Cytochrome P450 CYP81A12 and CYP81A21 Are Associated with Resistance to Two Acetolactate Synthase Inhibitors in *Echinochloa phyllopogon*[1][W]

Satoshi Iwakami, Masaki Endo, Hiroaki Saika, Junichi Okuno, Naoki Nakamura, Masao Yokoyama, Hiroaki Watanabe, Seiichi Toki, Akira Uchino*, and Tatsuya Inamura

Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606–8502, Japan (S.I., T.I.); Plant Genome Engineering Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba 305–8602, Japan (M.E., H.S., S.T.); The Japan Association for Advancement of Phyto-Regulators, Ushiku 300–1211, Japan (J.O., N.N., M.Y.); and Crop Production Systems Division, NARO Agricultural Research Center, Tsukuba 305–8666, Japan (H.W., A.U.)

Previous studies have demonstrated multiple herbicide resistance in California populations of *Echinochloa phyllopogon*, a noxious weed in rice (*Oryza sativa*) fields. It was suggested that the resistance to two classes of acetolactate synthase-inhibiting herbicides, bensulfuron-methyl (BSM) and penoxsulam (PX), may be caused by enhanced activities of herbicide-metabolizing cytochrome P450. We investigated BSM metabolism in the resistant (R) and susceptible (S) lines of *E. phyllopogon*, which were originally collected from different areas in California. R plants metabolized BSM through *O*-demethylation more rapidly than S plants. Based on available information about BSM tolerance in rice, we isolated and analyzed *P450* genes of the *CYP81A* subfamily in *E. phyllopogon*. Two genes, *CYP81A12* and *CYP81A21*, were more actively transcribed in R plants compared with S plants. Transgenic Arabidopsis (*Arabidopsis thaliana*) expressing either of the two genes survived in media containing BSM or PX at levels at which the wild type stopped growing. Segregation of resistances in the F2 generation from crosses of R and S plants suggested that the resistance to BSM and PX were each under the control of a single regulatory element. In F6 recombinant inbred lines, BSM and PX resistances cosegregated with increased transcript levels of *CYP81A12* and *CYP81A21*. Heterologously produced CYP81A12 and CYP81A21 proteins in yeast (*Saccharomyces cerevisiae*) metabolized BSM through *O*-demethylation. Our results suggest that overexpression of the two *P450* genes confers resistance to two classes of acetolactate synthase inhibitors to *E. phyllopogon*. The overexpression of the two genes could be regulated simultaneously by a single trans-acting element in the R line of *E. phyllopogon*.

Herbicides have become essential tools in modern agriculture for efficient weed control. Meanwhile, herbicide resistance has evolved through persistent herbicide selection exerted on huge weed populations across vast areas. The mechanisms responsible for herbicide resistance are generally grouped into two categories: target site resistance and nontarget site resistance (Yuan et al., 2007). Target site resistance, which is caused by alterations in the protein(s) that are targeted by the herbicide, accounts for a large majority of the reported cases of herbicide resistance (Powles and Yu, 2010). A single nucleotide polymorphism in a target site-encoding gene is the most frequently observed alteration, causing a structural change in the target site and reducing the ability of herbicides to interact with the target sites. Overproduction of target site proteins is another mechanism for target site resistance, as recently disclosed in glyphosate resistance (Gaines et al., 2010; Tranel et al., 2011; Salas et al., 2012). In these cases, amplification of target site-encoding genes results in overproduction of target site proteins. The molecular mechanisms of target site resistance are relatively easily analyzed, as most herbicides target specific enzymes (Yuan et al., 2007). Also, management of target site resistance can be readily achieved because resistant (R) plants can be controlled by herbicides with different modes of action.

A much less studied but more threatening mechanism is nontarget site resistance. Most nontarget site resistances are associated with enhanced metabolism and impaired translocation (Powles and Yu, 2010; Délye, 2013; Yu and Powles, 2014). In contrast to target site resistance, nontarget site resistances tend to affect multiple herbicides of different chemical classes and different modes of action (Délye, 2013), although they can be limited to several herbicides with the same mode of action (e.g. Jeffers et al., 1996; Iwakami et al., 2014b). Threatening aspects of nontarget site resistance thus are the unexpected resistances to other herbicides (Preston,

[1] This work was supported by the Pesticide Science Society of Japan (grant to S.I.) and the Japan Association for Advancement of Phyto-Regulators (grant to A.U.).

* Address correspondence to uchino@affrc.go.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Akira Uchino (uchino@affrc.go.jp).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.113.232843

2004). However, little is known about the molecular mechanisms of nontarget site resistance. Elucidating these mechanisms, including the identification of the molecular players involved, has been the leading challenge in herbicide resistance research (Powles and Yu, 2010; Délye, 2013).

Echinochloa phyllopogon, also known as *Echinochloa oryzicola*, is an allotetraploid ($2n = 4x = 36$) and predominantly self-pollinated grass species in the Panicoideae subfamily (Yamasue, 2001). *E. phyllopogon* is a noxious weed in rice (*Oryza sativa*) fields, and severe infestations cause large decreases in rice yield. In the late 1990s, R populations were found in the Sacramento Valley of California (Fischer et al., 2000a). R plants exhibited resistance to at least nine herbicides from seven different chemical groups: sulfonylurea, triazolopyrimidine, pyrimidinyl carboxy herbicides, aryloxyphenoxypropionate, thiocarbamate, quinclorac, and clomazone (Fischer et al., 2000b; Osuna et al., 2002; Ruiz-Santaella et al., 2006; Bakkali et al., 2007; Yasuor et al., 2009, 2010, 2012). Today, there seems to be only one herbicide left to control this weed in California (Yasuor et al., 2012). Thus, elucidating the molecular mechanism of resistance is urgently required.

The multiple herbicide-resistant *E. phyllopogon* exhibits resistance to bensulfuron-methyl (BSM), a sulfonylurea herbicide, and penoxsulam (PX), a triazolopyrimidine herbicide (Osuna et al., 2002; Yasuor et al., 2009), both of which are absorbed via roots (PX can also be absorbed via shoots) and inhibit acetolactate synthase (ALS), a key enzyme for the biosynthesis of the branched-chain amino acids Val, Leu, and Ile (Duggleby, 2005). Sensitivity of the ALS enzyme to BSM and PX did not significantly differ between R and susceptible (S) plants (Osuna et al., 2002; Yasuor et al., 2009), and the nucleotide sequences encoding ALS were identical in the R and S lines, lacking amino acid substitutions known to confer resistance (Iwakami et al., 2012). The BSM and PX resistances were shown to be reduced by cytochrome P450 inhibitors in R plants (Osuna et al., 2002; Yasuor et al., 2009). Also, the P450 inhibitors reduced PX metabolism in R plants to the level of that in S plants (Yasuor et al., 2009). These observations suggested that nontarget site resistance mediated by enhanced activities of P450s is involved in the mechanism of BSM and PX resistance.

P450s are a group of heme-thiolate monooxygenases that catalyze a wide variety of monooxygenation/hydroxylation reactions (Bak et al., 2011). Hundreds of *P450* genes exist in plant genomes, and each P450 participates in various biochemical pathways to produce primary and secondary metabolites (Mizutani and Ohta, 2010). Several herbicide-metabolizing P450s have been identified in a number of plant species (Siminszky, 2006). In rice, BSM is mainly metabolized through *O*-demethylation of the methoxy group at position 4 of the pyrimidine ring (Takeda et al., 1986). Purified rice microsomes catalyzed this reaction (Deng and Hatzios, 2002), and a *CYP81A6* knockout mutant of rice was susceptible to BSM (Pan et al., 2006). Therefore, P450 activity of CYP81A6 appears to catalyze the *O*-demethylation of BSM, although direct evidence for the reaction has yet to be reported. The CYP81A subfamily was found in *E. phyllopogon* as well as other species of the Panicoideae (Nelson, 2009; Iwakami et al., 2014a) but is not conserved in all plants (Nelson, 2009). Considering the putative BSM metabolizing function of the rice P450 and also the high level of BSM resistance in *E. phyllopogon* populations (Osuna et al., 2002; also our preliminary results), we scrutinized BSM resistance as a first step to the elucidation of multiple herbicide resistance of *E. phyllopogon*. This seemed a promising approach, although BSM resistance is not a significant practical problem, as BSM is not used to control *E. phyllopogon* because of its rather low herbicidal activity against *Echinochloa* spp. at the recommended dose in rice fields. In addition, we also studied PX resistance in *E. phyllopogon* as another type of ALS inhibitor resistance related to P450 activity; PX is frequently used to control *Echinochloa* spp. in rice fields. We characterized P450s of the CYP81A subfamily in *E. phyllopogon* and found that two *CYP81A P450* genes are associated with BSM and PX resistance in *E. phyllopogon*. This study provides a molecular basis for the understanding of the multiple herbicide resistance in *E. phyllopogon*, as well as of nontarget site resistance in weeds in general.

RESULTS

BSM Metabolism in *E. phyllopogon*

To test if BSM metabolism in the R line of *E. phyllopogon* is more active than in the S line and if the metabolic pathway is the same as in rice, we compared the amounts of BSM and *O*-demethylated BSM between the R and S lines of *E. phyllopogon* treated with BSM for 24 h. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detected BSM and *O*-demethylated metabolites in shoots and roots of R and S lines (Fig. 1). In roots, the amount of BSM in the R line was one-half that detected in the S line, while that of *O*-demethylated BSM was 2-fold higher. In shoots, the amounts of BSM and *O*-demethylated BSM were less than one-tenth of those in roots. Together, these data suggest that BSM was metabolized primarily in the roots of *E. phyllopogon* and that it was metabolized more rapidly in the R line via the same metabolic pathway as in rice.

The CYP81A Subfamily in *E. phyllopogon*

We isolated seven *CYP81A* genes from the R line of *E. phyllopogon*, in addition to the five *CYP81A* genes previously described (Iwakami et al., 2014a; Supplemental Fig. S1). The seven genes were named by the Cytochrome P450 Nomenclature Committee (Dr. David Nelson, University of Tennessee Health Science Center). Three *CYP81A* genes, *CYP81A13P*, *CYP81A19P*, and *CYP81A25P*, are pseudogenes encoding truncated proteins and therefore were not analyzed further. Comparison of the other nine

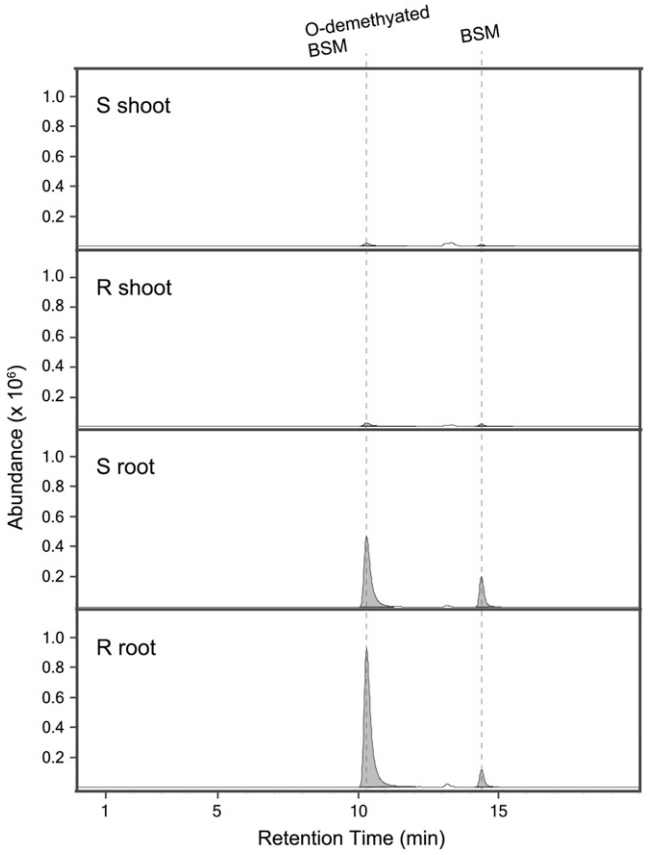

Figure 1. LC-MS/MS analyses of a BSM metabolite formed in the shoots and roots of R and S lines of *E. phyllopogon*. Roots of seedlings at the second leaf stage were treated with 10 μM BSM for 24 h before analysis.

genes between the R and S lines revealed nucleotide polymorphisms in four genes, *CYP81A12*, *CYP81A21*, *CYP81A22*, and *CYP81A26*. In all of them except for *CYP81A12*, at least one nonsynonymous substitution was found (Supplemental Fig. S2). Two nonsynonymous substitutions in CYP81A22 were predicted to be within the substrate recognition site, a key region that may influence the catalytic functions of P450s (Gotoh, 1992).

Transcript Levels of *CYP81A* Genes in R and S *E. phyllopogon*

Among the nine *CYP81A* genes, *CYP81A12* and *CYP81A21* transcripts were particularly abundant in shoots and roots of R line seedlings at the second leaf stage, both in BSM-treated and untreated plants (Fig. 2). The transcript levels differed between the lines by factors of 4 or more. The expression of these genes was not influenced by BSM treatment, and they were constitutively overexpressed in the R line. *CYP81A22* expression also was higher in roots of the R line than in those of the S line in BSM-treated as well as untreated plants. Transcript levels of *CYP81A22* in the shoots tended to be higher in the BSM-treated condition, although the differences were not significant statistically. The other genes did not exhibit higher transcription levels in the R line; they were more actively transcribed in the S line (*CYP81A14* and *CYP81A15*) or at similar levels in both lines.

We investigated the expression profiles of the genes with higher expression levels in the R line (*CYP81A12*, *CYP81A21*, and *CYP81A22*) in more detail, differentiating between shoots and roots at different developmental stages, etiolated shoots, and spikelets (Supplemental Fig. S3). The transcription of the three genes was significantly higher in the R line in all organs investigated, except for *CYP81A22* in shoots at the third-leaf stage, where the observed difference was not significant statistically.

Susceptibility of Transgenic Arabidopsis to BSM and PX

CYP81A12, *CYP81A21*, and *CYP81A22*, which had exhibited high transcript abundance in the R line, were introduced into Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia-0) under the control of the *Cauliflower mosaic virus* 35S promoter. For *CYP81A21* and *CYP81A22*, R as well as S line alleles were introduced because the alleles showed an amino acid polymorphism.

Five homozygous lines were selected for *CYP81A12* expression (Fig. 3A). Transcript accumulation levels of the transgene were quite different between the five lines, and BSM susceptibility also differed (Fig. 3C). The resistance indices of 12R-19 and 12R-21, defined as the factor by which the herbicide concentration inducing 50% growth reduction (GR_{50}) differed from the concentration required in the wild type, was more than 1,000. The BSM susceptibility was highly correlated to the transcription level of the introduced *P450* gene in the *CYP81A12* transgenic lines (Supplemental Fig. S4); lines with lower susceptibility corresponded to the lines with higher expression of *CYP81A12*. Thus, *CYP81A12* conferred a decrease in BSM susceptibility to transgenic Arabidopsis depending on its transcript abundance.

For *CYP81A21*, four homozygous lines each were selected for the R line allele and the S line allele (Fig. 3B). These lines accumulated various levels of the *CYP81A21* transcript. BSM susceptibilities of each line differed greatly, and four lines (21R-6, 21R-9, 21S-9, and 21S-20) had resistance indices greater than 1,000; in other words, their GR_{50} values were more than 1,000-fold higher than the wild-type value (Fig. 3D). The susceptibility to BSM was highly correlated to the transgene expression, similar to the *CYP81A12* transgenic lines discussed above (Fig. 3, B and D; Supplemental Fig. S4), and was independent of the inserted allele (Fig. 3, D and F; Supplemental Fig. S5). Thus, both alleles of *CYP81A21* conferred a decrease in BSM susceptibility to transgenic Arabidopsis that increased with the transcript level.

Transgenic Arabidopsis expressing the R line allele of *CYP81A22* did not exhibit a significant decrease in

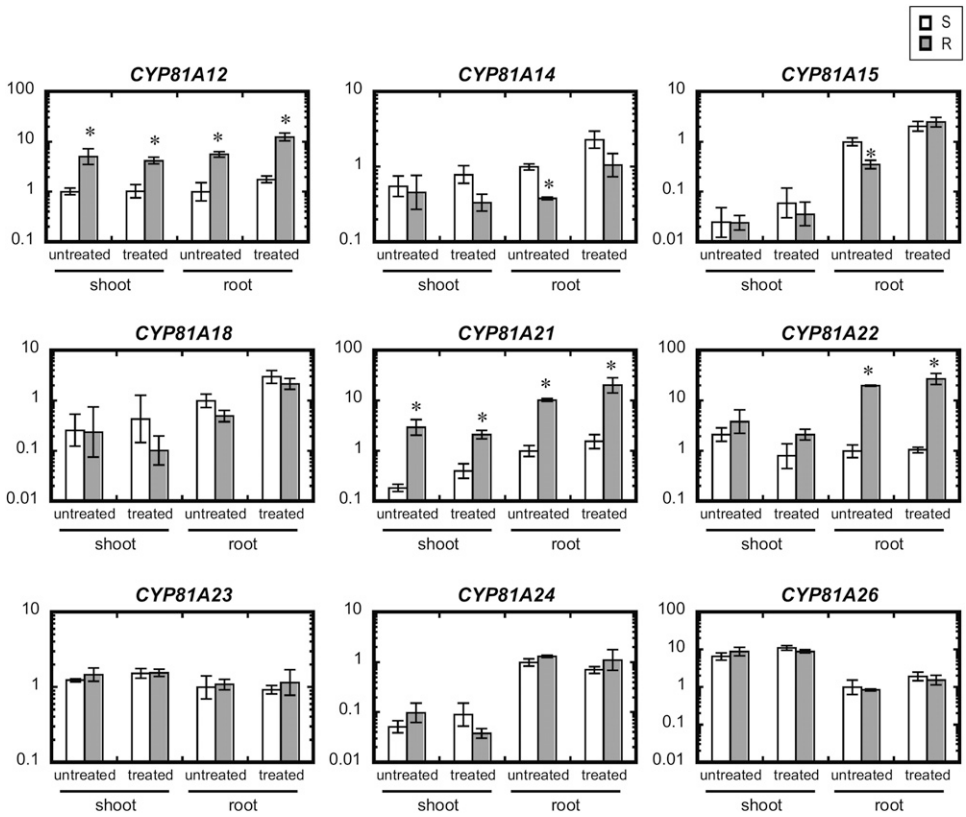

Figure 2. Transcript levels of *P450* genes in the shoots and roots of R and S lines of *E. phyllopogon*. Roots of seedlings at the second-leaf stage were treated with 10 μM BSM; control plants remained untreated. Transcript levels were compared between R and S lines at 24 h after BSM treatment by real-time RT-PCR using *EIF4B* and *RPII* as internal control genes. Transcript abundance was normalized to the level in the root of the untreated S line. Data shown are means ± SD of four biological replicates (Student's *t* test, $*P < 0.01$).

BSM susceptibility, in contrast to Arabidopsis expressing *CYP81A12* or *CYP81A21* (data not shown). Similarly, the S line allele of *CYP81A22* did not confer significantly decreased BSM susceptibility (data not shown).

We also examined the PX susceptibilities of the transgenic lines expressing either of *CYP81A12*, *CYP81A21*, and *CYP81A22*. The *CYP81A12*- and *CYP81A21*-expressing lines with low BSM susceptibility also exhibited low PX susceptibility, although the effect was less pronounced (Fig. 3, G and H). For example, in line 21R-6, the resistance index was 10 for PX compared with 4,000 for BSM (Fig. 3F; Supplemental Figs. S5 and S6). A correlation between mRNA accumulation and decreasing PX susceptibility was observed, similarly as with the BSM response (Fig. 3B; Supplemental Fig. S6). The lines with low transgene expression and relatively high BSM susceptibility (e.g. lines 12R-14 and 21R-4) did not show prominent decreases in PX susceptibility (data not shown). In lines expressing *CYP81A22*, no significant change in PX susceptibility was observed (data not shown).

Promoter Regions and Copy Numbers of *CYP81A12* and *CYP81A21*

The putative promoter sequences of *CYP81A12* and *CYP81A21* were characterized over approximately 2 kb upstream from the transcription start site. The 300-bp region upstream from the ATG starting codon was extremely similar in the two genes. Large differences between the R and S lines were not observed in the putative promoter regions, although the promoters of the R line alleles of *CYP81A12* and *CYP81A21* carried two single nucleotide polymorphisms and two insertions, respectively (Fig. 4A).

The copy numbers of *CYP81A12* and *CYP81A21* were surveyed in a genomic DNA-blot analysis using gene-specific probes designed from the regions around the 5′-untranslated region and the promoter sequences (Fig. 4A). The band patterns were not different between the R and S lines for *Sac*I and *Sph*I digestions; single bands of the same size were observed in both lines (Fig. 4B). The signal intensity of the detected bands for the two genes was similar between the R and S lines. These results indicate that the copy number of *CYP81A12* and *CYP81A21* was one in both the R and S lines.

Segregation Analyses of Herbicide Susceptibilities in R and S *E. phyllopogon*

Herbicide susceptibilities of R and S plants from Californian populations of *E. phyllopogon* were reported to differ by at least more than 25-fold for BSM and 5- to 9-fold for PX (Osuna et al., 2002; Yasuor et al., 2009). In our evaluation of plants growing on Murashige and Skoog media, the GR_{50} of BSM was estimated to be 0.65 and 716 μM for the S and R lines, respectively (Supplemental Fig. S7, A and B). Thus, the resistance index for BSM was more than 1,100, which is not inconsistent with the

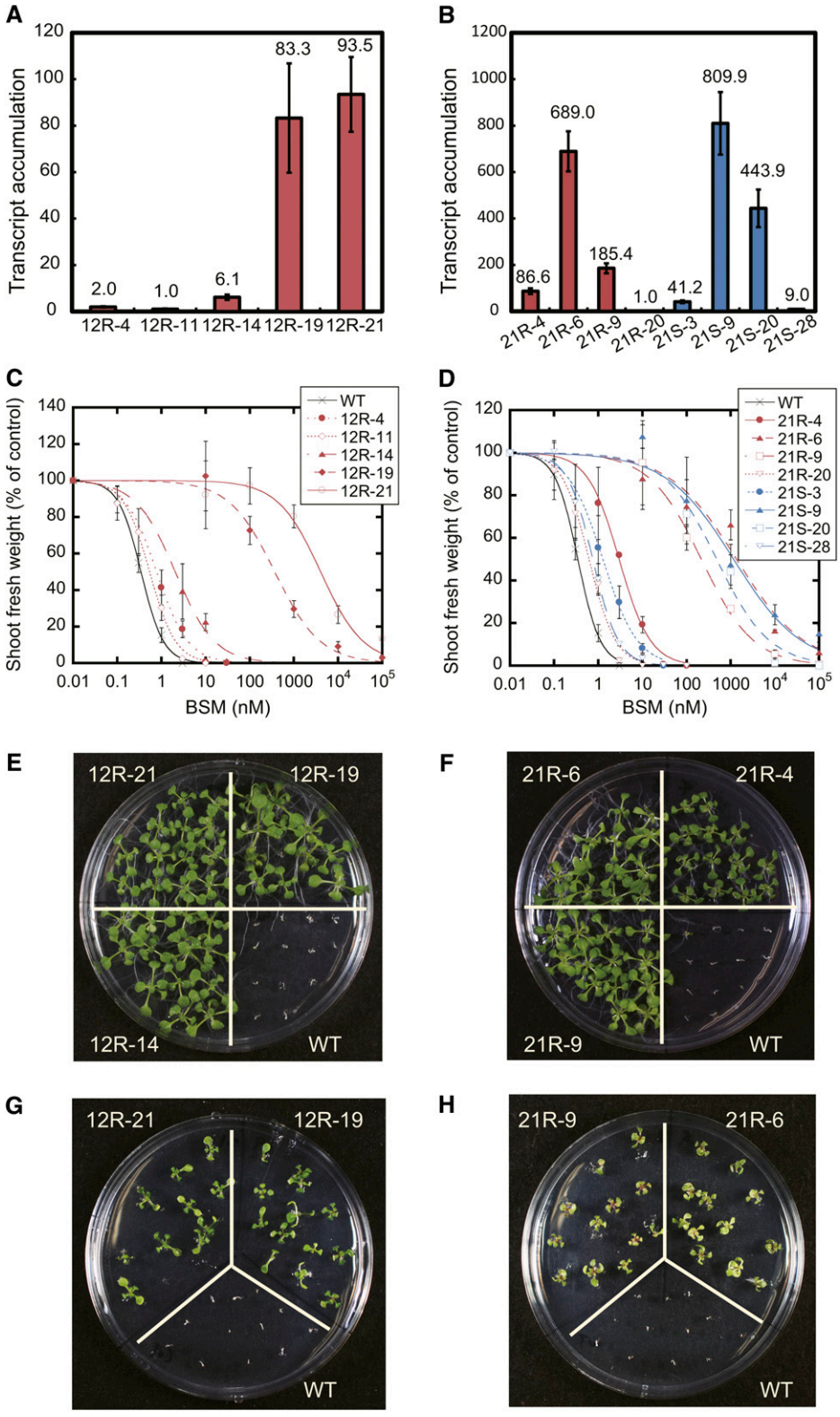

Figure 3. Transcript levels of transgenes and susceptibilities to BSM and PX in transgenic Arabidopsis expressing *CYP81A12* and *CYP81A21*. 12R represents transgenic Arabidopsis with the *CYP81A12* allele of an R line of *E. phyllopogon*. 21R and 21S represent transgenic Arabidopsis with the *CYP81A21* alleles of an R and S line of *E. phyllopogon*, respectively. A and B, mRNA levels of the transgene in independent transgenic lines. The transcript levels of *CYP81A12* and *CYP81A21* were quantified by real-time RT-PCR using *GAPDH* as an internal control gene. Data are expressed as values relative to 12R-11 (*CYP81A12*) and 21R-20 (*CYP81A21*). C and D, BSM susceptibility of independent transgenic lines. Susceptibilities were evaluated by relative growth (shoot fresh weight) on Murashige and Skoog media containing BSM. Bars represent SD (n = 4). E and F, Seedlings grown for 12 d on Murashige and Skoog media with 3 nM BSM. G and H, Seedlings grown for 12 d on Murashige and Skoog media with 10 nM PX.

previous report (at least more than 25-fold) that did not test high herbicide doses and therefore could not estimate GR_{50} accurately. On the other hand, the resistance index for PX, 6.2, was similar to published values (Supplemental Fig. S7, C and D).

Inheritance of BSM and PX resistance was assessed in the progeny of crosses between R and S plants. Resistance segregated in the F2 population on media containing 10 μM BSM or 0.3 μM PX, concentrations on which the S lines stopped growing at the first-leaf

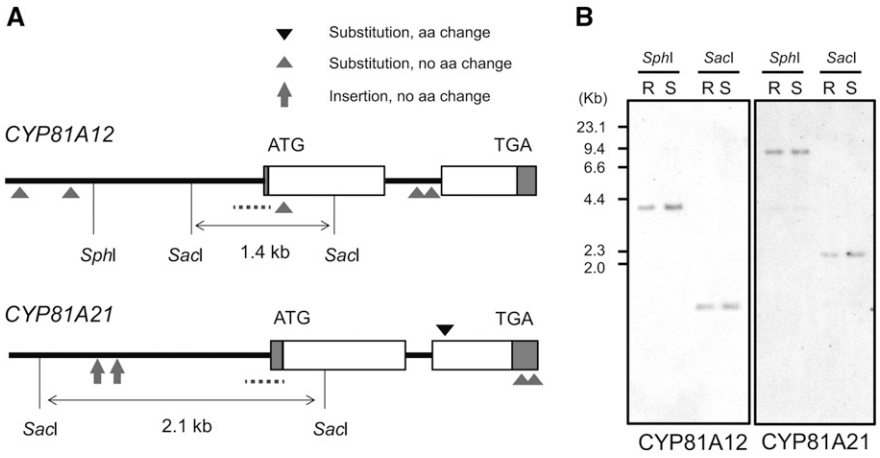

Figure 4. Molecular analysis of the *CYP81A12* and *CYP81A21* loci in R and S lines of *E. phyllopogon*. A, Schematic structure of *CYP81A12* and *CYP81A21* of the R and S lines. White and gray boxes represent coding regions and untranslated regions, respectively. *Sph*I and *Sac*I restriction sites, the translation start codon (ATG), and translation stop codon (TGA) are indicated. Dotted bars represent the positions of probes for genomic DNA-blot analysis. Triangles indicate the positions where DNA polymorphisms were observed. Arrows mark insertions observed in *CYP81A21* of the R line. B, DNA-blot analysis of *Sph*I/*Sac*I-digested genomic DNA from R and S plants. *CYP81A12* and *CYP81A21* were detected using the probes depicted in Figure 4A.

stage (Supplemental Fig. S7). The susceptibilities were assessed by measuring plant height 7 d after herbicide treatment. For BSM resistance, segregation approached a 1:2:1 ratio (R:intermediate:S = 15:25:14, χ^2 = 0.3333, P = 0.8465; Supplemental Fig. S8A). The segregation of PX resistance was not as clear, likely because of a much lower resistance level of the parental R line compared with BSM. Still, the ratio of the number of plants taller than the S-parent line to plants with a similar plant height as the S-approached 3:1 (R + intermediate:S parent line = 38:16, χ^2 = 0.6173, P = 0.4321; Supplemental Fig. S8B). These results suggested that the resistances to BSM and PX were each under the control of a single locus.

In the F6 population, 40 recombinant inbred lines (RILs) of the two reference lines were used to assess the linkage of the resistances to BSM and PX and the transcript levels of *CYP81A12*, *CYP81A21*, and *CYP81A22*. BSM and PX susceptibilities were fixed in almost all RILs and strongly linked to each other; 14 lines were resistant to BSM and PX, 25 lines were susceptible to BSM and PX, and only one line segregated for BSM and PX resistances (Fig. 5; Supplemental Table S1). Transcript levels of the three genes were determined in roots because the transcript levels differed most in the roots of the two parental lines (Supplemental Fig. S3). In the 14 R and 25 S RILs, high transcript levels of *CYP81A12* and *CYP81A21* perfectly cosegregated with BSM and PX resistances (Fig. 5). The transcript levels of the segregating lines were intermediate between those of the R and S groups, presumably because the levels were determined in bulk samples of seedling roots (n = 9). The transcript level of *CYP81A22* was not associated with the herbicide resistances (Supplemental Fig. S9), indicating that the increased expression of *CYP81A22* observed in the R parents plays no major role in herbicide resistance.

We also examined the alleles of the three genes in each RIL. The promoter sequences and coding sequences of *CYP81A12* and *CYP81A21* were not associated with herbicide resistance and high transcript levels (Fig. 5; Supplemental Table S1); plants that carried the S line allele of *CYP81A12* and/or *CYP81A21* could become resistant to both herbicides if their transcript levels were high and vice versa (Supplemental Table S1). The R and S alleles of *CYP81A12* and *CYP81A21* segregated independently (Supplemental Table S1), suggesting that *CYP81A12* and *CYP81A21* are located on different chromosomes. In contrast to *CYP81A12* and *CYP81A21*, the transcript levels of *CYP81A22* were perfectly associated with the allele (Supplemental Fig. S9). Alleles were also determined for *CYP81A26* because the two lines differ by an amino acid polymorphism in the gene products. The alleles did not cosegregate with BSM and PX susceptibilities in the F6 plants (Supplemental Table S1), indicating that the amino acid polymorphism of *CYP81A26* is mostly irrelevant for BSM and PX resistance.

BSM Metabolic Functions of CYP81A12 and CYP81A21

To evaluate the ability of CYP81A12 and CYP81A21 to metabolize BSM via *O*-demethylation, recombinant CYP81A12 and CYP81A21 proteins were expressed using a yeast (*Saccharomyces cerevisiae*) expression system that also carried the Arabidopsis NADPH-cytochrome P450 reductase gene *ATR1* (Pompon et al., 1996). Accumulation of CYP81A12 and CYP81A21 proteins in the transgenic yeast were confirmed by immunoblotting (Fig. 6). The immunoblot showed that more CYP81A12 than CYP81A21 accumulated in the transgenic yeast.

For the metabolism assay, BSM was added to the yeast culture media. Fifteen hours later, the media

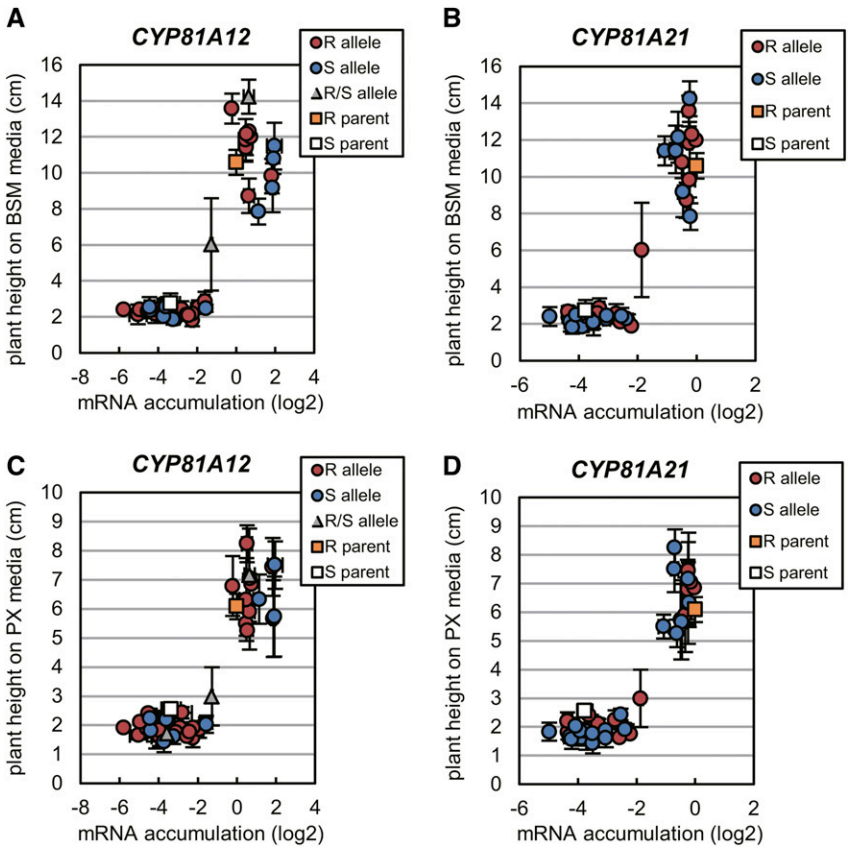

Figure 5. Transcript levels of *CYP81A12* and *CYP81A21* and susceptibilities to BSM (A and B) and PX (C and D) in F6 lines derived from a cross between an R-parent and an S-parent line of *E. phyllopogon*. Transcript accumulations in roots of each line ($n = 9$) were examined by real-time RT-PCR using *RPII* and *TUB* as internal control genes. Bars for mRNA levels represent the SD of three technical replicates in real-time RT-PCR. BSM and PX susceptibilities were examined by measuring the plant height of nine plants of each line. Bars for plant height show the SD of the nine biological replicates. R and S mark homozygous lines carrying alleles of R and S parents, respectively. R/S indicates a heterozygous line having R as well as S alleles from R and S parents.

were analyzed by LC-MS/MS. Although small, a new peak was detected in the media of yeast expressing CYP81A12 and CYP81A21, whereas no such peak occurred in the empty vector control (Fig. 6). The retention time of the new peak was 11.2 min, corresponding to the *O*-demethylated metabolite from BSM in the analysis of standards. The mass spectrum of the metabolite was the same as that of the *O*-demethylated BSM standard. These results indicated that CYP81A21 and CYP81A12 detoxify BSM via *O*-demethylation. The amounts of the metabolite were higher in the cultures transformed with *CYP81A12* than in those expressing *CYP81A21*. This difference probably is caused by higher expression levels of *CYP81A12* in the transformed yeast (Fig. 6), although further analysis is required to determine if BSM metabolic activities differ between the two P450s.

DISCUSSION

Our results showed that two *E. phyllopogon P450* genes, *CYP81A12* and *CYP81A21*, confer marked decreases in the susceptibilities to the two herbicides BSM and PX to Arabidopsis. The decrease in the susceptibilities was strongly correlated to the transcript levels of both genes in transgenic lines. The higher expression of the two genes in *E. phyllopogon* cosegregated completely with BSM and PX resistance in F6 RILs. CYP81A21 has amino acid polymorphisms that distinguish the R and S alleles, but the allelic variation had no influence on the susceptibilities to BSM and PX in transgenic Arabidopsis, and the alleles did not cosegregate with BSM and PX resistance in the F6 RILs of *E. phyllopogon*. Heterologously produced CYP81A12 and CYP81A21 proteins metabolized BSM into *O*-demethylated BSM, the highly accumulated metabolite in the R line of *E. phyllopogon*. These results indicate that enhanced expression of *CYP81A12* and *CYP81A21* plays a key role in the resistance to two different classes of ALS inhibitors in *E. phyllopogon*.

The resistance index observed in transformed Arabidopsis plants with high levels of *CYP81A12* and *CYP81A21* expression was more than 1,000 for BSM but only about 10 for PX (Fig. 3; Supplemental Fig. S6). BSM and PX responses of the R and S lines of *E. phyllopogon* were similar to those of the transformants; the resistance index for BSM was 1,100, and that for PX was 6.2 (Supplemental Fig. S7). The parallel herbicide responses between transformed Arabidopsis and *E. phyllopogon* supports the hypothesis that BSM and PX resistance in the R line of *E. phyllopogon* is caused by overexpression of these *P450* genes.

In this study, heterologously produced CYP81A12 and CYP81A21 degraded BSM into the *O*-demethylated metabolite. The ALS-inhibiting activity of the *O*-demethylated metabolite in vitro is approximately one-three thousandth of that of BSM, and its herbicidal activity is

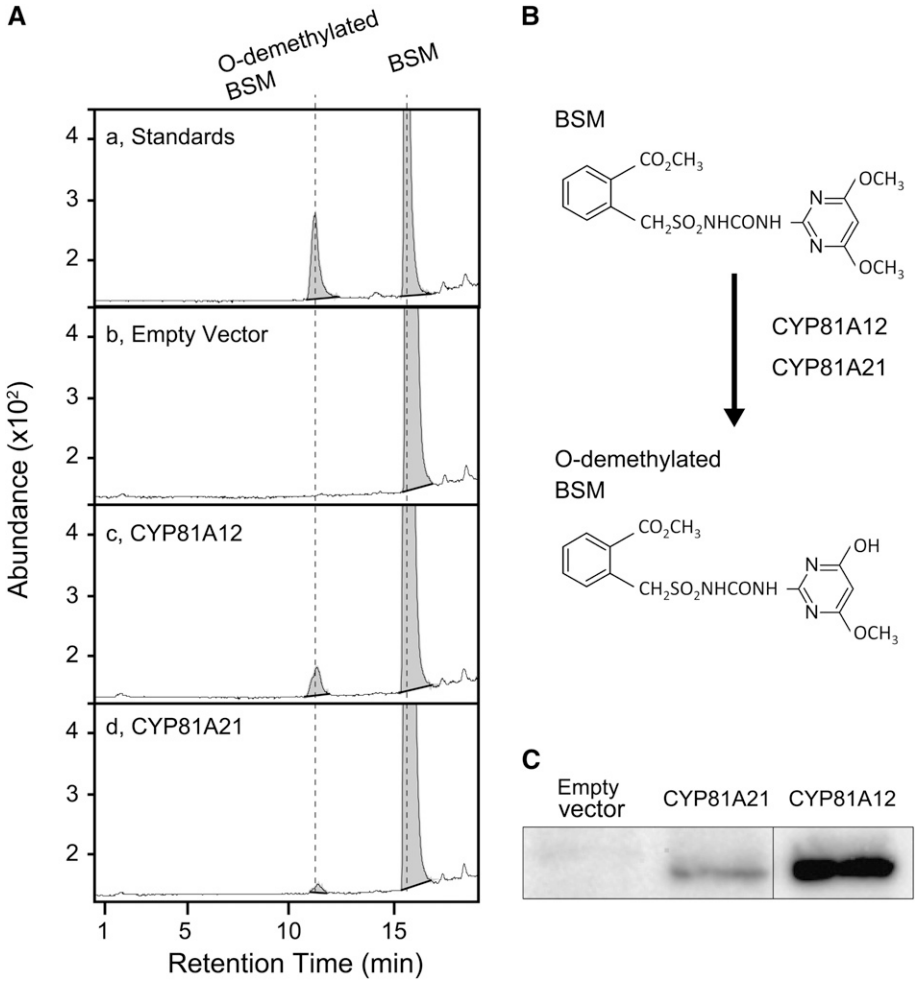

Figure 6. BSM metabolism catalyzed by CYP81A12 and CYP81A21. A, LC-MS/MS analyses of a BSM metabolite formed in yeast expressing *CYP81A12* and *CYP81A21*. a, Chromatogram of standards of BSM and BSM *O*-demethylated at position 4 of the pyrimidine ring. b, Chromatogram of yeast harboring the empty vector (pYeDP60) as a control. BSM was detected in the culture media. c, Chromatogram of yeast expressing an R line allele of *CYP81A12*. A new peak, corresponding to the *O*-demethylated BSM, was detected. d, Chromatogram for the yeast expressing an R line allele of *CYP81A21*. The peak corresponding to the *O*-demethylated BSM was small but clearly detectable. B, Changes in the structure of BSM following *O*-demethylation catalyzed by CYP81A12 and CYP81A21. C, Immunoblot of microsome fractions extracted from yeast expressing CYP81A12 and CYP81A21.

practically abolished in plants, including *E. phyllopogon* (Takeda et al., 1986). The catalysis of the *O*-demethylation reaction by CYP81A12 and CYP81A21 can explain the low susceptibility to BSM in transgenic Arabidopsis expressing either of the two *P450* genes. Importantly, the *O*-demethylated metabolite was the metabolite that accumulated more in the R line of *E. phyllopogon*. These results indicate that the two genes can confer BSM resistance to the R lines of *E. phyllopogon*. In rice, no direct evidence has been documented for *O*-demethylation of BSM by a specific P450, although P450s are known to be involved in BSM metabolism (Deng and Hatzios, 2002). Our data show that P450s in the CYP81A subfamily metabolize BSM into an *O*-demethylated metabolite. However, we have not investigated other metabolites that might be produced by the P450s, and further analyses using radioactive BSM might be required to fully characterize its metabolism.

We did not investigate the involvement of P450s in PX metabolism. PX is known to selectively kill wild-type *Echinochloa* spp. without damaging rice because of the rapid metabolic breakdown of PX in rice (Johnson et al., 2009). The major PX metabolite in rice is *O*-demethylated at one of the heterocyclic methoxy groups (Johnson et al., 2012). The reaction is similar to the *O*-demethylation of BSM. Previously, Yasuor et al. (2009) observed that PX was more rapidly metabolized into polar metabolites in the R line than in the S line of *E. phyllopogon*. Identification of the metabolites in *E. phyllopogon* and also in yeast expressing recombinant P450s will be future issues.

Our segregation study using an F2 population suggested that the resistances to BSM and PX resistances were controlled by a single genetic element each. Higher expression levels of *CYP81A12* and *CYP81A21* did not segregate in the F6 RILs, although the alleles of both genes including the promoter regions segregated independently (Fig. 5). The copy numbers of the two genes were not different between the R and S plants (Fig. 4B). These results suggest that a trans-element simultaneously regulates the expression of both genes in the R lines, and it appears unlikely that other mechanisms, such as gene amplification of the *P450* genes or cis-acting regulatory loci, regulate expression. In contrast to *CYP81A12* and *CYP81A21*, the magnitude of the expression levels of *CYP81A22* was associated with its alleles in F6 RILs, although it was not associated with herbicide resistance. The mechanisms

underlying the regulation of expression levels of *CYP81A22* are unknown but likely involve cis-elements because large indels were observed in its promoter regions when comparing the R and S lines (data not shown).

The similarity of functions observed in *CYP81A12* and *CYP81A21* is likely attributable to their origin; these genes are putative homeologs derived from the allotetraploid nature of *E. phyllopogon*. The identities of the coding sequences and protein sequences of the two genes were very high, 96.2% and 94.7%, respectively. Their short genetic distance in the phylogenetic tree of coding sequences strongly suggests that the two genes are homeologs (Supplemental Fig. S1). *CYP81A12* and *CYP81A21* segregated independently in the F6 RILs (Supplemental Table S1). This autonomy of inheritance refutes the possibility of tandem duplication and supports the homeologous relationship of the two genes. At present, it is unknown whether increased expression of either of the two *P450* genes is sufficient to acquire herbicide resistance in R plants of *E. phyllopogon* or whether both genes must be up-regulated.

The CYP81A subfamily belongs to the CYP81 family of P450s, a large protein family, in which lineage-specific duplication events, also known as a CYP bloom, frequently have occurred (Nelson and Werck-Reichhart, 2011). Apparently, the CYP81A subfamily is also lineage specific, because genes encoding this subfamily have only been reported in grasses (Nelson, 2009). In the CYP81A subfamily, large divergence is observed in the number and sequences of P450s in *E. phyllopogon*, maize (*Zea mays*), and *Sorghum bicolor*, three members of the Panicoideae subfamily (Supplemental Fig. S1). This divergence is much larger than in rice (subfamily Oryzoideae) and *Brachypodium distachyon* (subfamily Pooideae), indicating that lineage-specific duplication events may have occurred together with the diversification of the Panicoideae. Duplicated *P450* genes could be beneficial for fine-tuning the levels of the metabolites of their gene products via up- or down-regulation of each copy (subfunctionalization; e.g. Kushiro et al., 2004; Magome et al., 2013) and may enable the acquisition of new enzymatic functions (neofunctionalization; e.g. Frey et al., 2009; Prasad et al., 2012; Weng et al., 2012). The expression of *CYP81A22* in Arabidopsis failed to confer significant decreases in herbicide susceptibilities in contrast to *CYP81A12* and *CYP81A21*, supporting the notion that members of the CYP81A subfamily may perform different enzymatic functions. The functional characterization of the diverse *CYP81A* genes in *E. phyllopogon* is an important issue in the study of herbicide resistance.

To elucidate the evolution of multiple herbicide resistance in *E. phyllopogon*, it will be important to identify the element responsible for the overexpression of the two *CYP81A* genes, a causal gene for BSM and PX resistance. Our research suggested a single causal element that contrasts, however, with well-studied cases of nontarget site-resistant weeds such as *Lolium rigidum* or *Alopecurus myosuroides*. In these species, the resistance to each herbicide is frequently regulated by multiple genes (e.g. Neve and Powles, 2005a, 2005b; Petit et al., 2010; Busi et al., 2011, 2013). This discrepancy can be explained by the mode of pollination. Outcrossing in *L. rigidum* and *A. myosuroides* can facilitate the evolution of resistance through the accumulation of minor genes, where individual genes make limited contributions to herbicide resistance but can cooperatively cause significant levels of resistance. The mode of pollination in *E. phyllopogon*, however, is self-crossing; outcrossing is limited in the study populations as demonstrated by Tsuji et al. (2003), and accumulations of minor resistance genes are unlikely to occur. The self-pollinating nature of this species might have favored the selection of a major gene causing a large decrease in herbicide susceptibility.

Moreover, it will be necessary to establish whether the resistance to herbicides other than BSM and PX is based on similar mechanisms. Previous studies suggested that resistances to different herbicides can be based on distinct mechanisms. For example, in the resistance to aryloxyphenoxy-propionate herbicides, significant contributions of glutathione and Cys conjugation seem to be involved (Ruiz-Santaella et al., 2006; Bakkali et al., 2007). In quinclorac resistance, insensitivity of ethylene synthesis pathways that usually respond to quinclorac and enhanced activity of β-cyanoalanine synthase were suggested (Yasuor et al., 2012). In bispyribac-sodium resistance, bispyribac-sodium-induced P450s appear involved (Yun et al., 2005), and slightly increased expression of two *P450* genes (not *CYP81A21* or *CYP81A21*) has been reported under bispyribac-sodium stress (Iwakami et al., 2014a).

Herbicides exert strong selection pressure in agroecosystems, resulting in the evolution of herbicide-resistant plant lines. P450-mediated herbicide resistance has been implicated in several weed species (Preston, 2004; Yuan et al., 2007; Powles and Yu, 2010; Beckie and Tardif, 2012). Despite their threatening impact on agriculture, the molecular mechanisms of P450-mediated resistance remained unknown for more than 25 years (Kemp and Caseley, 1987). In this study, we identified *P450* genes associated with herbicide resistance in weeds causing serious problems in agricultural fields. Our findings provide a new perspective on P450-mediated herbicide resistance in weeds and will inform further studies on herbicide resistance.

MATERIALS AND METHODS

Origin of Materials and Seed Preparation

Lines 511 and 401 were used as the R and S lines of *Echinochloa phyllopogon*, respectively. These lines were originally collected from rice (*Oryza sativa*) fields in the Sacramento Valley of California in 1997 and were self-pollinated for three successive generations (Tsuji et al., 2003). BSM susceptibility of 401 was similar to those of other susceptible accessions collected from California and lacked multiple herbicide resistance, indicating that the BSM response of 401 is within the response range of wild-type *E. phyllopogon*. The two lines and F3 populations resulting from a crossing of 401 and 511 were provided by Dr. Yuji Yamasue (Kyoto University). The crossing procedure was described

in Tsuji et al., 2003. The F3 populations were further self-pollinated for three successive generations to obtain F6 seeds. Another cross between the S and R lines was generated according to the procedure in Tsuji et al., 2003, followed by selfing to produce F2 populations. The seeds were sterilized with 0.5% (w/v) sodium hypochlorite for 15 min, further sterilized with 0.04% (w/v) sodium hypochlorite for 30 min, and then washed three times in sterile water. Seeds were germinated on wet paper for 2.5 d in a growth chamber at 25°C under constant fluorescent light (approximately 300 μE m^{-2} s^{-1}) and then transferred to solid media.

Sample Preparation for BSM Metabolism and Transcript Analysis under BSM Stress

Germinated seeds of the R and S lines of *E. phyllopogon* were planted on solid one-half-strength Murashige and Skoog medium and grown under the same conditions used for germination. Four days after planting, the plants were transplanted onto one-half-strength Murashige and Skoog solid media with or without 10 μM BSM. After 24 h, 1 g of shoots or roots was harvested, washed in distilled water, and stored at –20°C for BSM metabolism analysis. For transcript analysis, the shoot of a plant and roots of three plants were harvested with four replications, frozen in liquid N_2, and stored at –80°C. Total RNA was isolated from the frozen samples. Complementary DNA (cDNA) was synthesized from the RNA, and transcript levels were determined by real-time reverse transcription (RT)-PCR (described below).

LC-MS/MS Analysis of BSM Metabolites

For the analysis of BSM metabolism in *E. phyllopogon*, the stored samples were homogenized in 100 mL of acetone and filtered through glass fiber filters (Kiriyama), and the filtrate was dissolved in 200 mL of acetone. Five milliliters of water was added to 20 mL of extract, and the acetone was evaporated. The samples (10 μL) were loaded onto a solid-phase extraction cartridge (InertSep PLS-2, styrene-divinylbenzene; GL Sciences), followed by washing with 5 mL of water:acetic acid:formic acid (80:20:1). BSM and its metabolites were eluted using 10 mL of water:acetic acid:formic acid (20:80:1).

HPLC analysis was performed on an Agilent 1200 Series, and tandem mass spectrometry was performed on an Agilent 6410 Triple Quad LC/MS with a Zorbax Eclipse Plus C18 analytical column at 40°C (Agilent Technologies). As internal standards, *O*-demethylated BSM [methyl-α-(4-hydroxy-6-methoxypyrimidin-2-yl) carbamoylsulfamoyl-*o*-toluate], a gift from DuPont, and BSM (Wako) were used. Separations were performed with mobile phases consisting of methanol (solution A) and water with 0.01% (v/v) formic acid (solution B). The gradient increased linearly from 70% (v/v) solution A and 30% (v/v) solution B to 100% solution B over 15 min with a flow rate of 0.2 mL min^{-1}. The molecules were ionized at 350°C. Collision voltages for BSM and *O*-demethylated BSM were 20 and 15 V, respectively. Data were analyzed using Mass Hunter software (Agilent Technologies) and quantified based on standard curves.

For the analysis of recombinant P450s in yeast (*Saccharomyces cerevisiae*), the stored samples were diluted 10-fold with water and filtered through 0.2-μm polytetrafluoroethylene membranes (Whatman). LC-MS/MS analysis was performed as described above.

Nucleic Acid Extraction and cDNA Synthesis

Genomic DNA was extracted from leaves of *E. phyllopogon* using a DNeasy Plant Mini Kit (Qiagen) or a Nucleon Phytopure extraction kit (Amersham Pharmacia Biotech). The plants were grown in a greenhouse at Tsukuba, Japan.

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) with on-column DNase I digestion (RNase-Free DNase Set, Qiagen). The RNAs were further treated with a TURBO DNA-free kit (Applied Biosystems) for complete elimination of genomic DNA. Total RNA (1 μg) was reverse transcribed using a PrimeScript II First-Strand cDNA Synthesis Kit with oligo(dT) primer (TaKaRa). The plants from which RNA was extracted are described below.

Isolation of *CYP81A* Genes

For the isolation of *CYP81A* subfamily genes from *E. phyllopogon*, degenerate PCR was conducted using cDNA prepared from the RNA extracted from third-leaf stage shoots of the R line. Primers used are listed in Supplemental Table S2. The plants used for RNA extractions were grown on soil in a growth chamber with the same environmental conditions as described above for seed germination. PCR amplification was carried out using TaKaRa LA Taq or PrimeSTAR GXL DNA Polymerase (TaKaRa) with the primers listed in Supplemental Table S2. The amplicons were cloned using a pGEM-T Easy Vector Systems Kit (Promega) and sequenced as described previously (Iwakami et al., 2012). The full-length *CYP81A* gene was obtained by RACE as described previously (Iwakami et al., 2012). The 5′-flanking sequences of *CYP81A12*, *CYP81A21*, and *CYP81A22* were determined by thermal asymmetric interlaced PCR (Liu et al., 1995; Liu and Whittier, 1995).

Real-Time RT-PCR

Real-time RT-PCR of *E. phyllopogon* was carried out as described previously (Iwakami et al., 2012). mRNA quantitation was performed by the ΔΔCT method. Gene expression was normalized to the expression of eukaryotic translation initiation factor 4B (*EIF4B*) and RNA polymerase II (*RPII*) in the mRNA quantitation under BSM stress and to *RPII* and tubulin (*TUB*) in the analysis of F6 RILs. The stability of these genes was evaluated using geNorm^{PLUS} software (Vandesompele et al., 2002). Primers used in real-time PCR are listed in Supplemental Table S2.

Transgene expression in Arabidopsis (*Arabidopsis thaliana*) was quantified using the standard curve method of real-time RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal control.

Arabidopsis Transformation and Characterization of Transgenic Plants

The coding regions of *CYP81A12*, *CYP81A21*, and *CYP81A22* were amplified by PCR with the primer pairs listed in Supplemental Table S2 using cDNA prepared from third-leaf stage shoots. The amplicons were subcloned into a pENTR/D-TOPO cloning vector (Invitrogen) to yield entry vectors. Binary vectors were produced in an LR Clonase-catalyzed reaction (Invitrogen) with the entry clones and the pB2GW7 vector (Karimi et al., 2002) for transformation of Arabidopsis. The binary vectors were transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Arabidopsis (ecotype Columbia-0) was transformed with the floral dip method using the transformed *A. tumefaciens* strains (Clough and Bent, 1998). The transformants were selected by bialaphos (25 mg L^{-1}) treatment.

Four to five 12-d-old seedlings of T3 or T4 homozygous lines grown on Murashige and Skoog solid medium were harvested, frozen in liquid nitrogen, and stored for RNA extraction. The cDNAs were synthesized as described above.

BSM susceptibilities of the transgenics were evaluated by growth on Murashige and Skoog solid medium containing BSM. All assays were conducted at 22°C with a 12-h photoperiod and a light intensity of about 70 μE m^{-2} s^{-1}. Seeds were planted on Murashige and Skoog solid media containing BSM in petri dishes and allowed to grow for 12 d. The position of each dish in the growth chamber was changed every 1 or 2 d. The fresh weights of 10 plants were measured immediately after removing fluid (four replicates), and the GR_{50} value was calculated using a nonlinear computer analysis based on a log-logistic model (Seefeldt et al., 1995).

Genomic DNA-Blot Analysis

DNA (3 μg) of the R and S lines of *E. phyllopogon* was digested with *Sph*I or *Sac*I (Roche). The digested DNA samples were electrophoresed on a 0.8% (w/v) agarose gel, and DNA fragments were transferred onto a positively charged nylon membrane (Roche). Gene-specific DNA probes of approximately 300 bp were prepared using a PCR DIG Probe Synthesis Kit (Roche). For probe synthesis, cloned DNA sequences of *CYP81A12* and *CYP81A21* were used as templates with a forward primer (5′-CCTCTCTTCCCCTCCCTGAC-3′) and a reverse primer (5′-GCCACGTAGGCCTTATCCAT-3′). Hybridization was performed according to the DIG Application Manual (Roche). Hybridization signals were detected on an LPR-400EX chemiluminescence detection analyzer (Taitec).

Inheritance of Transcript Levels and Herbicide Resistances

Transcript levels of F6 RILs were determined by real-time RT-PCR using RNAs extracted from first leaf stage roots of nine plants. Germination was conducted as described above, and plants were grown under the same

conditions as described for germination. At the first leaf stage, roots from nine plants were harvested, frozen in liquid nitrogen, and stored for RNA extraction. cDNA was synthesized as described above.

BSM and PX susceptibilities of the F6 RILs were evaluated by growth on one-half-strength Murashige and Skoog media containing 10 μM BSM and 0.3 μM PX, respectively. Nine germinated seeds were planted on one-half-strength Murashige and Skoog media containing a herbicide. Seven days after transplanting, plant height was measured. Germination was conducted as described above. Plants grew at 25°C under constant fluorescent light (approximately 300 μE m^{-2} s^{-1}).

Alleles of each line were determined by sequencing PCR products amplified from DNA extracted from F5 plants. Primers used for the amplification of coding sequence and promoter regions are listed in Supplemental Table S2.

Yeast Transformation and P450 Expression

For the expression of P450s, the WAT11 yeast strain and the pYeDP60 vector system was used (Pompon et al., 1996). The WAT11 strain contains an Arabidopsis NADPH-P450 reductase gene (*ATR1*); pYeDP60 is an expression vector with the Gal-inducible and Glc-repressed yeast hybrid promoter *GAL10-CYC1*. *Kpn*I and *Eco*RI sites were introduced by PCR just upstream of the ATG and downstream of the stop codon of the full-length coding sequences, respectively. In addition, the FLAG epitope sequence was fused just downstream of the ATG using the primers listed in Supplemental Table S2. The amplicons were subcloned into pGEM-T Easy (Promega). The subclones were digested with *Kpn*I and *Eco*RI and then inserted into pYeDP60. The vectors were transformed into the yeast strain using a LiCl method according to the manufacturer's instructions provided with the pYES2 vector (Invitrogen). *P450* expression was induced according to the modified two-stage cultivation method using modified SLI medium (Jiang and Morgan, 2004). For construction of the expression vector, original nucleotide sequences of *E. phyllopogon P450* genes were used because no significant improvements were observed by recoding the N-terminal part of the *P450* genes according to the procedure described by Hehn et al. (2002).

BSM was added to the culture medium at a final concentration of 100 μM at 9 h after the induction of *P450* by Gal. At 24 h after the start of Gal induction, the culture solution was centrifuged at 500*g* for 5 min. The supernatant was filtered through a polyvinylidene difluoride membrane (0.22-μm pore size, Merck Millipore) and stored at –20°C until LC-MS/MS analysis.

Immunoblot Analysis

Microsomes of transgenic yeast were prepared according to Pompon et al. (1996), and 30 mg of the microsomes was electrophoresed on 10% (w/v) SDS polyacrylamide gels (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Merck Millipore) by semidry blotting. Membranes were blocked overnight at 4°C in 5% (w/v) nonfat dried milk and then incubated in 1:4,600 dilutions of anti-flag M2 monoclonal antibody for 1 h at room temperature (Sigma-Aldrich). Blots were washed in Tris-buffered saline plus Tween 20 (12.5 mM Tris–HCl, 137 mM NaCl, 0.1% [v/v] Tween 20, pH 7.5) and incubated for 1 h at room temperature in a 1:2,500 dilution of goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific) in Tris-buffered saline plus Tween 20. The blot was stained using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Signals were detected on an LPR-400EX chemiluminescence analyzer (Taitec).

The cDNA sequences of the R strain alleles are cataloged under the following accession numbers: *CYP81A13P*, AB733993; *CYP81A14*, AB733994; *CYP81A15*, AB733995; *CYP81A18*, AB733996; *CYP81A19P*, AB733997; *CYP81A23*, AB734000; *CYP81A24*, AB734001; *CYP81A25P*, AB734002; and *CYP81A26*, AB734003. The cDNA sequences of the S strain alleles are cataloged under the accession number *CYP81A26*, AB818465. The accession numbers for the genomic DNA and cDNA sequences of *CYP81A12* are AB818461 for the R line allele and AB818460 for the S line allele. The accession numbers for genomic DNA and cDNA sequences of *CYP81A21* are AB818462 for the R line allele and AB818463 for the S line allele. The accession numbers for the genomic DNA sequences of *CYP81A22* are AB872309 for the R line allele and AB872310 for the S line allele. Genes used as reference genes for real-time RT-PCR in *E. phyllopogon* were *EIF4B*, AB720070; *RPII*, AB775462; and *TUB*, AB872311; in Arabidopsis, *GAPDH*, AT1G13440 was used.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phenogram of CYP81A subfamily proteins from various Poaceae.

Supplemental Figure S2. Alignment of protein sequences of the CYP81A subfamily of an R line.

Supplemental Figure S3. Transcript levels of *CYP81A12*, *CYP81A21*, and *CYP81A22* in several organs of an R and an S line of *E. phyllopogon*.

Supplemental Figure S4. Relationship of transcript level and BSM susceptibility in transgenic Arabidopsis expressing *CYP81A12* or *CYP81A21*.

Supplemental Figure S5. BSM and PX responses of transgenic Arabidopsis expressing an S line allele of *CYP81A21*.

Supplemental Figure S6. PX susceptibilities in transgenic Arabidopsis expressing *CYP81A12* or an R line allele of *CYP81A21*.

Supplemental Figure S7. BSM and PX susceptibilities of an R and an S line of *E. phyllopogon*.

Supplemental Figure S8. Segregation of BSM and PX resistances in an F2 population derived from a cross between an R and an S line of *E. phyllopogon*.

Supplemental Figure S9. Transcript levels of *CYP81A22* and susceptibilities to BSM and PX in F6 lines derived from a cross between an R parent and an S parent line of *E. phyllopogon*.

Supplemental Table S1. Linkage between herbicide resistances and genotypes in the RILs.

Supplemental Table S2. Primers used in this study.

ACKNOWLEDGMENTS

We thank Drs. Tomonori Fujioka and Tsutomu Shimizu for technical advice on heterologous expression of P450s in yeast; Ayako Nishizawa-Yokoi for help in western-blot analysis; DuPont for providing BSM and the analytical standards of BSM metabolites; Dr. David Nelson for classifying and naming the P450s; Drs. Danièle Werck-Reichhart and Denis Pompom for providing the pYeDP60 vector and WAT11 yeast strain; Dr. Hiroyuki Shibaike for sharing experimental facilities; Sachiko Inanuma, Hiroko Kato, Kiyoko Amagai, Rieko Aoto, Chiharu Furusawa, Akemi Nagashii, Etsuko Ozawa, and Fukuko Suzuki for technical assistance; and Dr. Yuji Yamasue for providing the opportunity to conduct this research and for providing the seeds of *E. phyllopogon* used in the study.

Received November 22, 2013; accepted April 22, 2014; published April 23, 2014.

LITERATURE CITED

Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S, Werck-Reichhart D (2011) Cytochromes p450. Arabidopsis Book **9:** e0144

Bakkali Y, Ruiz-Santaella JP, Osuna MD, Wagner J, Fischer AJ, De Prado R (2007) Late watergrass (*Echinochloa phyllopogon*): mechanisms involved in the resistance to fenoxaprop-*p*-ethyl. J Agric Food Chem **55:** 4052–4058

Beckie HJ, Tardif FJ (2012) Herbicide cross resistance in weeds. Crop Prot **35:** 15–28

Busi R, Neve P, Powles S (2013) Evolved polygenic herbicide resistance in *Lolium rigidum* by low-dose herbicide selection within standing genetic variation. Evol Appl **6:** 231–242

Busi R, Vila-Aiub MM, Powles SB (2011) Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. Heredity (Edinb) **106:** 817–824

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Délye C (2013) Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Manag Sci **69:** 176–187

Deng F, Hatzios KK (2002) Characterization of cytochrome P450-mediated bensulfuron-methyl *O*-demethylation in rice. Pestic Biochem Physiol **74:** 102–115

Duggleby RG (2005) Suicide inhibition of acetohydroxyacid synthase by hydroxypyruvate. J Enzyme Inhib Med Chem **20:** 1–4

Fischer AJ, Ateh CM, Bayer DE, Hill JE (2000a) Herbicide-resistant *Echinochloa oryzoides* and *E. phyllopogon* in California *Oryza sativa* fields. Weed Sci **48:** 225–230

Fischer AJ, Bayer DE, Carriere MD, Ateh CM, Yim KO (2000b) Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. Pestic Biochem Physiol **68:** 156–165

Frey M, Schullehner K, Dick R, Fiesselmann A, Gierl A (2009) Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochemistry **70:** 1645–1651

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, Nissen SJ, Patzoldt WL, Tranel PJ, Culpepper AS, et al (2010) Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci USA **107:** 1029–1034

Gotoh O (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. J Biol Chem **267:** 83–90

Hehn A, Morant M, Werck-Reichhart D (2002) Partial recoding of P450 and P450 reductase cDNAs for improved expression in yeast and plants. Methods Enzymol **357:** 343–351

Iwakami S, Uchino A, Kataoka Y, Shibaike H, Watanabe H, Inamura T (2014a) Cytochrome P450 genes induced by bispyribac-sodium treatment in a multiple-herbicide-resistant biotype of *Echinochloa phyllopogon*. Pest Manag Sci **70:** 549–558

Iwakami S, Uchino A, Watanabe H, Yamasue Y, Inamura T (2012) Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. Pest Manag Sci **68:** 1098–1106

Iwakami S, Watanabe H, Miura T, Matsumoto H, Uchino A (2014b) Occurrence of sulfonylurea resistance in *Sagittaria trifolia* L., a basal monocot species, based on target-site and non-target-site resistance. Weed Biol Manage **14:** 43–49

Jeffers GM, O'Donovan JT, Hall LM (1996) Wild mustard (*Brassica kaber*) resistance to ethametsulfuron but not to other herbicides. Weed Technol **10:** 847–850

Jiang H, Morgan JA (2004) Optimization of an in vivo plant P450 monooxygenase system in *Saccharomyces cerevisiae*. Biotechnol Bioeng **85:** 130–137

Johnson TC, Mann RK, Schmitzer PR, Gast RE, deBoer GJ (2012) Triazolopyrimidines. *In* W Krämer, U Schirmer, P Jeschke, W Matthias, eds, Modern Crop Protection Compounds, Ed 2, Vol 1. Wiley-VCH, Weinheim, Germany, pp 99–117

Johnson TC, Martin TP, Mann RK, Pobanz MA (2009) Penoxsulam: structure-activity relationships of triazolopyrimidine sulfonamides. Bioorg Med Chem **17:** 4230–4240

Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci **7:** 193–195

Kemp MS, Caseley JC (1987) Synergistic effects of 1-aminobenzotriazole on the phytotoxicity of chlorotoluron and isoproturon in a resistant population of black-grass (*Alepurus myosuroides*). *In* 1987 British Crop Protection Conference: Weeds. BCPC Publications, Surrey, UK, pp 895–899

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. EMBO J **23:** 1647–1656

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J **8:** 457–463

Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics **25:** 674–681

Magome H, Nomura T, Hanada A, Takeda-Kamiya N, Ohnishi T, Shinma Y, Katsumata T, Kawaide H, Kamiya Y, Yamaguchi S (2013) CYP714B1 and CYP714B2 encode gibberellin 13-oxidases that reduce gibberellin activity in rice. Proc Natl Acad Sci USA **110:** 1947–1952

Mizutani M, Ohta D (2010) Diversification of P450 genes during land plant evolution. Annu Rev Plant Biol **61:** 291–315

Nelson D, Werck-Reichhart D (2011) A P450-centric view of plant evolution. Plant J **66:** 194–211

Nelson DR (2009) The cytochrome p450 homepage. Hum Genomics **4:** 59–65

Neve P, Powles S (2005a) High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. Heredity (Edinb) **95:** 485–492

Neve P, Powles S (2005b) Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*. Theor Appl Genet **110:** 1154–1166

Osuna MD, Vidotto F, Fischer AJ, Bayer DE, De Prado R, Ferrero A (2002) Cross-resistance to bispyribac-sodium and bensulfuron-methyl in *Echinochloa phyllopogon* and *Cyperus difformis*. Pestic Biochem Physiol **73:** 9–17

Pan G, Zhang X, Liu K, Zhang J, Wu X, Zhu J, Tu J (2006) Map-based cloning of a novel rice cytochrome P450 gene *CYP81A6* that confers resistance to two different classes of herbicides. Plant Mol Biol **61:** 933–943

Petit C, Duhieu B, Boucansaud K, Délye C (2010) Complex genetic control of non-target-site-based resistance to herbicides inhibiting acetyl-coenzyme A carboxylase and acetolactate-synthase in *Alopecurus myosuroides* Huds. Plant Sci **178:** 501–509

Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol **272:** 51–64

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol **61:** 317–347

Prasad KV, Song BH, Olson-Manning C, Anderson JT, Lee CR, Schranz ME, Windsor AJ, Clauss MJ, Manzaneda AJ, Naqvi I, et al (2012) A gain-of-function polymorphism controlling complex traits and fitness in nature. Science **337:** 1081–1084

Preston C (2004) Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci **52:** 448–453

Ruiz-Santaella JP, De Prado R, Wagner J, Fischer AJ, Gerhards R (2006) Resistance mechanisms to cyhalofop-butyl in a biotype of *Echinochloa phyllopogon* (Stapf) Koss. from California. J Plant Dis Prot **XX:** 95–100

Salas RA, Dayan FE, Pan Z, Watson SB, Dickson JW, Scott RC, Burgos NR (2012) *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag Sci **68:** 1223–1230

Seefeldt SS, Jensen JE, Fuerst EP (1995) Log-logistic analysis of herbicide dose-response relationships. Weed Technol **9:** 218–227

Siminszky B (2006) Plant cytochrome P450-mediated herbicide metabolism. Phytochem Rev **5:** 445–458

Takeda S, Erbes DL, Sweetser PB, Hay JV, Yuyama T (1986) Mode of herbicidal and selective action of DPX-F5384 between rice and weeds. J Weed Sci Tech **31:** 157–163

Tranel PJ, Riggins CW, Bell MS, Hager AG (2011) Herbicide resistances in *Amaranthus tuberculatus*: a call for new options. J Agric Food Chem **59:** 5808–5812

Tsuji R, Fischer AJ, Yoshino M, Roel A, Hill JE, Yamasue Y (2003) Herbicide-resistant late watergrass (*Echinochloa phyllopogon*): similarity in morphological and amplified fragment length polymorphism traits. Weed Sci **51:** 740–747

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:** H0034, 0011

Weng JK, Li Y, Mo H, Chapple C (2012) Assembly of an evolutionarily new pathway for α-pyrone biosynthesis in Arabidopsis. Science **337:** 960–964

Yamasue Y (2001) Strategy of *Echinochloa oryzicola* Vasing. for survival in flooded rice. Weed Biol Manage **1:** 28–36

Yasuor H, Milan M, Eckert JW, Fischer AJ (2012) Quinclorac resistance: a concerted hormonal and enzymatic effort in *Echinochloa phyllopogon*. Pest Manag Sci **68:** 108–115

Yasuor H, Osuna MD, Ortiz A, Saldaín NE, Eckert JW, Fischer AJ (2009) Mechanism of resistance to penoxsulam in late watergrass (*Echinochloa phyllopogon* [Stapf] Koss.). J Agric Food Chem **57:** 3653–3660

Yasuor H, Zou W, Tolstikov VV, Tjeerdema RS, Fischer AJ (2010) Differential oxidative metabolism and 5-ketoclomazone accumulation are involved in *Echinochloa phyllopogon* resistance to clomazone. Plant Physiol **153:** 319–326

Yu Q, Powles SB (January 20, 2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Manag Sci http://dx.doi.org/10.1002/ps.3710

Yuan JS, Tranel PJ, Stewart CNJ Jr (2007) Non-target-site herbicide resistance: a family business. Trends Plant Sci **12:** 6–13

Yun MS, Yogo Y, Miura R, Yamasue Y, Fischer AJ (2005) Cytochrome P-450 monooxygenase activity in herbicide-resistant and -susceptible late watergrass (*Echinochloa phyllopogon*). Pestic Biochem Physiol **83:** 107–114