conducted using males from F_1(S) or F_1(R) lines. Each genetic combination was conducted twice with new *A. tuberculatus* plants, thus constituting a complete replication of the experiment. Crosses were conducted in growth chambers maintained at 28/22°C day/night with fluorescent and incandescent bulbs providing 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux at the plant canopy, programmed for a 16-hr photoperiod.

Evaluation of F_2 and BC Lines. Inheritance of PPO inhibitor resistance was determined by evaluating R or S responses of plants from F_2 and BC lines 15 days after treatment with lactofen at 110 grams of active ingredient per hectare (g ai·ha^{-1}) plus 1% (vol/vol) crop oil concentrate (COC). From each F_2 or BC line, 50 plants from each cross (including replicated crosses) were assessed in a completely randomized design. The entire experiment was conducted twice, with a total of 100 plants being assessed from each cross. Responses for each cross were subjected to χ^2 analysis to determine whether the responses were due to a single genetic unit of inheritance. Because we observed no differences among replications of the same cross, data obtained from similar crosses were combined.

cDNA Sequencing. Total RNA was isolated by using young leaf tissue from a single plant from each of the R and S biotypes (32), followed by purification of mRNA (Promega, Madison, WI). Purified mRNA was used to obtain full-length sequences of *PPX1* or *PPX2* by using 5′ and 3′ RACE (Invitrogen, Carlsbad, CA). Primers were designed based on conserved regions of nucleotide sequences of *PPX1* or *PPX2* from numerous plant species (9, 10, 33). Sequencing of the resultant fragments facilitated the design of gene-specific primers for *A. tuberculatus PPX1* and *PPX2* that were used to obtain their full-length sequences. Total RNA was individually isolated from three *A. tuberculatus* plants each of the R or S biotypes and used to create cDNA in reactions with reverse transcriptase (Invitrogen). PCR was used to amplify *PPX1*, *PPX2*, or *PPX2L* with the following primers: *PPX1*, forward 5′-GAGAGAGTGCGAGAGAGATGAG-3′ and reverse 5′-CAAGATGCTGGAGCCCTATTGAC-3′; *PPX2*, forward 5′-GCCATCGCCATTGTCAGTTTAC-3′ and reverse 5′-GAATTACGCGGTCTTCTCATCCAT-3′; and *PPX2L*, forward 5′-GACAAAATTGGATTCAGAATTTAGC-3′ and reverse 5′-GAATTACGCGGTCTTCTCATCCAT-3′. PCRs contained 1 μl of cDNA; 400 nM each of forward and reverse primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1.5 mM $MgCl_2$; and 1.0 unit of high-fidelity *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN) with a 1× concentration of supplied buffer in a final volume of 25 μl. The reactions were subjected to a 3-min incubation at 95°C; 35 cycles of 0.5 min at 95°C, 1 min at 58°C, and 1.5 min at 72°C; and then 5 min at 72°C. Resultant PCR products were isolated by gel electrophoresis, sequenced (34), and compared by using both Sequencher 4.1 and online software (35). Because sequences among plants from the same biotypes were similar,

only a single sequence is presented for each gene/biotype combination.

Southern Blot Analysis. gDNA was isolated from young leaves of *A. tuberculatus* plants from the S or R biotypes (36). PPO inhibitor responses of each plant were confirmed by treatment with lactofen at 175 g ai·ha^{-1} plus 1% (vol/vol) COC. Samples were prepared by digesting 7.5 μg of gDNA with 100 units of either EcoRI or HindIII to completion, followed by separation in a 1% (wt/wt) agarose gel, and then were transferred to a nylon membrane (Roche Molecular Biochemicals). The membrane was probed with a digoxigenin-labeled (Roche Molecular Biochemicals) PCR fragment of *PPX2L* amplified from gDNA isolated from a single S plant. Hybridization and probe detection were performed in accordance with the manufacturer's instructions.

PCR-Based Molecular Markers. Inheritance of *PPX1* and *PPX2L* alleles in BC_S progeny was studied by treating plants with lactofen at 110 g ai·ha^{-1} plus 1% (vol/vol) COC. Prior to lactofen applications, tissue samples were obtained from each plant to isolate DNA (37). PCR-based molecular markers were used to identify the parental origin (R or S) of the *PPX* alleles contributed by the F_1 male to the BC_S progeny. To differentiate R or S *PPX1* alleles, a fragment of genomic *PPX1* was amplified by PCR using the forward primer 5′-TGATAAGTCGCTCAATGGAGA-3′ and reverse primer 5′-AGATTTGTAGCACCTCCAATG-3′, followed by BspDI digestion to identify S alleles (i.e., S *PPX1* alleles contain a recognition sequence for BspDI, whereas R alleles do not). To identify parent-specific *PPX2L* alleles, a fragment of genomic *PPX2L* was amplified by PCR using the forward primer 5′-AAGAGACCTCTTGAGGGCTTC-3′ and the reverse primer 5′-GAATTACGCGGTCTTCTCATCCAT-3′, followed by TfiI digestion to identify S alleles (i.e., S *PPX2L* alleles contain a recognition sequence for TfiI, whereas R alleles do not). PCRs contained 40 ng of total DNA; 400 nM each of forward and reverse primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 2.0 mM $MgCl_2$; and 1 unit of *Taq* polymerase (Invitrogen) with a 1× concentration of supplied buffer in a final volume of 20 μl. The reactions were subjected to a 3-min incubation at 95°C; 40 cycles of 0.5 min at 95°C, 1 min at 60°C or 64°C (for reactions with *PPX1* or *PPX2L* primers, respectively), and 1.5 min at 72°C; and then 5 min at 72°C. After PCR amplification, a mixture containing 0.5 unit of the appropriate restriction enzyme with a 1× concentration of supplied buffer in a final volume of 10 μl was added to each reaction. Digests with BspDI were incubated at 37°C for 4 hr; digests with TfiI were incubated at 65°C for 2 hr. PCR products were fractionated in a 1% (wt/wt) agarose gel containing 0.5 μg·ml^{-1} ethidium bromide and were visualized with UV light.

***PPX2L* gDNA Sequencing.** gDNA was isolated from leaf tissue of S or R plants (37) to sequence a portion of genomic *PPX2L*. Primers were designed that flanked the G210 codon of PP02L; then, subsequent sequencing of amplified fragments facilitated the design of new primers until the exon containing the G210 codon was identified. Primer sets (A–D, forward and reverse), starting with the largest fragment, were as follows: A, 5′-GCCATCGCCATTGTCAGTTTAC-3′ and 5′-GGAGCAGTGACAACCACAGCATCA-3′; B, 5′-ATCGATGATCTTGGGCTTCGTG-3′ and 5′-AATGGTAAGGAGTCGCACCAAC-3′; C, 5′-CTTCAAATCCCGCTGCACTA-3′ and 5′-TACTTCTGGAAATGTATGG-3′; and D, 5′-GAGAAAACACAATGCTACTGAA-3′ and 5′-ACAGCCTCCAGAAAATGTTG-3′. PCR amplification, sequencing, and analysis were performed similar to the method used for cDNA sequencing of *PPX* genes.

Functional Complementation. A shortened version of *PPX2L* from the S *A. tuberculatus* biotype was cloned into a pBAD-TOPO expression vector (Invitrogen) so that translation began at the second ATG start codon (+91). *PPX2L* cDNA was PCR-amplified by using the forward primer 5′-CAGGAATAAGTAATGGGCAACATTTCTGAG-3′, containing both a ribosome binding site (AGGA) and an ATG start codon, and the reverse primer 5′-GAAGAATTACGCGGTCTTCTCATC-3′ containing a stop codon. To create putative PPO-inhibitor R and S plasmids that would encode proteins differing only in the presence/absence of G210, *PPX2L* was PCR-amplified from multiple cDNA samples and a region of the gene encompassing an ≈500-bp XhoI/DraIII fragment was sequenced. The 3-bp polymorphism corresponding to the ΔG210 mutation was within this XhoI/DraIII fragment. Two XhoI/DraIII fragments were identified that were identical except for the presence/absence of the G210 codon and a C/T nucleotide polymorphism that was in the third position of a serine codon (and therefore did not alter the encoded protein). These two fragments were each used to replace the corresponding fragment in the pBAD-TOPO *PPX2L* construct. The region encompassing the replaced fragment was sequenced from the two resulting constructs to confirm the existence of the 3-bp polymorphism and that no other polymorphisms were created during the cloning process. Susceptible and putative R PPO plasmids were used to transform a *hemG* mutant strain of *E. coli*, SASX38 (24), kindly provided by Harry Dailey (University of Georgia, Athens, GA). The SASX38 *E. coli* strain was maintained on LB medium supplemented with 20 μg·ml^{-1} hematin. Transformation-competent *E. coli* were prepared by using $CaCl_2$ (38). Transformed colonies of SASX38, and non-transformed controls, were tested for their ability to grow on LB medium alone or supplemented with 20 μg·ml^{-1} hematin or with the PPO inhibitor lactofen ranging from 0.01 to 100 μM, and incubated at 37°C for 14 hr.

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