Simultaneous overexpression of multiple herbicide-metabolizing genes for broad-spectrum resistance in an agricultural weed *Echinochloa phyllopogon*

Hiroe Suda¹, Yusuke Yoshimoto¹, Tomomi Kubo¹, Keisuke Tanaka², Satoru Tanaka³, Takuya Yamaguchi⁴, Masahiro Miyashita¹, Tohru Tominaga¹, Satoshi Iwakami¹

¹ Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

² NODAI Genome Research Center, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

³ Faculty of Regional Environment Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

⁴ Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

Corresponding Author: Satoshi Iwakami

Tel: +81 75 753 6064

Email: iwakami.satoshi.2v@kyoto-u.ac.jp

Summary

The use of herbicides in agricultural fields has driven the evolution of weeds for resistance, causing a grave threat to the current agriculture. One big mystery of weed resistance involves multiple-herbicide resistance (MHR) concomitant to enhanced herbicide metabolism. Previous research unveiled that the overexpression of catalytically promiscuous cytochrome P450s underlies the metabolism-based cross-resistance in multiple species. However, the concept of activation of promiscuous enzymes does not fully accommodate the resistance to diverse herbicides in MHR *Echinochloa phyllopogon* although the genetic inheritance of MHR was suggested as under a single gene control. Here, we show that the high-level resistance to diclofop-methyl in *E. phyllopogon* is caused by the simultaneous overexpression of CYP81A12/21, the previously identified promiscuous P450s, and a novel P450 CYP709C69. We found that the MHR line rapidly produced two distinct hydroxylated-diclofop-acid, only one of which was the major metabolite produced by CYP81As. RNA-seq followed by real-time PCR in the crossed progeny of MHR and sensitive lines identified several P450 genes whose overexpressions were associated with MHR. Gene functional characterization revealed that only CYP709C69 conferred diclofop-methyl resistance in rice calli and produced another hydroxylated-diclofop-acid in yeast, reinforcing the relatively low activity of CYP81As to diclofop-methyl. Plants transformed with CYP709C69 had unchanged sensitivity to 46 herbicides except for clomazone, where transgenic plants became more susceptible. The present findings establish a novel concept that simultaneous overexpression of herbicide-metabolizing genes enhances and broadens the profile of metabolic resistance in weeds.

Key words: cytochrome P450, herbicide resistance, herbicide metabolism, resistance evolution, metabolism-based cross-resistance

Introduction

The current crop production relies heavily on herbicides to maximize crop yield by removing weeds from agricultural fields in a labor-saving way. However, herbicides, in turn, have acted as a selective pressure on a vast number of weed individuals in agricultural fields, resulting in the evolution of herbicide resistance.¹ Resistance evolution is occurring on a global scale and poses a grave threat to current food security. Weed populations have evolved diverse mechanisms of resistance from well-characterized mutations in herbicide target gene (target-site resistance, TSR) to the other much-less-understood mechanisms (non-target-site resistance, NTSR).² The devastating impact on agriculture can be brought about by enhanced herbicide-metabolism, one of the major NTSR mechanisms. Weeds with enhanced herbicide-metabolism often exhibit a large scope of resistance to chemically unrelated herbicides, making chemical control extremely difficult.³ Despite the serious threat of metabolism-based resistance, the details for the expression of multiple-herbicide resistance (MHR) remain largely unknown. A greater understanding of the evolutionary processes and physiological mechanisms of MHR is urgently required.

Recently, cytochrome P450 monooxygenases (P450s) belonging to CYP81A subfamily were reported to encompass pleiotropic expression of MHR in *Echinochloa phyllopogon*,⁴ an allotetraploid ($2x=4x=36$) and predominantly self-fertilizing weed in rice cultivation.⁵ Resistant populations found in California in 1997 mysteriously exhibited resistance not only to the herbicides used in the fields at that time but also to many other herbicides commercialized later.⁶ Intensive analyses of this MHR population, mainly from biochemical and molecular approaches, have led to the discovery that the overexpression of two CYP81A cytochrome P450 monooxygenases (P450s) are responsible for the cross-resistance to many herbicides such as acetolactate synthase (ALS) inhibitors,^{4,7} acetyl-CoA carboxylase (ACCase) inhibitors,⁸ a 1-deoxy-D-xylulose 5-phosphate synthase (DXS) inhibitor,⁹ and a synthetic auxin (unpublished data). The two P450s, CYP81A12 and CYP81A21, exhibit catalytic promiscuity against diverse herbicide chemistries, accommodating the resistance to multiple herbicides in the *E. phyllopogon* population.⁴ Through genetic segregation study, the overexpression of the two P450s was suggested to be controlled by an unidentified single element.⁷ A series of research provided molecular evidence that the overexpression of promiscuous enzyme at least partly accommodates MHR that is often accompanied in weeds with enhanced herbicide metabolism.

While the identification of the two responsible genes advanced the understanding of metabolic resistance in weeds, the mystery of MHR in the Californian population is not fully

understood, among which is the high-level resistance to diclofop-methyl. Diclofop-methyl is the first commercialized acetyl-CoA carboxylase (ACCase) inhibiting herbicide. ACCase inhibitors are solely active to Poaceae plants by inhibiting the plastidic form of ACCase present in Poaceae.¹⁰ Previously, we found that the MHR line is highly resistant to diclofop-methyl, and the resistance is inherited following the Mendelian segregation ratio as in other herbicides.⁸ The subsequent genetic and molecular studies revealed that the overexpression of the two *CYP81A* genes is linked with resistance in the crossed-progeny of sensitive (S) and MHR lines, and the yeast lines expressing either of the two *CYP81As* produced hydroxylated form of diclofop-methyl. However, we also noticed that the resistance level of transgenic rice lines expressing *CYP81A12/21* to diclofop-methyl was not prominently higher than that of other ACCase herbicides (tralkoxydim and pinoxaden), which is inconsistent with the observation that the resistance level of the MHR line to diclofop-methyl is much higher than the two herbicides.⁸ Thus, we reasoned that an additional element specific to diclofop-methyl metabolism underlies the high-level diclofop-methyl resistance. Here, we report that the overexpression of a novel diclofop-methyl metabolizing P450, CYP709C69, is involved in high-level resistance to diclofop-methyl. The P450 hydroxylates diclofop-acid, the active body of diclofop-methyl, in a manner different from *CYP81As*. In stark contrast to the catalytic promiscuity observed in *CYP81As*, CYP709C69 exhibited little metabolic activity to other herbicides except that it may be involved in the activation of a DXS inhibitor clomazone. This study highlights that simultaneous overexpression of herbicide-metabolizing genes encoding enzymes with different enzymatic properties is involved in MHR in weeds. The concept is distinct from the accumulations of multiple genes for broad or high-level resistance and serves as a model for future studies of metabolic resistance in weeds.

Results

Two distinct hydroxylated metabolites of diclofop-acid are highly accumulated in MHR *E. phyllopogon*

To gain clues for the high-level resistance to diclofop-methyl in the MHR line, we investigated the metabolism of diclofop-methyl in *E. phyllopogon*. Diclofop-methyl is a pro-herbicide that is activated by endogenous esterase as is the case for most aryloxyphenoxy-propanoate herbicides.¹¹ Following the de-esterification, the resultant active body diclofop-acid is inactivated by P450-mediated hydroxylation followed by further glycosylation (Fig. 1A).^{12–14} In the metabolism scheme,

diclofop-acid hydroxylation is the rate-limiting step for diclofop-methyl inactivation in plants.¹⁵ Our previous study showed that both the MHR and S lines converted the majority of diclofop-methyl into diclofop-acid by 3 h after treatment for 30 min.⁸ In this study, 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), which correspond 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). The signal intensity of M2 was higher than that of M1 under this condition. In line with our study, a variety of hydroxylated diclofop-acid metabolites were reported in wheat,^{16,17} suggesting the presence of at least partially overlapping metabolic pathway for diclofop-methyl between wheat and *E. phyllopogon*.

We next compared the amount of each hydroxylated metabolite between the MHR and S lines. To accurately evaluate the hydroxylation activity, possible glycosylated metabolites were converted into hydroxylated diclofop-acid by treating β -glycosidase to the extract of the diclofop-methyl treated plants (Fig. 1A), which was then subjected to comparative quantification between the lines. As a result, the amounts of both hydroxylated metabolites were higher in MHR plants at 0 and 1 HAT although no difference was observed at 3 and 6 HAT (Fig. 1D).

To investigate whether the metabolites are produced by CYP81A12 and CYP81A21, the P450s responsible for the resistance to the majority of herbicides in the MHR line,⁴ we analyzed the *in vivo* diclofop-methyl metabolism of yeast carrying *CYP81A12/21*. In the yeast system, another peak (M3), which was not clearly separated from M1, was observed in addition to M1 and M2. Furthermore, the chromatographic profile of M1 and M2 differed between *E. phyllopogon* and yeast. The intensity of M2 was higher in *E. phyllopogon*, while that of M1 was prominently higher in yeast (Fig. 1E). The result suggests that an additional enzyme that catalyzes hydroxylation to form M2 is involved in diclofop-methyl resistance in the MHR line.

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

To identify the additional diclofop-hydroxylating enzyme, we performed RNA-seq analysis using the recently released draft genome of *E. phyllopogon* with 66,521 gene models.¹⁸ Among the gene models, we identified 549 putative P450 genes. A transcriptome comparison detected some P450 genes highly expressed in the MHR line. A closer look at the sequences of these P450 genes found

that at least two of them (Contig817_pilon.380 and Contig 222_pilon.21) were misannotated due to the existence of similar genes next to each other (Fig. S1). Thus, we replaced the respective coding sequences with two correct sequences and mapped the reads to the revised transcriptome.

Finally, we detected 463 genes that were highly expressed in the MHR line (Fig. 2A, B, C). Among the genes, 18 P450 genes were detected including previously reported multiple-herbicide metabolizing *CYP81A12* and *CYP81A21* taking the top four and five positions, respectively, among all the highly-expressed P450 genes in the MHR line (Fig. 2D, E, F). All but one of the identified genes were among the not-highly expressed genes in the S line with transcripts per million (TPM) being less than the mean of 15.032. On the other hand, the transcript levels of nine out of 18 P450 genes were placed among the highly expressed genes in the transcriptome of the MHR line (Fig. 2D). Note that they were also among the most highly expressed genes in the CYPome of the MHR line (Fig. 2E). These results suggest that the identified overexpressed genes may have an important role in MHR *E. phyllopogon*. Four of them respectively encode a truncated protein: Contig168_pilon.233 (*CYP81A25P*), Contig1257_pilon.115 (*CYP81A19P*), Contig222_pilon21.b (*CYP704A*), and Contig774_pilon.23 (*CYP71E*); thus, they were excluded from further analyses.

The candidate overexpressed genes were further screened by real-time PCR for their co-segregation with MHR using the F6 recombinant inbred lines from a cross between the MHR and S lines (Fig. 2G). The analysis revealed that *CYP72A122*, *CYP72A252v1*, *CYP72A252v2*, and *CYP709C69* co-segregated with diclofop-methyl resistance, suggesting that they are the prime candidates for metabolite M2-producing P450(s). Thus, we subjected the four genes to subsequent functional characterization. We also tested the function of *CYP92A198* and *CYP78C* as we failed to evaluate them by real-time PCR due to failures in designing appropriate primers.

Identification of novel diclofop-methyl metabolizing P450

For the evaluation of the diclofop-metabolizing activity of each gene, we used the rice transformation system as employed previously.⁸ We expressed the six candidate genes in rice calli with the pCAMBIA1390 vector, where the transgene was placed under the control of Cauliflower mosaic virus 35S (CaMV35S) promoter. The calli were tested for growth on the media supplemented with diclofop-methyl. Among the six genes, only *CYP709C69* conferred marked resistance at the lethal dose of diclofop-methyl (0.4 μ M) to rice calli (Fig. S2A). No difference was observed in the coding sequence of *CYP709C69* between the S and MHR lines. When 12 independent transformed lines

were subjected to diclofop-methyl, all the lines with *CYP709C69* stopped growing at 6 μ M (Fig. 3A). The diclofop-methyl resistance level was equivalent to the cases of *CYP81A12* and *CYP81A21*.

We also tested diclofop-methyl sensitivity of *Arabidopsis thaliana* transformed with *CYP709C69* although the major plastidic form of ACCase is insensitive to ACCase inhibitors. Using the same vector, we transformed *A. thaliana* and selected the highest expressing line among the three T3 homozygous lines (Fig. 3B). The highest expressing line (#31-5) was tested for diclofop-methyl sensitivity together with previously described *A. thaliana* lines with high-level expression of *CYP81A12* or *CYP81A21*, which exhibit high-level resistance to many herbicides.^{4,7} Although diclofop-methyl is an ACCase inhibitor specifically effective to Poaceae plants, a high dose of diclofop-acid inhibits the growth of *A. thaliana*.¹⁹ In the current study, the similar growth inhibition in wild-type *A. thaliana* was observed after the treatment with diclofop-methyl at 3 to 30 μ M. On the other hand, the lines with the P450 genes were less affected although only marginal resistance was observed in the *CYP81A12* line. Thus, we confirmed that *CYP709C69* protects the species where the dominant form of the herbicide target-site is insensitive to it.

We then transformed *CYP709C69* in yeast and investigated diclofop-methyl metabolism *in vivo* as performed previously.⁸ LC-MS/MS analysis revealed that M2 was produced as the main peak (Fig. 3C). To determine the hydroxylation position in the OH-metabolites of diclofop-acid, we purified M1 and M2 from yeast culture applied with diclofop-methyl using high-performance liquid chromatography (HPLC), which were then subjected to nuclear magnetic resonance (NMR) analysis. Since the NMR structural analysis of three OH-metabolites of diclofop-methyl has been reported previously,²⁰ we compared the chemical shift patterns in the aromatic regions of M1 and M2 with the reported data. The results showed that M1 has a similar pattern with the metabolite containing a 2,5-dichloro-4-hydroxyphenoxy moiety and M2 with that containing a 2,4-dichloro-5-hydroxyphenoxy moiety (Fig. S3). The position of one of the chlorine atoms in M1 is different from the original position, which is thought to be formed by intramolecular migration during enzymatic hydroxylation of the aromatic ring (so-called NIH-shift) as previously reported (Fig. 3D).²⁰ This implies that *CYP81A12* and *CYP81A21* mediate the NIH-shift reaction in diclofop metabolism. In this study, we could not determine the structure of M3 due to the limited amount available from *CYP81A* expressing yeast (Fig. 1E, 3C). Considering that wheat produces the hydroxylated diclofop with a 2,3-dichloro-4-hydroxyphenoxy moiety in addition to the metabolites shown above, M3 may carry this moiety (Fig. 3D) although further experiments are required.

We further tested the diclofop-methyl metabolizing activity of other CYP709Cs in *E. phyllopogon* although no association with resistance was observed in the gene expression analyses (Fig. 2G). We isolated five more genes, i.e., *CYP709C67* (Contig12_pilon.29), *CYP709C68* (Contig817_pilon.380.a), *CYP709C70* (Contig960_pilon.352), *CYP709C71* (Contig817_pilon.378), and *CYP709C* (Contig960_pilon.355), and transformed them in rice calli. In stark contrast to *CYP709C69*, only a marginal activity was observed in *CYP709C68/70/71*, where some callus lines slightly grew only at 0.4 μ M (Fig. S2B). The genome holds six more putative *CYP709C* genes (Contig1380_pilon.11, Contig12_pilon.1, Contig291_pilon.387, Contig1182_pilon.1, Contig291_pilon.396, and Contig120_pilon.313) although the annotated protein sequences other than Contig1380_pilon.11 and Contig291_pilon.387 were too short (<331) for a canonical P450 sequence. Together, *CYP709C69* is likely the major contributor to the production of M2 metabolite in *E. phyllopogon*.

CYP709C69 has a narrow substrate specificity

To investigate the role of *CYP709C69* in the metabolism of other herbicides, we tested the herbicide sensitivity of *CYP709C69*-expressing *A. thaliana*. We also used *CYP709C69*-expressing rice calli for the evaluation of its metabolizing activity to ACCase inhibitors (Fig. 4A). Among a total of 46 herbicides from at least 15 modes of action, a DXS inhibitor clomazone was the only herbicide where a marked alteration in the sensitivity of the transgenic plants was observed (Fig. S4). The *A. thaliana* lines expressing *CYP709C69* became more vulnerable to clomazone in inverse proportion to the expression level (Fig. 4B, Fig. 3B), in contrast to the *A. thaliana* transformed with *CYP81As*.⁹ Clomazone is a pro-herbicide where P450 was proposed as the enzyme to convert 5-OH clomazone, which is then further converted to the active form 5-keto clomazone (Fig. 4C).⁶ Thus, *CYP709C69* may be involved in this sequential bio-activation reaction.

Distantly located herbicide-metabolizing genes are simultaneously overexpressed

Finally, to gain insights into the mechanism of overexpression of the three herbicide-metabolizing P450 genes (*CYP81A12*, *CYP81A21*, and *CYP709C69*), we estimated the genomic loci of the three genes. Since the current draft genome of *E. phyllopogon* is contig-based, the physical relationships of the three P450 genes in the chromosome are unclear. Thus, we allocated the loci of the three P450 genes in the chromosome-scale genome of diploid *Echinochloa* sp. (*E. haploclada*).¹⁸ The synteny

around the *CYP81A* regions (Contig168 and Contig1257) and *CYP709C* regions (Contig817 and Contig960) is highly conserved in the *E. haploclada* genome (Fig. 5A). The syntenic dot plot also shows that the two contig sets are highly conserved (Fig. 5B), providing strong evidence that each set represents a part of the homeologous chromosome. The *CYP81A* and *CYP709C* loci respectively correspond to chromosomes 1 and 3 in the *E. haploclada* genome (Fig. 5C). The data strongly suggest that the three P450 genes are physically unlinked. Considering that the MHR including diclofop-methyl resistance is inherited as a Mendelian trait ^{7,8} and that the overexpression of the three genes is genetically linked (Fig. 2), a single genetic element is behind the MHR of the Californian population of *E. phyllopogon*. The causal element may directly or indirectly regulate the expression of the P450s (Fig. 5D).

Discussion

Molecular mechanism of multiple-herbicide resistance

The expression of MHR in metabolism-based resistance is one of the biggest mysteries in herbicide resistance in weeds. Previous studies have uncovered that *E. phyllopogon* carries enzymes with low substrate specificity, namely *CYP81A12* and *CYP81A21* that when overexpressed, lead to resistance to many herbicides ranging from multiple modes of action. ⁸ On the other hand, MHR *E. phyllopogon* exhibits resistance even to some herbicides to which the *CYP81As* have little or no activity, making the overexpression of *CYP81As* not the prime explanation to the expression of MHR trait. In this study, by focusing on the discrepancy between the resistance level of diclofop-methyl in the Californian MHR line and the metabolic activity of *CYP81A12/21* to diclofop-methyl, we revealed that the overexpression of a novel P450 *CYP709C69* is genetically linked with MHR in the crossed progeny, where the overexpression of *CYP81A12* and *CYP81A21* has also been observed. Although the diclofop-methyl metabolizing activity of *CYP709C69* inferred from the rice transformation experiments is not distinctly different from those of *CYP81As*, the simultaneous actions of these P450s would result in high-level resistance to diclofop-methyl. The findings add another layer in the understanding of metabolic resistance that herbicide-metabolizing enzymes can act simultaneously on herbicides.

Our findings show that MHR *E. phyllopogon* neutralizes many herbicides by simultaneous overexpression of several herbicide-detoxifying genes with different enzymatic properties, expanding the resistance window. This simultaneous overexpression of the multiple genes is strongly suggested to be under a single switch in this MHR line, which differs from a mere stepwise accumulation of

multiple mechanisms as suggested in other species.^{21,22} While in obligate cross-pollinating weeds outcrossing and the resultant mechanism accumulation can easily occur, cross-pollination in self-pollinating species such as *E. phyllopogon* rarely occurs, making the stepwise accumulation of multiple mechanisms unlikely, although not excluded. Intriguingly, co-segregations of overexpression and MHR were observed in at least three more P450 genes in the F6 lines, i.e., *CYP72A122*, *CYP72A252v1*, and *CYP72A252v2* (Fig. 2G). Similarly, the overexpression of other genes related to herbicide resistance such as glutathione S-transferases and transporters may also co-segregate with resistance although we have not investigated the possibility. If any of the genes carry herbicide-metabolizing function, the scope of resistance of the MHR line would be expanded. This study provides an answer to the evolutionary mystery of MHR in agricultural weeds: MHR is caused by the result of "simultaneous" activation of genes encoding herbicide-detoxifying enzymes with different enzymatic properties. It also highlights once again the threat of resistant weeds, where a single evolutionary step can lead to the evolution of resistance to multiple herbicides.

The major remaining challenge is the identification of the element responsible for the overexpression of the three P450 genes. In insecticide resistance, *cis*-mutation including gene amplification is often reported as a major mechanism of overexpression of insecticide-metabolizing P450 genes.²³ In the case of MHR *E. phyllopogon*, *cis*-mutation is excluded as a mechanism for *CYP81A12/21*: some of F6 lines with the S-line allele of *CYP81A12/21* exhibited resistance.⁷ We did not present the demonstrative evidence to exclude *cis*-element as the cause of *CYP709C69* overexpression due to the difficulties of the analysis as there are no nucleotide polymorphisms within and around the gene between the S and MHR lines. However, multiple observations, e.g., Mendelian segregation of MHR, co-segregation of the overexpression of the three P450 genes, and physically unlinked genetic loci of the three genes, form circumstantial evidence to support the role of *trans*-element for the overexpression of *CYP709C69*. *Trans*-elements that cause the overexpression of genes for resistance have remain unidentified in pesticide resistance, making their identification an important issue in the comprehensive understanding of pesticide resistance.

In the case of MHR in *E. phyllopogon*, we are speculating the possibility that the regulatory system of P450 gene expression, which was originally evolved in *E. phyllopogon* for some function, may have been evolutionarily co-opted to counter herbicide stress. In aphids, overexpression of a P450 gene that enabled host shift to tobacco by inactivating nicotine has caused neonicotinoid-insecticide resistance.^{24,25} Similarly, the regulatory system of the three P450 genes of *E.*

phyllopogon may have some physiological or ecological function in *E. phyllopogon*. As CYP81As in maize are involved in the biosynthesis of zealexins, antimicrobial substances,²⁶ CYP81As in *E. phyllopogon* may have a similar function. Elucidation of the regulatory system and the endogenous function of each P450 may provide insight into the fitness costs associated with resistance expression and hints for the control of MHR plants that are hardly controlled by herbicide chemistries. In addition, the deeper understanding may also provide insight into the generality and the likelihood of this agronomically-devastating evolutionary mechanism.

New aspects on the modes of herbicide metabolism in plants

The current study enlightens new aspects of the modes of P450-mediated herbicide-metabolism in plants. Firstly, our results show that a single chemistry is subjected to independent hydroxylation reactions by multiple P450s *in planta*: i.e., the active body of diclofop-methyl, diclofop-acid, is hydroxylated at the different positions of its aromatic ring by two types of P450 enzymes. Similarly, the involvement of more than one P450 for a single herbicide metabolism was suggested in other species although their influence on herbicide metabolism *in planta* remains untapped: e.g., Jerusalem artichoke (*Helianthus tuberosus*) for phenylurea herbicides by CYP76B1, CYP73A1, and CYP81B1^{27–29} and *indica* rice for bensulfuron-methyl by CYP81A6 and CYP72A31.^{30–32} In addition to providing evidence of simultaneous inactivating-actions to diclofop-methyl by multiple enzymes (CYP81A12/21 and CYP709C69), our results also suggest a possibility that these enzymes also act on clomazone both in activating (CYP709C69) and inactivating (CYP81A12/21) ways in *E. phyllopogon*. Clomazone is one of the herbicides that undergoes complexed multiple oxidative pathways in plants.³³ Our previous studies showed that *A. thaliana* expressing CYP81A12 or CYP81A21 became resistant to clomazone⁹ and that they oxidize clomazone to an unidentified metabolite.⁴ Although the detailed role *in planta* remains to be explored, the three P450s likely take part in clomazone metabolism in *E. phyllopogon*. Note that the Californian MHR *E. phyllopogon* exhibits resistance to clomazone,³⁴ suggesting that CYP81A12 and CYP81A21 are the greater influence in the balance of the activating and inactivating forces by CYP709C69 and CYP81As, respectively. The higher transcript levels of CYP81A12/21 in the MHR line (Fig. 2) may play a role in the expression of resistance phenotype to clomazone. While some herbicides are metabolized through a single major metabolic pathway, many herbicides follow multiple metabolic pathways in plants. Connecting the dynamics of herbicide metabolism in plants with the factors involved in each metabolic step is important for better

understanding of not only the evolution of weed resistance but also the basis of herbicide selectivity and mode of action.

In addition, we disclosed that CYP81As mediate chlorine migration reaction in the process of diclofop hydroxylation. The hydroxylation of the aromatic ring can cause the migration of the original functional group such as halogen, alkyl group, and carboxyl group, to the adjacent carbon, which is called NIH-shift. ³⁵ P450s are among the major enzymes involved in NIH-shift with the detailed molecular mechanisms of the migration reaction not fully understood. ³⁶ 2,4-D and diclofop-methyl are the two major herbicides involving NIH-shift in their metabolism *in planta*. ³⁷ It will be interesting to see whether CYP81A also induces NIH-shift for 2,4-D.

Negative cross-resistance by P450 genes

The negative aspect of CYP709C69 that we found in this study is of particular interest. When a mechanism that confers resistance to one herbicide increases susceptibility to another herbicide, the phenomenon is called negative cross-resistance. While negative cross-resistance has been documented in the case of TSR mechanisms such as photosystem II inhibitors, ³⁸ phytoene desaturase inhibitors, ³⁹ and tubulin assembly inhibitors, ⁴⁰ it is rarely associated with NTSR. A similar phenomenon has been observed for *CYP81A12/21*, which increases sensitivity to ALS inhibiting herbicide pyriftalid when expressed in *A. thaliana* ⁴. The results suggest that the P450 genes act as the Achilles' heel of metabolic resistance. The MHR line from California is resistant to both clomazone and pyriftalid, ⁴ which is likely due to the influence of other genes as discussed above in the case of clomazone. Simpler resistance mechanisms such as gene amplification in one of the three P450 genes would be interesting to further investigate the sensitivity to these herbicides.

The insights from this study may offer potential novel targets for the generation of genetically modified herbicide-resistant crops. The introduction of herbicide-resistant crops has been instrumental in efficient weed management by enabling new uses of existing herbicide technologies. These herbicide-resistant crops have been produced mainly by transforming a herbicide-resistance gene from other species or introducing a point mutation into the TSR genes through random mutagenesis ⁴¹. If the herbicide-activation functions observed in CYP709C and CYP81A of *E. phyllopogon* are conserved in crops, knocking out the corresponding crop genes may result in the generation of herbicide-resistant cultivars, providing an additional approach to genetically modified herbicide-resistant crop technology.

Conclusion

Our study presents a novel concept that metabolic MHR is brought about by the simultaneous activation of herbicide-metabolizing genes. This concept goes beyond the previously established understanding of MHR where concomitant resistance is caused by the activation of catalytically promiscuous enzymes, underscoring the complexity of the mechanism of metabolism-based MHR. The genes in the same regulatory system may not be limited to *CYP709C69* but may also include other herbicide metabolism-related genes such as P450s, glutathione S-transferases, glycosyltransferases, and transporters. Depending on the herbicide-metabolizing function of these genes, the range of resistance to herbicides would be further extended. MHR *E. phyllopogon* also shows resistance to herbicides that cannot be explained by the three P450 genes, such as fenoxaprop-*P*-ethyl,⁴² which may be brought about by the gene(s) simultaneously activated. The simultaneous activation of herbicide metabolic genes proposed in this study needs to be tested in various populations and species.

METHODS

Origin of plant material

S and MHR lines of *E. phyllopogon* were originated from Sacramento Valley in California.⁴³ The F6 progeny of the two lines was derived from a single seed descent method.⁷ The herbicide responses of F6 lines were evaluated previously.^{7-9,44}

Analysis of diclofop metabolites in plants

E. phyllopogon were cultured hydroponically to around 2.5 leaf-stage at 25°C with a 12-h photoperiod (approximately 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$) as described previously.⁸ The shoots of hydroponically cultured *E. phyllopogon* (2.5 leaf stage) were dipped in 30 μM diclofop-methyl solution supplemented with Tween 20 (0.01%) for 30 min. The shoots of 10 plants (approximately 300 mg) were collected at 0, 1, 3, and 6 h after application. On collection, they were rinsed in distilled water containing 20% methanol and 0.2% Triton X-100, and snap-frozen in liquid nitrogen. The plant tissue was ground into powder in liquid nitrogen with a mortar and pestle and extracted with 6 ml of 80% cold methanol, followed by centrifugation at 9,000 g for 10 min at 4°C. The pellet was further extracted twice with 2 ml of 80% cold methanol. 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, 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 spectrometric 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 (N₂) of 2.0 l/min, and drying gas at 15 l/min. 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-100V (2 mm ID x 150 mm, 3 μ m, Tosoh, Tokyo, Japan). The column was eluted with a linear gradient from 30 to 90% mobile phase B (0.1% formic acid in acetonitrile) in mobile phase A (0.1% formic acid in water) for 20 min at a flow rate of 0.2 ml/min at 40°C. The injection volume was 5 μ L.

RNA extraction and cDNA synthesis

Plants were cultivated as described above. 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 μ g) 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 MHR lines were used for RNA-seq analysis with four 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)⁴⁵ with the following options: TRAILING:25, SLIDINGWINDOW:4, MINLEN:80. The reads were mapped against coding sequences of the draft genome of *E. phyllopogon*¹⁸ using bowtie2 (ver 2.3.5.1) with the following options: '-X 900-very-sensitive-no-discordant-no-mixed-dpad 0-gbar 99999999'. The mapped reads were counted using RSEM.⁴⁶ Differential expression analysis was performed using edgeR (ver 3.28.0) with glmQLFTest⁴⁷ in R.⁴⁸ Contigs were annotated by BLASTX (Blast+ v.2.9.0)⁴⁸ against rice protein sequences (*Oryza sativa* MSU release 7) and rice P450 protein sequences in Cytochrome P450 Homepage (<https://dmnelson.uthsc.edu/>).

Real-time PCR

Real-time PCR was performed as described previously⁴⁹ using primers listed in Table S1. Some of the primer sets were developed elsewhere⁴⁹⁻⁵¹. Eukaryotic translation initiation factor 4B (*EIF4B*) was used as an internal control gene and data was analyzed by the $\Delta\Delta C_t$ method.⁵²

Isolation of P450 genes

The full-length sequence of each P450 gene was amplified from the cDNA of *E. phyllopogon* (line 511) using primers listed in 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 pCAMBIA1390 vector with the In-Fusion DH Cloning Kit (TaKaRa, Kusatsu, Japan) or SLICE reaction⁵³ using primers listed in Table S1. The binary construct was introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze and thaw method.⁵⁴ Rice calli (*Oryza sativa* cv. Nipponbare) were transformed as previously described.^{8,55} *A. thaliana* (ecotype Columbia-0) was transformed by floral dip method⁵⁶ with the selection of hygromycin B (20 mg l⁻¹). 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 previously⁸. The calli were cultured at 30°C for 3 weeks. For the *A. thaliana* sensitivity assay, the surface-sterilized seeds were placed on Murashige and Skoog solid media.⁵⁷ After three days of stratification at 4°C, the media were placed at a climate chamber with 22°C, 12-h photoperiod, and 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ 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.⁵⁸ The herbicides and the doses were summarized in Table S2.

Heterologous expression of P450s in yeast and whole-cell herbicide metabolism assay

Expression in yeast (*Saccharomyces cerevisiae*) was conducted using WAT11 strain and pYeDP60 vector system.⁵⁹ The coding regions of other P450s were cloned into the pYeDP60 vector with the yeast Kozak sequence inserted as described before.⁸ WAT11 was transformed using the lithium acetate method.⁶⁰ A whole-cell assay for diclofop-methyl metabolism was performed according to Iwakami et al.⁸ with the modification of diclofop-methyl concentration to 300 μM . After incubation for 24 h, yeast cells were centrifuged at 13,500 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 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 x 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) in mobile phase A (0.1% formic acid in water) 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. ¹H-NMR spectra of each metabolite were recorded on a Bruker Ascend 400 MHz spectrometer (Billerica, MA, USA) with tetramethylsilane as an internal standard.

Synteny analysis

The genome of *E. haploclada*¹⁸ was used for the synteny analysis. The local synteny around the *CYP81As* and *CYP709Cs* was visualized with clinker (ver 0.0.21).⁶¹ The synteny of putative homeologous contigs was analyzed with D-GENIES.⁶²

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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.T.; RNA-sequencing, K.T.; Bioinformatics, S.I., H.S., T.K.; LC-MS/MS and NMR analysis, H.S., Y.Y., M.M.; Gene characterization, H.S., S.I.; Writing, S.I., H.S., M.M.; Visualization, S.I., H.S., T.K.; Supervision, S.I., T.T.; Funding acquisition, S.I.

DATA ACCESSIBILITY:

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure legends

Figure 1. Two distinct hydroxylated metabolites of diclofop-acid are highly accumulated in multiple-herbicide resistant (MHR) *Echinochloa phyllopogon*

(A) Diclofop-methyl metabolism in plants. The pro-herbicide diclofop-methyl converted to active form diclofop-acid in plants by esterase. Diclofop-acid is inactivated by cytochrome P450 mediated oxidation at the aromatic ring and further conjugated with glucose. In the quantification of OH-diclofop-acid, diclofop-acid-O-Gluc was inverted to OH-diclofop-acid by β -glucosidase treatment. (B) Schematic representation of sample preparation for diclofop-methyl metabolism study. The shoots of hydroponically grown 2.5-leaf stage plants were dipped in diclofop-methyl solution (30 μ M) for 30 min. The shoots were collected and stored at 0, 1, 3, or 6 h after treatment (HAT). (C) Detection of OH-diclofop-acid in the MHR line of *E. phyllopogon* on LC-MS/MS. The extract of 3 HAT was analyzed. Two metabolites (M1 and M2) were detected. (D) Comparison of M1 and M2 metabolite amounts between sensitive (S) and MHR lines. Diclofop-acid-O-Gluc was converted to OH-diclofop-acid with β -glucosidase. The error bars and the values on each graph represent SEM and *P* values (Student's *t*-test), respectively. (E) *In vivo* diclofop metabolism assay using yeast harboring empty vector (pYeDP60), *CYP81A12*, or *CYP81A21*.

Figure 2. Candidate genes for high-level diclofop-methyl resistance in *Echinochloa phyllopogon* were identified by gene expression study

(A) Multi-dimensional scaling (MDS) plot of the RNA-seq libraries of the sensitive (S) and multiple-herbicide resistant (MHR) lines. (B) Volcano plot of the S and MHR lines. Differentially expressed genes (fold-change>3, FDR<0.05) are shown in dark gray. Upregulated P450 genes are shown in yellow. (C) The number of differentially expressed genes. (D) Expression rank of P450 genes in the transcriptome of MHR line. P450s genes are shown in yellow. Larger yellow plots represent differentially expressed P450 genes. (E) The transcription level of P450 genes in S and MHR lines. P450 genes with upregulation in the MHR line are connected by lines. Three diclofop-methyl metabolizing P450 genes are shown in yellow. (F) The annotations of P450 genes that were upregulated in the MHR line. Progeny association study was not conducted on Contig490_pilon.258 and Contig1194_pilon.5091 since the appropriate primers were not designed. $\checkmark$, frame shift was not observed in sequence analysis or association between herbicide response and gene expression was observed in the progeny. x, association between herbicide response and gene

expression was not observed in the progeny. Highlight in gray indicates that the gene passed the tests. *, calculated in edgeR. ^a, Iwakami et al.⁷ ^b, Iwakami et al.⁸ (G) Progeny association of the mRNA levels of the candidate P450 genes. mRNA levels were quantified by real-time PCR in 16 F6 lines. The signal for CYP71C35 was not detected in the S line. Thus, the values were set as 40 cycles for visualization. F6-S, sensitive F6 lines; F6-R, resistant F6 lines.

Figure 3. Identification of novel diclofop-methyl metabolizing P450

(A) Diclofop-methyl sensitivity of rice calli transformed with P450 genes of *Echinochloa phyllopogon*. Twelve independent calli were grown for three weeks. (B) Diclofop-methyl sensitivity of *Arabidopsis thaliana* transformed with P450 genes of *E. phyllopogon*. Transcript levels of CYP709C69 in the transgenic *A. thaliana* were evaluated by real-time PCR. Bars represent SEM. 81A21#12-10 and 81A21#6-1 were the lines previously established. ⁷ (C) *In vivo* diclofop-methyl metabolism assay using yeast harboring empty vector (pYeDP60), CYP81A12, or CYP709C69. (D) The role of P450s in diclofop-acid hydroxylation. The structures of M1 and M2 were determined by nuclear magnetic resonance analysis. M1 metabolite was produced by the NIH-shift reaction. The arrows indicate migration of chlorine. The structure of M3 metabolite was estimated to have a 2,3-dichloro-4-hydroxyphenoxy moiety as reported in wheat. ²⁰

Figure 4. CYP709C69 has a narrow substrate specificity

(A) Rice calli and *Arabidopsis thaliana* transformed with CYP709C69 were tested for their sensitivity to 46 herbicides from at least 15 modes of action. The tested herbicides were listed in Table S2. (B) Clomazone sensitivity of *A. thaliana* transformed with CYP709C69. (C) The pro-herbicide clomazone converted to the active form 5-keto clomazone by the reaction of plant P450s. CYP709C69 may be involved in this reaction.

Figure 5. The genetic model of multiple-herbicide resistance in *Echinochloa phyllopogon*

(A) The local synteny of CYP81A and CYP709C regions in the draft genome of *E. phyllopogon* (2n=4X=36) and *E. haploclada* (2n=2X=18). ¹⁸ (B) The genomic synteny of the CYP81A and CYP709C contigs in *E. phyllopogon*. The two sets of contigs in *E. phyllopogon* likely forms homeologous pairs. (C) The loci of CYP81A12, CYP81A21, and CYP709C69 orthologs in diploid *E. haploclada*. (D) A working model for multiple-herbicide resistance in *E. phyllopogon*. A causal

743 mutation activates the expression of at least three herbicide-metabolizing P450s through unknown
744 gene-regulating systems in *E. phyllopogon*. CYP81A12/21 promiscuously metabolize many
745 herbicides at least from five modes of action. CYP709C69 is the more specific enzyme, which
746 simultaneously metabolizes diclofop-methyl together with CYP81A12/21.

A **B** **C** **D**