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Research Article

Transcriptionally linked simultaneous overexpression of P450 genes for broad-spectrum herbicide resistance

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Abstract

Broad-spectrum herbicide resistance (BSHR), often linked to weeds with metabolism-based herbicide resistance, poses a threat to food production. Past studies have revealed that overexpression of catalytically promiscuous enzymes explains BSHR in some weeds; however, the mechanism of BSHR expression remains poorly understood. Here, we investigated the molecular basis of high-level resistance to diclofop-methyl in BSHR late watergrass (*Echinochloa phyllopogon*) found in the United States, which cannot be solely explained by the overexpression of promiscuous cytochrome P450 monooxygenases CYP81A12/21. The BSHR late watergrass line rapidly produced 2 distinct hydroxylated diclofop acids, only 1 of which was the major metabolite produced by CYP81A12/21. RNA-seq and subsequent reverse transcription quantitative PCR (RT-qPCR)-based segregation screening identified the transcriptionally linked overexpression of a gene, *CYP709C69*, with *CYP81A12/21* in the BSHR line. The gene conferred diclofop-methyl resistance in plants and produced another hydroxylated diclofop acid in yeast (*Saccharomyces cerevisiae*). Unlike CYP81A12/21, CYP709C69 showed no other herbicide-metabolizing function except for a presumed clomazone-activating function. The overexpression of the 3 herbicide-metabolizing genes was also identified in another BSHR late watergrass in Japan, suggesting a convergence of BSHR evolution at the molecular level. Synteny analysis of the P450 genes implied that they are located at mutually independent loci, which supports the idea that a single *trans*-element regulates the 3 genes. We propose that transcriptionally linked simultaneous overexpression of herbicide-metabolizing genes enhances and broadens the metabolic resistance in weeds. The convergence of the complex mechanism in BSHR late watergrass from 2 countries suggests that BSHR evolved through co-opting a conserved gene regulatory system in late watergrass.

Introduction

The heavy utilization of herbicides for weed control in agricultural fields has resulted in the evolution of herbicide-resistant weeds (Powles and Yu 2010). To effectively manage these resistant weeds and curb the evolution of resistance, a thorough understanding of the molecular mechanisms underlying resistance is imperative. To date, the most extensively characterized mechanism is the mutation of herbicide target-site proteins, known as target-site resistance. These causal DNA mutations have been identified in various weed species over the past several decades (Gaines et al. 2020). However, mechanisms other than target-site

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resistance, referred to as nontarget-site resistance, remain poorly understood (Délye 2013). Among these nontarget-site mechanisms, metabolism-based resistance is the most commonly observed, with resistant weeds posing the greatest threat to crop production due to their frequent expression of broad-spectrum herbicide resistance (BSHR), including resistance to yet-to-be-discovered chemicals (Yu and Powles 2014; Dimaano and Iwakami 2021). While it has recently been found that overexpression of catalytically promiscuous enzymes well explains BSHR in some weed species (Iwakami et al. 2019; Han et al. 2021; Pan et al. 2022), the mechanism underlying BSHR expression in weeds is still poorly understood.

We have studied the molecular mechanism of BSHR in late watergrass (*Echinochloa phyllopogon*) (syn. *Echinochloa oryzicola*). Late watergrass is an allotetraploid (2× = 4× = 36) and predominantly self-fertilizing noxious weed occurring in paddy fields (Yamasue 2001). Resistance in late watergrass was reported in California in the late 1990s (Fischer et al. 2000), where resistance was exhibited not only to the herbicides used in the fields at that time but also to many other herbicides commercialized later (Nandula et al. 2019). Intensive analyses of this BSHR population led to the discovery that the overexpression of 2 enzymatically identical herbicide-metabolizing cytochrome P450s, CYP81A12 and CYP81A21, was responsible for this cross-resistance to many herbicides (Iwakami, Endo, et al. 2014; Chayapakdee 2019; Guo et al. 2019; Iwakami et al. 2019; Dimaano et al. 2020). While the overexpression mechanism of the 2 genes remains elusive, genetic analysis strongly suggests that these genes are regulated by a single *trans*-element (Iwakami, Endo, et al. 2014). The presence of a regulatory factor driving this overexpression of the 2 CYP81As might broaden the range of resistance if other herbicide-metabolizing genes are under such regulatory factor.

In this study, we focused on the high-level resistance in BSHR late watergrass to the acetyl-CoA carboxylase (ACCase) inhibitor diclofop-methyl. We previously noticed that the resistance level of transgenic rice (*Oryza sativa*) lines expressing *CYP81A12/21* to diclofop-methyl was not substantially higher than that to other ACCase herbicides (tralkoxydim and pinoxaden), which is inconsistent with the observation that the resistance level of the BSHR line to diclofop-methyl was much higher than that to the 2 herbicides (Iwakami et al. 2019). Considering that diclofop-methyl resistance is inherited, following the Mendelian segregation ratio as in other herbicides (Iwakami et al. 2019), we hypothesized that an additional element specific to diclofop-methyl metabolism underlies the high-level diclofop-methyl resistance. Here, we report that the overexpression of a diclofop-methyl metabolizing P450, CYP709C69, leads to high-level resistance to diclofop-methyl. The gene is transcriptionally linked with the catalytically promiscuous *CYP81A12/21*. Our findings provide a novel model of metabolic resistance, in which a single evolutionary event can lead to the development of BSHR with high-level resistance through the simultaneous overexpression of multiple herbicide-metabolizing genes.

Results

Analysis of diclofop acid metabolism in BSHR late watergrass

To understand the high-level resistance to diclofop-methyl in the BSHR line, we investigated the metabolism of diclofop-methyl in late watergrass. Diclofop-methyl is a proherbicide that is activated by endogenous esterase, as is the case for most aryloxyphenoxy–propanoate herbicides (Wenger et al. 2012). Following de-esterification, the resultant active form diclofop acid is inactivated by P450-mediated hydroxylation, followed by further glucosylation (Fig. 1A) (Shimabukuro et al. 1979, 1987; McFadden et al. 1989). In the metabolism scheme, diclofop acid hydroxylation is the rate-limiting step for diclofop-methyl inactivation in wheat (*Triticum aestivum*); this occurrence has also been suggested in other plants (Owen 2000). Our previous study showed that both BSHR and sensitive (S) lines converted the majority of diclofop-methyl into diclofop acid by 3 h after treatment (HAT) for 30 min (Iwakami et al. 2019).

Here, we further analyzed the dynamics of putative OH metabolites of diclofop acid in the extract of plants prepared as before (Fig. 1B). Two peaks with retention times of 11.3 and 12.4 min (M1 and M2, respectively), corresponding to putative OH metabolites, were detected by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis using a multiple reaction monitoring (MRM) mode (Fig. 1C). We next compared the amount of each hydroxylated metabolite between the BSHR and S lines. To accurately evaluate the hydroxylation activity, possible glucosylated metabolites were converted into hydroxylated diclofop acid by adding β-glucosidase to the extract of the diclofop-methyl treated plants (Fig. 1A), which was then subjected to comparative quantification between the lines. The results showed that the amounts of both hydroxylated metabolites were higher in BSHR plants at 0 and 1 HAT (Fig. 1D). At later time points, no significant difference was observed. This is likely because the initial diclofop acid-OH was rapidly turned over to further metabolites, which should have prevented their accumulation later than 3 HAT.

To investigate whether CYP81A12 and CYP81A21 are responsible for producing both M1 and M2, we conducted an analysis of the whole-cell diclofop-methyl metabolism in yeast carrying these enzymes. Notably, yeast carrying CYP81A12/21 produced M1 as the major metabolite while also producing M2 and an additional metabolite (M3) as minor metabolites (Fig. 1E). Given that M2 was produced in late watergrass at comparable levels to M1 (Fig. 1D), it is reasonable to hypothesize that an additional enzyme capable of producing M2 is involved in conferring diclofop-methyl resistance in the BSHR late watergrass. We urge further

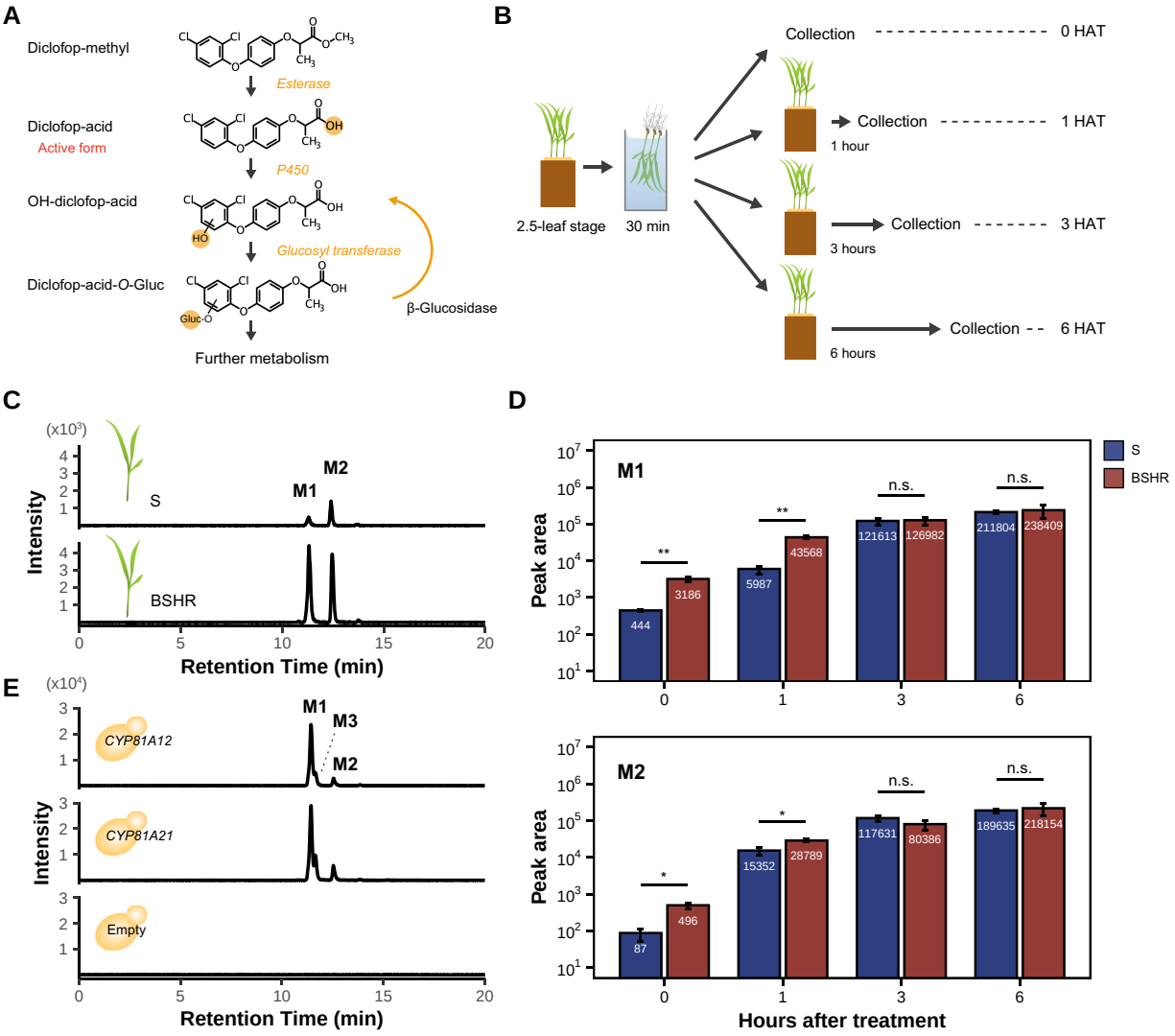

Figure 1. Characterization of diclofop acid metabolism in BSHR *E. phyllopogon*. **A)** Diclofop-methyl metabolism in plants. The enzymes estimated to be involved in each metabolism step are shown. **B)** Schematic representation of sample preparation for the diclofop-methyl metabolism study. The shoots were dipped in diclofop-methyl solution (30 μM) for 30 min, which were collected and stored at 0, 1, 3, or 6 HAT. **C)** Detection of OH-diclofop acid in S and BSHR lines of *E. phyllopogon* on LC–MS/MS. The extract of 3 HAT was analyzed. **D)** Comparison of M1 and M2 metabolite amounts between S and BSHR lines. Diclofop-acid-*O*-Gluc was converted to OH-diclofop acid with β-glucosidase as shown in **A)**. The error bars, SEM ($n = 3$). * and ** show significant differences of $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). n.s., not significant. **E)** Whole-cell diclofop metabolism assay using yeast harboring empty vector (pYeDP60), *CYP81A12*, or *CYP81A21*.

investigation to test this hypothesis and gain a deeper understanding of the mechanisms of herbicide resistance in the BSHR late watergrass.

Candidate genes for high-level diclofop-methyl resistance identified by gene expression analysis

To identify the additional diclofop-hydroxylating enzyme, we performed RNA-seq analysis using the recently released draft genome of late watergrass (Ye et al. 2020), with some manual curations leading to 66,523 gene models (see Materials and methods). We detected 463 genes that were highly expressed in the BSHR line, including 18 P450 genes (Figs. 2, A and B, and S1). Among these, *CYP81A12* and *CYP81A21* were identified as the fourth and fifth most highly expressed genes, respectively, among all 551 P450 genes in the BSHR line (Fig. 2C). Four out of the 18 P450 genes encode a truncated protein: Contig168_pilon.233 (*CYP81A25P*), Contig1257_pilon.115 (*CYP81A19P*), Contig222_pilon21.b (*CYP704A*), and Contig 774_pilon.23 (*CYP71E*); thus, they were excluded from further analyses.

The candidate overexpressed genes were further screened by reverse transcription quantitative PCR (RT-qPCR) for their cosegregation with BSHR using the F6 recombinant inbred lines from a cross between the BSHR and S lines (Fig. 2D). The analysis revealed that *CYP72A122*, *CYP72A252v1*, *CYP72A252v2*, and *CYP709C69* cosegregated with diclofop-methyl resistance (Fig. 2E), suggesting they are the prime

A

Gene ID	Annotation	LogFC[a]	FDR[b]	TPM		Rank in transcriptome		Rank in CYPome		Protein sequence	Progeny association
				S	BSHR	S	BSHR	S	BSHR		
Contig363_pilon.90	CYP72A122	1.64	4.11E-09	65.64	210.46	2407	651	4	1		Yes
Contig168_pilon.232	CYP81A12	2.98	3.58E-10	13.88	112.18	8983	1390	42	4		Yes[c,d]
Contig1257_pilon.113	CYP81A21	4.30	9.18E-11	4.13	83.76	14348	1890	111	5		Yes[c,d]
Contig168_pilon.233	CYP81A25P	3.12	7.18E-11	6.96	62.00	12465	2556	80	7	Frame shift	Not tested
Contig1257_pilon.115	CYP81A19P	2.41	1.63E-09	10.87	59.79	10345	2656	58	8	Frame shift	Not tested
Contig363_pilon.94	CYP72A252v1	1.91	7.30E-08	13.17	50.81	9288	3100	48	11		Yes
Contig817_pilon.380.b	CYP709C69	6.83	5.00E-12	0.38	46.06	17366	3413	256	12		Yes
Contig90_pilon.215	CYP72A252v2	3.05	4.51E-10	5.29	45.46	13611	3457	98	13		Yes
Contig1299_pilon.29	CYP71C35	3.10	4.62E-11	3.08	27.21	15169	5510	126	25		No
Contig12_pilon.29	NGS709C67	1.66	7.85E-06	0.55	5.65	17207	13492	233	93		No
Contig10_pilon.131	CYP71S	3.29	1.94E-08	0.55	5.62	17207	13492	233	93		No
Contig960_pilon.352	CYP709C70	3.43	1.40E-06	0.39	4.63	17351	14119	253	107		No
Contig1257_pilon.112	CYP81A22	2.19	6.57E-06	0.92	4.47	16869	14220	204	108		No[c]
Contig222_pilon21.b	CYP704A	2.33	1.68E-05	0.65	3.51	17127	14891	222	122	Frame shift	Not tested
Contig490_pilon.258	CYP92A198	6.10	6.69E-08	0.04	3.10	17675	15206	340	128		Not tested
Contig1138_pilon.9	CYP88A	3.23	2.78E-06	0.28	2.71	17455	15504	269	136		×
Contig1194_pilon.509	CYP78C	1.71	9.21E-03	0.54	1.79	17213	16195	234	165		Not tested
Contig774_pilon.23	CYP71E	1.71	2.44E-03	0.43	1.49	17319	16447	248	177	Frame shift	No

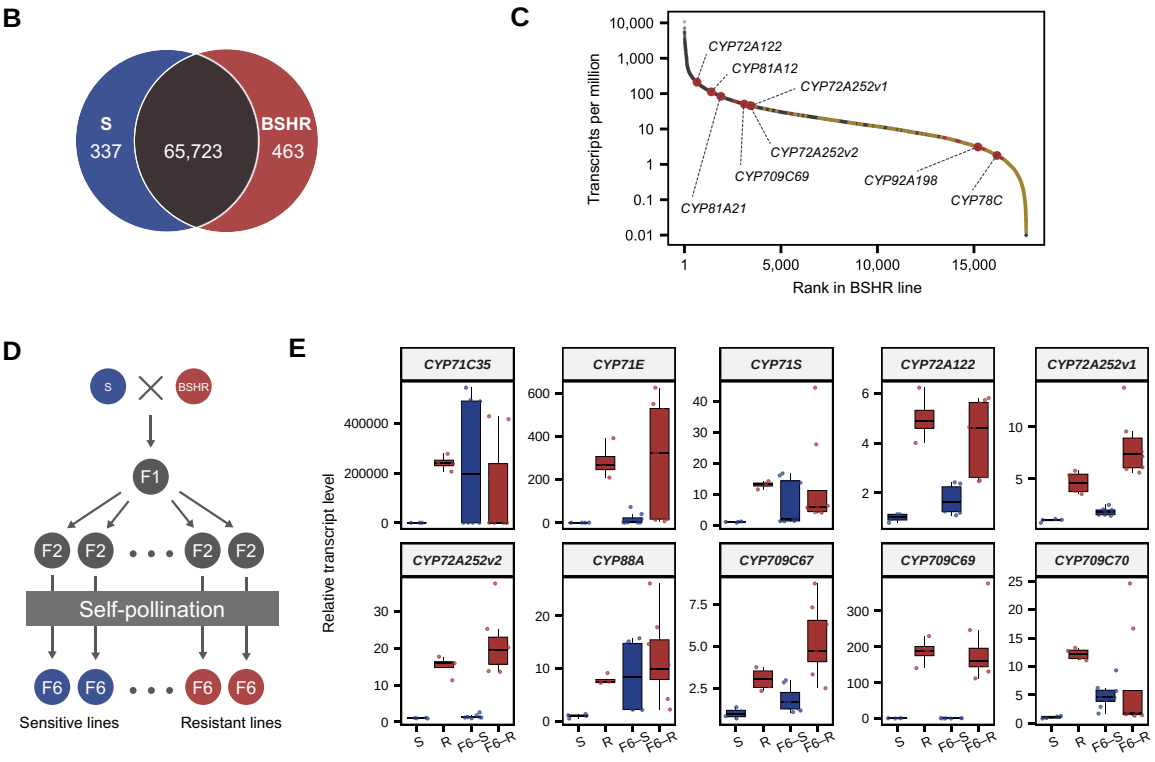

Figure 2. Candidate genes for high-level diclofop-methyl resistance in *E. phyllopogon* were identified by gene expression study. **A)** Annotations of P450 genes that were upregulated in the BSHR line. Genes are ordered according to BSHR expression rank. Progeny association study was not conducted on Contig490_pilon.258 and Contig1194_pilon.509 since the appropriate primers were not designed. The genes in gray highlight were the candidates that were further tested in rice transformation experiment. [a]Log2 fold-change. [b]False discovery rate. [c,d]The progeny-association experiments were conducted in Iwakami, Endo, et al. (2014) or Iwakami et al. (2019). **B)** Number of differentially expressed genes. **C)** Expression rank of P450 genes in the transcriptome of BSHR line. P450s genes are shown in yellow and red. Red plots represent differentially expressed P450 genes. Larger red plots are the genes shown in gray in **A)**. **D)** F6 lines used in the association study. **E)** Progeny association of the mRNA levels in the shoots (4 plants in bulk for each line) of the candidate P450 genes. mRNA levels were quantified by RT-qPCR in 16 F6 lines. The values for *CYP71C35* in the S and some F6 lines were set as 40 cycles since the signal was not detected. Each boxplot shows median (the center horizontal), interquartile range (upper and lower edges of the box), and 1.5 times the interquartile range (whisker). F6-S, sensitive F6 lines; F6-R, resistant F6 lines.

candidates for metabolite M2-producing P450(s). Thus, we subjected the 4 genes to subsequent functional characterization. In addition, we also tested *CYP92A198* and *CYP78C*, since we could not evaluate them in the RT-qPCR study as we failed to generate the standard curves, most likely due to their lower expression (Fig. 2C).

Identification of a diclofop-methyl metabolizing P450

For the evaluation of the diclofop-metabolizing activity of each gene, we used the rice transformation system as employed previously (Iwakami et al. 2019). We expressed the 6 candidate genes isolated from the BSHR line in rice calli under the control of the cauliflower mosaic virus 35S promoter. The

calli were tested for growth on the media supplemented with diclofop-methyl. Among the 6 genes, only *CYP709C69* conferred marked resistance at the lethal dose of diclofop-methyl (0.4 μM) supplied to rice calli (Supplemental Fig. S2). No difference was observed in the coding sequence of *CYP709C69* between the S and BSHR lines. When 12 independent transformed lines were subjected to diclofop-methyl, all the lines with *CYP709C69* stopped growing at 6 μM (Fig. 3A). The diclofop-methyl resistance level was equivalent to the cases of *CYP81A12* and *CYP81A21*. A decrease in diclofop-methyl sensitivity was also observed in an *Arabidopsis* (*Arabidopsis thaliana*) line transformed with *CYP709C69*, as well as in those transformed with *CYP81A12* or *CYP81A21* (Supplemental Fig. S3).

We then transformed *CYP709C69* in yeast and investigated whole-cell diclofop-methyl metabolism as performed previously (Iwakami et al. 2019). LC–MS/MS analysis revealed that M2 was produced as the main peak (Fig. 3B). To determine the hydroxylation position in the OH metabolites of diclofop acid, we purified M1 and M2 from yeast culture treated with diclofop-methyl using HPLC, which were then subjected to NMR analysis. Since the NMR structural analysis of 3 OH metabolites of diclofop-methyl has been reported in wheat previously (Tanaka et al. 1990), we compared the chemical shift patterns in the aromatic regions of M1 and M2 with the reported data. The results showed that M1 and M2 had a similar pattern with the metabolite, containing a 2,5-dichloro-4-hydroxyphenoxy moiety and 2,4-dichloro-5-hydroxyphenoxy moiety, respectively (Supplemental Fig. S4).

Notably, wheat CYP709C1, a CYP709C characterized in plants, was reported not to harbor diclofop acid-metabolizing activity (Kandel et al. 2005). Thus, we investigated a few of the other CYP709Cs in rice and late watergrass to investigate the functional conservation among CYP709Cs. In contrast to CYP709C1 in wheat, we detected M2-producing activity in CYP709C68 in late watergrass and CYP709C5 and CYP709C9 in rice in yeast whole-cell assay (Supplemental Fig. S5). These results suggest that the ability to metabolize diclofop is at least partially conserved in CYP709C-containing members.

Herbicide-metabolizing profile of CYP709C69

To investigate the role of CYP709C69 in the metabolism of other herbicides, we assessed the herbicide sensitivity of *CYP709C69*-expressing *Arabidopsis*. We also used *CYP709C69*-expressing rice calli for the evaluation of its

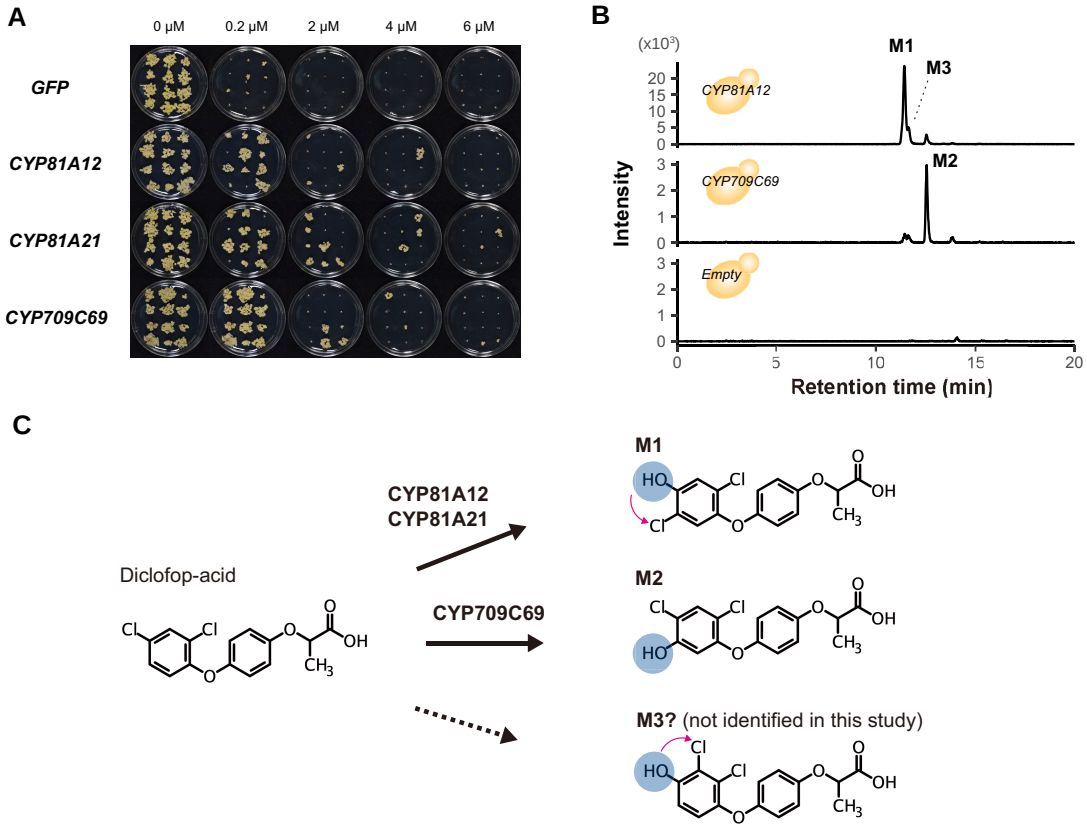

Figure 3. Identification of a diclofop-methyl metabolizing P450. **A)** Diclofop-methyl sensitivity of rice calli transformed with P450 genes of *E. phyllopogon*. Twelve secondary calli derived from 12 independent transformation events were cultivated for 3 weeks. **B)** Whole-cell diclofop-methyl metabolism assay using yeast harboring empty vector (pYeDP60), *CYP81A12*, or *CYP709C69*. **C)** The role of P450s in diclofop acid hydroxylation. The structures of M1 and M2 were determined by nuclear magnetic resonance analysis. The arrows indicate migration of chlorine. The structure of M3 was not determined in this study but likely carries a 2,3-dichloro-4-hydroxyphenoxy moiety.

metabolizing activity to ACCase inhibitors. Among a total of 46 herbicides with at least 15 modes of action, a DXS inhibitor clomazone was the only herbicide for which a marked alteration in the sensitivity of transgenic plants was observed (Supplemental Fig. S6). The *Arabidopsis* lines expressing *CYP709C69* became more vulnerable to clomazone in inverse proportion to the gene expression level (Fig. 4, A and B), in contrast to the *Arabidopsis* transformed with *CYP81As* (Guo et al. 2019). Clomazone is a proherbicide for which P450 was proposed as the enzyme to convert 5-OH clomazone and to further convert to the active 5-keto clomazone form (Fig. 4C) (Nandula et al. 2019). Thus, CYP709C69 may be involved in either of the sequential bioactivation reactions.

Overexpression of the 3 P450 genes observed in a BSHR population in Japan

To investigate the generality of the simultaneous overexpression of the 3 genes in BSHR in late watergrass, we analyzed another BSHR population. Recently, an ACCase inhibitor cyhalofop-butyl–resistant population (Eoz1814) was identified from a paddy field in Niigata Prefecture, Japan (Fig. 5A). Greenhouse experiments with commercial herbicide formulations revealed that the line purified from the population showed BSHR to ALS inhibitors (pyrimisulfan, propyrisulfuron, and penoxsulam) and synthetic auxin (quinclorac) (Supplemental Fig. S7), as was the case of the BSHR population found in California (Ruiz-Santaella et al. 2006; Chayapakdee et al. 2020; Dimaano et al. 2020). We further investigated the diclofop-methyl sensitivity of the Japanese BSHR line, together with an S line from Japan and BSHR and S lines from California. In this assay, the Japanese BSHR line showed similar resistance levels to the Californian BSHR line (Fig. 5B). Analyses of the genes encoding herbicide target sites, ACCase (*ACC1* to *ACC4*) and ALS (*ALS1* and *ALS2*), in Eoz1814 revealed no mutations involved in target-site resistance mechanisms.

We then analyzed the transcription of *CYP81A12*, *CYP81A21*, and *CYP706C69* by RT-qPCR. The Japanese BSHR line also exhibited significantly higher mRNA levels as in the case of the Californian BSHR line, although they were less pronounced (Fig. 5C). The results suggest that the BSHR lines in California and Japan share the same nontarget-site resistance mechanism. Meanwhile, similar diclofop-methyl resistance levels in the 2 BSHR lines, despite the difference in mRNA levels, imply the presence of additional mechanism(s) in the Japanese line.

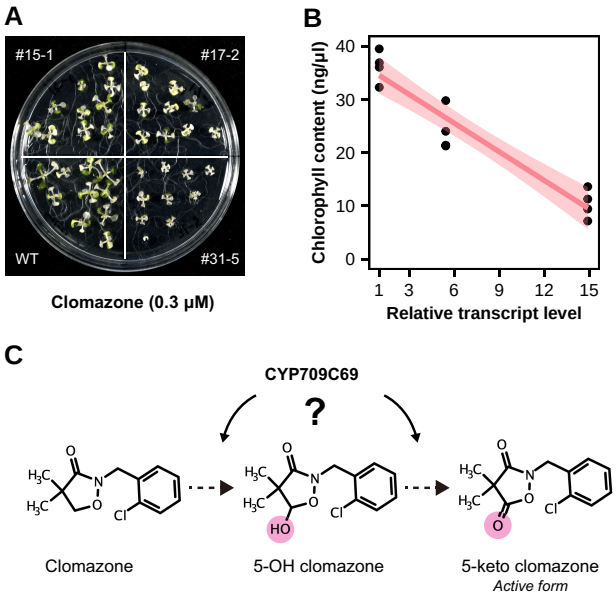

Figure 4. *CYP709C69* increased clomazone sensitivity in *Arabidopsis* (*A. thaliana*). **A)** Clomazone (0.3 μM) response of *Arabidopsis* lines transformed with *CYP709C69*. The mRNA level of each line was evaluated in Supplemental Fig. S4. **B)** Association of transcript level of *CYP709C69* ($n = 4$) and chlorophyll content in the 0.3 μM clomazone treated *Arabidopsis* lines. Linear regression line is shown with shaded regions representing the 95% confidence interval. **C)** The proherbicide clomazone is converted to the active form 5-keto clomazone through 5-OH clomazone in plants. CYP709C69 may be involved in either of the reaction.

Transcriptionally linked overexpression of distantly located herbicide-metabolizing genes

To gain further insight into the mechanism of overexpression of the 3 herbicide-metabolizing P450 genes (*CYP81A12*, *CYP81A21*, and *CYP709C69*), we estimated their genomic loci. Since the draft genome of late watergrass is contig based, the physical relationships of these genes in the chromosome are unclear. Therefore, we assigned 3 P450 gene loci to the genome of diploid *Echinochloa* sp. (*Echinochloa haploclada*) (Ye et al. 2020), the only member of the genus *Echinochloa* in which chromosome-scale genome assembly has been achieved. The *CYP81A* and *CYP709C* loci correspond to chromosomes 1 and 3, respectively, in the *E. haploclada* genome (Fig. 6A). The synteny around the *CYP81A* (Contig168 and Contig1257) and *CYP709C* regions (Contig817 and Contig960) is highly conserved in the *E. haploclada* genome (Fig. 6B), implying that the contigs are fragments of homoeologous chromosomes. The synteny structure shows that *CYP81A12* and *CYP81A21* represent a homoeologous relationship, as previously suggested by their phylogenetic relationships and herbicide-metabolizing functions (Iwakami, Endo, et al. 2014; Dimaano et al. 2020). A homoeologous gene for *CYP709C69* was not identified on Contig960, consistent with the phylogenetic analysis (Supplemental Fig. S5A). Considering that the BSHR including diclofop-methyl resistance is inherited as a Mendelian trait (Iwakami, Endo, et al. 2014; Iwakami et al. 2019) and that the overexpression of the 3 genes is transcriptionally linked (Fig. 2), a single genetic element is suggested to be responsible for the BSHR of the Californian population of late watergrass. This causal element may directly or indirectly regulate the expression of the P450s (Fig. 7).

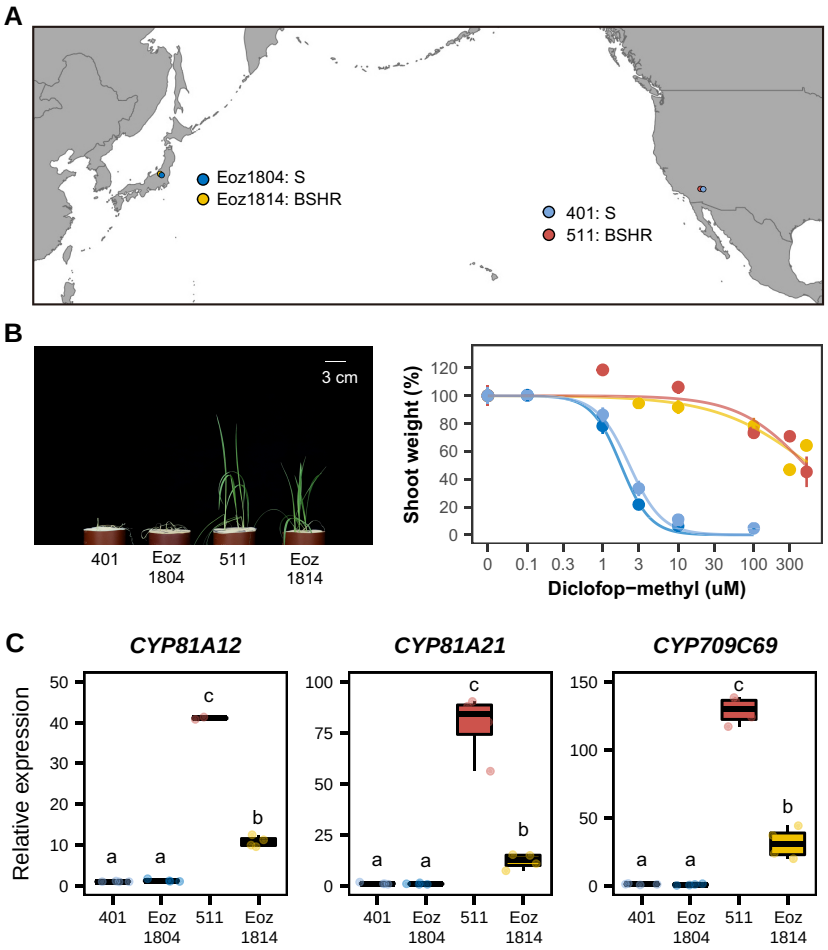

Figure 5. Overexpression of the 3 P450 genes was observed in a BSHR population found in Japan. **A)** Sampling sites of the five populations. Eoz1804 and Eoz1814 are S and BSHR, respectively. The 2 Californian populations (401 and 511) were used as controls. **B)** Diclofop-methyl response of the 4 lines. Diclofop-methyl was applied to 2.5-leaf stage plants. The plant appearance 9 days after diclofop-methyl treatment (100 μM) is shown. **C)** Transcription of the 3 P450 genes in the shoot of 2.5-leaf stage plants. Each boxplot shows median (the center horizontal), interquartile range (upper and lower edges of the box), and 1.5 times the interquartile range (whisker). Statistically significant differences evaluated using Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) are indicated by different letters ($n = 4$).

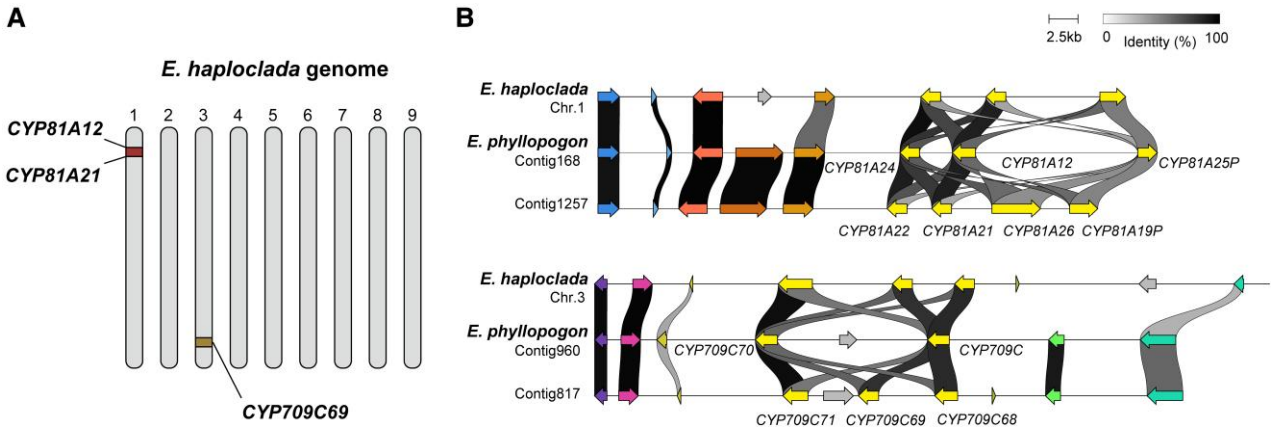

Figure 6. The loci of the 3 P450 genes estimated from the genome of diploid *Echinochloa* sp. **A)** Loci of *CYP81A12*, *CYP81A21*, and *CYP709C69* putative orthologs in diploid *E. haploclada*. **B)** Local synteny of *CYP81A* and *CYP709C* regions in the draft genome of *E. phyllopogon* (2n = 4X = 36) and *E. haploclada* (2n = 2X = 18). Yellow arrows represent P450 genes.

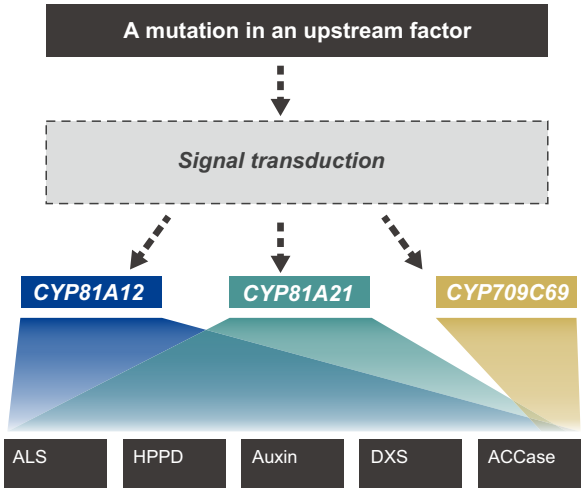

Figure 7. A working model for BSHR in *E. phyllopogon*. The overexpression of 3 P450s causes broad-spectrum resistance in *E. phyllopogon*. A causal mutation activates the expression of at least 3 herbicide-metabolizing P450s through an unknown gene-regulating system. ALS, acetolactate synthase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; DXS, deoxyxylulose 5-phosphate synthase; ACCase, acetyl-CoA carboxylase.

Discussion

We previously identified the overexpression of 2 catalytically promiscuous P450s, CYP81A12 and CYP81A21, involved in the BSHR that likely resulted from a single evolutionary event in late watergrass (Iwakami, Endo, et al. 2014; Iwakami et al. 2019). That analysis proposed the existence of a *trans*-element that drives these P450s, further implying that a number of genes may work downstream of this *trans*-element. Here, by focusing on the discrepancy between the resistance level of diclofop-methyl in the Californian BSHR line and the metabolic activity of CYP81A12/21 to diclofop-methyl, we successfully identified a diclofop-metabolizing gene by RNA-seq and genetic segregation screening. The newly discovered CYP709C69 metabolized a different position of diclofop acid from that metabolized by CYP81As, causing higher accumulation of a hydroxylated metabolite, in addition to the other hydroxylated metabolites derived from CYP81As. The data indicate that transcriptionally linked simultaneous overexpression of multiple enzymes with different substrate properties broadens the range of herbicide resistance.

CYP81A12/21 and CYP709C69 hydroxylate different positions of diclofop acid, producing M1 and M2, respectively (Fig. 3C). The position of one of the chlorine atoms in M1 is different from its original position, caused by intramolecular migration during enzymatic hydroxylation of the aromatic ring (the so-called NIH-shift), as previously reported (Tanaka et al. 1990). These hydroxylated positions are further glucosylated by glucosyltransferases in plants. If each glycosylation reaction is brought about by a different enzyme, partitioning the metabolic pathway by hydroxylating different positions of diclofop acid may allow metabolic reactions to proceed more efficiently by avoiding saturation of enzyme activity. Thus, the simultaneous activation of enzymes that hydroxylate different positions may have more than just an additive effect on the resistance level. Further research on the role of glucosyltransferases in the BSHR late watergrass may provide a more comprehensive understanding of metabolic resistance in weeds.

In the study of diclofop-methyl metabolism in wheat, 3 hydroxylated metabolites were identified, 2 of which are the M1 and M2 detected here. The third metabolite identified in wheat has a 2,3-dichloro-4-hydroxyphenoxy moiety (Tanaka et al. 1990). These findings suggest that the M3 detected as a minor peak in our yeast assay has this moiety (Fig. 3C), although we could not determine the structure of M3 due to a limited available amount of CYP81A-expressing yeast (Fig. 1, E and B). Notably, M3 was not detected in late watergrass (Fig. 1C), possibly due to differences between the experimental yeast and late watergrass systems. The yeast system was subjected to a much higher substrate concentration of the enzymes (300 μM diclofop-methyl solution for 24 h) than the late watergrass system (30 μM diclofop-methyl solution for 30 min), possibly forcing an enzymatic reaction to produce M3 in the yeast system. It is interesting to examine the possible production of the M3 metabolite in late watergrass, including comparisons with wheat, since the production of diverse hydroxyl metabolites may be associated with the resistance levels abovementioned.

In this study, we uncovered another aspect of BSHR: the simultaneous transcriptional overexpression of herbicide-metabolizing genes leads to an enhancement and broadening of metabolic resistance. This deeper understanding could provide insight into unanswered questions surrounding BSHR in Californian late watergrass. Previous studies established that overexpression of CYP81A12 and CYP81A21 underlies much of BSHR (Dimaano and Iwakami 2021) but not the resistance to bispyribac-sodium, fenoxaprop-ethyl, and cyhalofop-butyl (Fischer et al. 2000; Ruiz-Santaella et al. 2006; Bakkali et al. 2007). The presence of unidentified herbicide-metabolizing genes, which are regulated by the same system as these 3 P450 genes, may account for the resistance.

It is important to note that in uncovering BSHR in weeds, other genes that operate in concert with herbicide-metabolizing genes may weaken or counteract their individual effects. For example, CYP81A12/21 inactivates clomazone (Guo et al. 2019; Dimaano et al. 2020), while CYP709C69 increases herbicidal activity (Fig. 4). The fact that the BSHR late watergrass line exhibits clomazone resistance (Yasuor et al. 2008, 2010; Guo et al. 2019) suggests that the clomazone-inactivating force of CYP81A12/21 is stronger than the clomazone-activating force of CYP709C69. As such, the sensitivity of plants to herbicides is the result of multiple genes acting in concert, emphasizing the importance of understanding the full regulatory system of these herbicide-metabolizing genes.

Convergence across species and populations was noted for CYP81A-mediated metabolism-based resistance (Han et al. 2021). This study further disclosed that even the seemingly unlikely evolution of simultaneous overexpression of 3 genes on different chromosomes crosses population boundaries. Notably, the 2 late watergrass populations have never been selected by the herbicide diclofop-methyl. Thus, overexpression of diclofop-methyl-metabolizing CYP709C69 need not occur in the 2 populations, suggesting that it is an incidental phenomenon resulting from a mutation(s) leading to the overexpression of other enzymes, such as CYP81A12/21, required for adaptation to other herbicides. Although the mode of BSHR inheritance in the Japanese population and the differences in the causal locus between the populations need to be analyzed, an identical gene regulatory system was likely employed for resistance evolution in the 2 populations. In outcrossing weeds, the rapid evolution of nontarget-site resistance is caused by the accumulation of minor genes through pollen transfer (Yu and Powles 2014). In addition, the molecular patterns of gene accumulations often differ among resistant populations, as observed in *Ipomoea purpurea* (Van Etten et al. 2020) and *Alopecurus myosuroides* (Cai et al. 2023). This is because many genes with small effects can exist in genomes, allowing populations of outcrossing weeds to overcome herbicide stress through different combinations of genes. However, in self-pollinating weeds like late watergrass, gene accumulation through pollen transfer is less likely than that in outcrossing weeds. Therefore, a gene that endows sufficient level of resistance by itself should be necessary to evolve resistance in a short period of time. The similar BSHR mechanism in the 2 late watergrass populations may indicate that there are a limited number of genetic materials in the genome of late watergrass that can be used to drastically alter sensitivity.

The convergence of the BSHR mechanism suggests that the BSHR in the 2 populations evolved by co-opting a conserved gene regulatory system in late watergrass, rather than creating a new one. CYP81As is involved in the synthesis of the antimicrobial substance zealexin in maize (*Zea mays*) (Ding et al. 2020), and CYP709C1 harbors subterminal hydroxylation activity of C18 fatty acids in wheat (Kandel et al. 2005). The 3 P450s involved in BSHR may have originally played a role in the synthesis of these substances, and their enzymatic properties may have been co-opted for herbicide resistance evolution. Identification of the DNA mutation responsible for BSHR late watergrass and the subsequent elucidation of the downstream regulatory system will provide insight into the mechanism of BSHR expression and the likelihood of similar evolutionary events. It may also shed light on the biological costs of BSHR expression from co-opting the preexisting gene regulatory system. Interestingly, simultaneous overexpression of CYP81A and CYP709C was described in herbicide-resistant *Echinochloa* (Fang et al. 2019; Yan et al. 2019) and observed in rice under a herbicide and a safener treatment (Xu et al. 2015; Brazier-Hicks et al. 2020). This suggests that the coregulating system of the P450 genes is widely conserved in Poaceae. CYP81A and CYP709C have been found exclusively in Poaceae, suggesting that the regulatory system for these genes has likely been constructed during Poaceae evolution. Further study on the relationship between Poaceae evolution and metabolism-based resistance may provide perspectives on plant adaptation evolution to harsh environment.

Materials and methods

Origin of late watergrass (*E. phyllopogon*)

S and BSHR lines (i.e. 401 and 511, respectively) originating from the Sacramento Valley in California (Fischer et al. 2000; Tsuji et al. 2003) were used. The F6 progeny of the 2 lines was derived from a single seed descent method (Iwakami, Endo, et al. 2014). The herbicide responses of F6 lines were evaluated previously (Iwakami, Endo, et al. 2014; Guo et al. 2019; Iwakami et al. 2019; Chayapakdee et al. 2020), and 8 BSHR and 8 S lines were randomly selected for RT-qPCR experiment. In 2017, seeds from 2 populations (Eoz1804 and Eoz1814) were collected from 2 paddy fields in Niigata Prefecture, where farmers had reported poor control of late watergrass. The populations were tested for their sensitivity to cyhalofop-butyl, revealing that Eoz1814 had insufficient control with no resistance-segregating responses. A single seed from each population was self-pollinated and tested for sensitivity to commonly used *Echinochloa* herbicides in Japan. The Eoz1804 population was found to be sensitive to these herbicides. Thus, we used the population as a S control. Eoz1814 was confirmed to have resistance to cyhalofop-butyl and penoxsulam, an ALS inhibitor. A plant from each population was further self-pollinated before being used in this study. The full-length sequences of ALS genes (2 copies) and carboxyltransferase domain of ACCase genes (4 copies) were amplified in a copy-specific way and directly sequenced as previously described (Iwakami et al. 2012).

Analysis of diclofop metabolites in plants

Late watergrass were cultured hydroponically to around the 2.5-leaf stage at 25 °C under a 12-h photoperiod (~300 μmol $m^{-2}s^{-1}$), as described previously (Iwakami et al. 2019). The shoots of hydroponically cultured late watergrass (2.5-leaf stage) were dipped in 30 μM diclofop-methyl solution supplemented with Tween 20 (0.01% [v/v]) for 30 min. The shoots of 10 plants (~300 mg) were collected at 0, 1, 3, and 6 HAT. On collection, they were rinsed in distilled water containing 20% methanol (v/v) and 0.2% Triton X-100 (v/v) and snap-frozen in liquid nitrogen. The plant tissue was ground into a powder in liquid nitrogen using a mortar and pestle. Diclofop metabolites were extracted with 6 ml of 80% cold methanol (v/v), followed by centrifugation at 9,000 × g for 10 min at 4 °C. The pellet was further subjected to extraction twice with 2 ml of 80% cold methanol (v/v). The extract was combined, evaporated to dryness, and dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 5.0). The solution was treated with 0.3 mg of almond β-glucosidase

(BGH-101, Toyobo, Osaka, Japan) and incubated for 24 h at 37 °C. After the addition of 1 ml of acetonitrile, the solution was vortexed, left for 15 min at 4 °C, and then centrifuged at 1,700 × *g* for 15 min. The supernatant was collected and evaporated to remove acetonitrile. The sample was loaded onto a Sep-Pak C18 cartridge (Waters, Tokyo, Japan) and washed with 10 ml of water containing 0.1% formic acid (*v*/*v*), followed by elution with 4 ml of acetonitrile. The fraction eluted with acetonitrile was evaporated to dryness and redissolved in acetonitrile (0.5 ml/300 mg of fresh weight of plant tissue), which was subjected to mass spectrometric analysis.

Mass spectrometry analysis

Hydroxylated metabolites of diclofop acid were analyzed using a liquid chromatography–tandem mass spectrometer (Shimadzu LCMS-8030, Kyoto, Japan) equipped with an electrospray ionization source. The MS parameters used were as follows: interface voltage of 4.5 kV, desolvation line temperature of 250 °C, heat block temperature of 400 °C, nebulizing gas (N_2) of 2.0 L min^{-1}, and drying gas at 15 L min^{-1}. MRM was used to quantitate the hydroxylated metabolites of diclofop acid under the following conditions: negative ion mode with MRM transition of *m*/*z* 341 to *m*/*z* 269 (CE = 15 V, Q1 Pre Bias = 15 V, Q3 Pre Bias = 19 V). Separation of analytes was carried out using a reversed-phase column, TSK gel ODS-100 V (2 mm ID × 150 mm, 3 μm, Tosoh, Tokyo, Japan). The column was eluted with a linear gradient from 30% to 90% mobile phase B (0.1% formic acid in acetonitrile [*v*/*v*]) in mobile phase A (0.1% formic acid in water [*v*/*v*]) for 20 min at a flow rate of 0.2 ml min^{-1} at 40 °C. The injection volume was 5 μl.

RNA extraction and cDNA synthesis

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) followed by the application of TURBO DNA-*free* kit (Thermo Fisher Scientific, Tokyo, Japan) to eliminate genomic DNA. Total RNA (1 μg) was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

RNA-seq analysis

RNAs extracted from the shoot of 2.5-leaf stage plants of S and BSHR lines were used for RNA-seq analysis with 4 biological replications. RNA-seq libraries were prepared with TruSeq RNA Library Preparation Kit v2 (Illumina, USA), followed by pair-end sequencing on HiSeq 2500 sequencer (Illumina). Reads were filtered using TRIMMOMATIC (ver 0.36) (Bolger et al. 2014) with the following options: TRAILING:25, SLIDINGWINDOW:4, and MINLEN:80. The reads were mapped against coding sequences of the draft genome of late watergrass (Ye et al. 2020) using bowtie2 (ver 2.3.5.1) with the following options: "-X 900–very-sensitive–no-discordant–no-mixed–dpad 0–gbar 999999 99". The mapped reads were counted using RSEM (Li and Dewey 2011). Differential expression analysis was performed using edgeR (ver 3.28.0) with glmQLFTest (Robinson et al. 2010) in R (R Core Team 2022). Late watergrass genes were annotated by BLASTX (Blast+ v.2.9.0) (Camacho et al. 2009) against rice (*O. sativa*) protein sequences (*O. sativa* MSU release 7) and rice P450 protein sequences in Cytochrome P450 Homepage (https://drnelson.uthsc.edu/).

During the analysis, we noticed that 2 of the P450 genes (Contig817_pilon.380 and Contig 222_pilon.21) were misannotated due to the existence of similar genes next to each other (Supplemental Fig. S8). Thus, we replaced the respective coding sequences with correct sequences and mapped the reads to the revised transcriptome.

RT-qPCR

RT-qPCR was performed as described previously (Tanigaki et al. 2021) using primers listed in Supplemental Table S1. Some of the primer sets were developed elsewhere (Czechowski et al. 2005; Iwakami, Uchino, et al. 2014; Tanigaki et al. 2021). Eukaryotic translation initiation factor 4B (*EIF4B*) was used as an internal control gene, and data were analyzed using the ΔΔCt method (Schmittgen and Livak 2008).

Isolation of P450 genes

The full-length sequence of each P450 gene was amplified from the cDNA of late watergrass (line 511) using primers listed in Supplemental Table S1 with KOD FX Neo (TOYOBO) or PrimeSTAR GXL DNA Polymerase (Takara). The amplicons were subcloned using pGEM-T Easy Vector Systems Kit (Promega, Tokyo, Japan) or Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's instructions.

Plant transformation

The coding region of each P450 gene was cloned into the pCAMBIA1390 vector with the In-Fusion DH Cloning Kit (TaKaRa, Kusatsu, Japan) or SLiCE reaction (Motohashi 2015) using primers listed in Supplemental Table S1. The binary construct was introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze and thaw method (Hofgen and Willmitzer 1988). Rice calli (*O. sativa* cv. Nipponbare) were transformed as previously described (Toki 1997; Iwakami et al. 2019). *Arabidopsis* (*A. thaliana*, ecotype Col-0) was transformed via the floral dip method (Clough and Bent 1998), with hygromycin B (20 mg L^{-1}) for selection. T3 homozygous lines were selected based on the segregation ratio.

Herbicide sensitivity assay

Independently transformed calli were placed on N6D solid media supplemented with a herbicide as described previously (Iwakami et al. 2019). The secondary calli were cultured at 30 °C for 3 weeks. For the *Arabidopsis* sensitivity assay, the surface-sterilized seeds were placed on Murashige and Skoog solid media (Murashige and Skoog 1962). After 3 days of stratification at 4 °C, the media were placed in a climate chamber (22 °C, 12-h photoperiod, and 70 μE m^{-2} s^{-1}

light intensity) for 12 days. In the case of the diclofop-methyl assay, the plates were kept in the chamber for 14 days. Chlorophyll was extracted and measured as described previously (Endo et al. 2021). The herbicides and the doses are summarized in Supplemental Table S2.

The diclofop-methyl response of late watergrass was determined as described previously (Iwakami et al. 2019). Briefly, hydroponically cultured 2.5-leaf stage plants were dipped in diclofop-methyl solution for 30 min as described above. The plants were further cultivated for nine days. Dose–response curves were drawn with the drc package (ver 3.0) (Ritz et al. 2015).

Heterologous expression of P450s in yeast (*S. cerevisiae*) and whole-cell herbicide metabolism assay

Expression in yeast was conducted using WAT11 strain and pYeDP60 vector system (Pompon et al. 1996). The coding regions of other P450s were cloned into the pYeDP60 vector with the yeast Kozak sequence inserted as described before (Iwakami et al. 2019). WAT11 was transformed using the lithium acetate method (Ito et al. 1983). A whole-cell assay for diclofop-methyl metabolism was performed according to Iwakami et al. (2019), with the modification of diclofop-methyl concentration to 300 μM. After incubation for 24 h, yeast cells were centrifuged at 13,500 $\times g$ for 10 min. The supernatant was cleaned up using a Sep-Pak C18 cartridge as described above and subjected to LC–MS/MS analysis.

Structure determination of diclofop metabolites

The yeast expressing *CYP81A12* or *CYP709C69* in whole-cell assay buffer (350 ml) was incubated with diclofop-methyl (300 μM) for 24 h. The culture was centrifuged at 1,500 $\times g$ for 5 min to collect the supernatant. The yeast cells were resuspended in 1/10 volume of the buffer without diclofop-methyl. After incubation for an additional 12 h, the supernatant was collected by centrifugation, which was repeated twice. The whole procedure was repeated 30 times. After combining all the supernatants, the pH of the solution was adjusted to 1 by hydrochloric acid. The solution was extracted four times with diethyl ether (0.7 volume). After evaporating diethyl ether in vacuo, the resultant residue was dissolved in acetonitrile. HPLC (LC-10ADvp and SPD-M10Avp, Shimadzu) separation was carried out on a reversed-phase column (TSK gel ODS-100, 4.6 mm ID × 250 mm, 5 μm, Tosoh, Tokyo, Japan) with the following conditions: linear gradient from 30% to 72% mobile phase B (0.1% formic acid in acetonitrile [*v*/*v*]) in mobile phase A (0.1% formic acid in water [*v*/*v*]) for 35 min at a flow rate of 0.8 ml/min at 40 °C. The elution was monitored by UV absorbance at 215 and 280 nm. The fractions containing each hydroxylated metabolite of diclofop acid were evaporated to dryness and dissolved in deuterated chloroform. ^{1}H-NMR spectra of each metabolite were recorded on a Bruker Ascend 400 MHz spectrometer (Billerica, MA, USA) with tetramethylsilane as an internal standard.

Phylogenetic analysis

Deduced protein sequences were aligned with MAFFT (ver 7.475) (Katoh and Standley 2013). The alignments were used to estimate evolutionary topology in MEGA X (Kumar et al. 2018) using Neighbor-Joining method. JTT matrix-based method was used to compute the evolutionary distances.

Synteny analysis

The genome of *E. haploclada* (Ye et al. 2020) was used for the synteny analysis. The local synteny around the *CYP81A*s and *CYP709C*s was visualized with clinker (ver 0.0.21) (Gilchrist and Chooi 2021).

Statistical analysis

The comparison of 2 data sets was analyzed with Student's *t*-test. Tukey's HSD test was employed for multiple comparisons. Both were performed using R (R Core Team 2022). Other statistical analyses were described in each section.

Accession numbers

Sequence data from this article can be found at http://ibi.zju.edu.cn/RiceWeedomes/Echinochloa/under gene IDs listed in Fig. 2A.

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Author contributions

Conceptualization: S.I.; Research design: S.I., H.S., M.M., Y.Y., T.Y., K.T., S.T., T.K., and T.T.; RNA-sequencing: K.T.; Bioinformatics: S.I., H.S., and T.K.; LC–MS/MS and NMR: H.S., Y.Y., and M.M.; Japanese line characterization: T.K., A.U., S.A., and M.H.; Gene characterization: H.S., S.I., T.Y., and T.K.; Writing: S.I., H.S., M.M., and A.U.; Visualization: S.I., H.S., and T.K.; Supervision: S.I. and T.T.; and Funding acquisition: S.I., A.U., and T.K.

Supplemental data

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

Supplemental Figure S1. Detection of differentially expressed genes by RNA-seq.

Supplemental Figure S2. Diclofop-methyl sensitivity of P450 genes in late watergrass (*E. phyllopogon*).

Supplemental Figure S3. Diclofop-methyl sensitivity of *Arabidopsis* (*A. thaliana*) transformed with P450 genes in late watergrass (*E. phyllopogon*).

Supplemental Figure S4. ^{1}H-NMR spectra for OH-diclofop acid metabolites.

Supplemental Figure S5. Diclofop-methyl metabolizing activity of CYP709Cs.

Supplemental Figure S6. Herbicide metabolizing activity of CYP709C69.

Supplemental Figure S7. Herbicide sensitivity of late watergrass (*E. phyllopogon*) in Japan.

Supplemental Figure S8. Annotation correction.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. The summary of herbicides used for the functional characterizations of CYP709C69.

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Conflict of interest statement. The authors declare no competing interests.

Data availability

The mRNA-Seq data have been deposited in the DDBJ Sequence Read Archive (DRA) database (accession number DRA013092).

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