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

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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

44 **Introduction**

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

58 Recently, cytochrome P450 monooxygenases (P450s) belonging to CYP81A subfamily were
59 reported to encompass pleiotropic expression of MHR in *Echinochloa phyllopogon*,[4] an allotetraploid
60 (2x=4x=36) and predominantly self-fertilizing weed in rice cultivation. [5] Resistant populations found in
61 California in 1997 mysteriously exhibited resistance not only to the herbicides used in the fields at that
62 time but also to many other herbicides commercialized later. [6] Intensive analyses of this MHR
63 population, mainly from biochemical and molecular approaches, have led to the discovery that the
64 overexpression of two CYP81A cytochrome P450 monooxygenases (P450s) are responsible for the
65 cross-resistance to many herbicides such as acetolactate synthase (ALS) inhibitors, [4,7] acetyl-CoA
66 carboxylase (ACCase) inhibitors, [8] a 1-deoxy-D-xylulose 5-phosphate synthase (DXS) inhibitor, [9] and
67 a synthetic auxin (unpublished data). The two P450s, CYP81A12 and CYP81A21, exhibit catalytic
68 promiscuity against diverse herbicide chemistries, accommodating the resistance to multiple
69 herbicides in the *E. phyllopogon* population. [4] Through genetic segregation study, the overexpression
70 of the two P450s was suggested to be controlled by an unidentified single element. [7] A series of
71 research provided molecular evidence that the overexpression of promiscuous enzyme at least partly
72 accommodates MHR that is often accompanied in weeds with enhanced herbicide metabolism.

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

75 understood, among which is the high-level resistance to diclofop-methyl. Diclofop-methyl is the first
76 commercialized acetyl-CoA carboxylase (ACCase) inhibiting herbicide. ACCase inhibitors are solely
77 active to Poaceae plants by inhibiting the plastidic form of ACCase present in Poaceae. [10] Previously,
78 we found that the MHR line is highly resistant to diclofop-methyl, and the resistance is inherited
79 following the Mendelian segregation ratio as in other herbicides. [8] The subsequent genetic and
80 molecular studies revealed that the overexpression of the two *CYP81A* genes is linked with
81 resistance in the crossed-progeny of sensitive (S) and MHR lines, and the yeast lines expressing
82 either of the two CYP81As produced hydroxylated form of diclofop-methyl. However, we also noticed
83 that the resistance level of transgenic rice lines expressing *CYP81A12/21* to diclofop-methyl was not
84 prominently higher than that of other ACCase herbicides (tralkoxydim and pinoxaden), which is
85 inconsistent with the observation that the resistance level of the MHR line to diclofop-methyl is much
86 higher than the two herbicides. [8] Thus, we reasoned that an additional element specific to
87 diclofop-methyl metabolism underlies the high-level diclofop-methyl resistance. Here, we report that
88 the overexpression of a novel diclofop-methyl metabolizing P450, CYP709C69, is involved in
89 high-level resistance to diclofop-methyl. The P450 hydroxylates diclofop-acid, the active body of
90 diclofop-methyl, in a manner different from CYP81As. In stark contrast to the catalytic promiscuity
91 observed in CYP81As, CYP709C69 exhibited little metabolic activity to other herbicides except that it
92 may be involved in the activation of a DXS inhibitor clomazone. This study highlights that
93 simultaneous overexpression of herbicide-metabolizing genes encoding enzymes with different
94 enzymatic properties is involved in MHR in weeds. The concept is distinct from the accumulations of
95 multiple genes for broad or high-level resistance and serves as a model for future studies of
96 metabolic resistance in weeds.
97
98 **Results**
99 **Two distinct hydroxylated metabolites of diclofop-acid are highly accumulated in MHR *E.***
100 ***phyllopogon***
101 To gain clues for the high-level resistance to diclofop-methyl in the MHR line, we investigated the
102 metabolism of diclofop-methyl in *E. phyllopogon*. Diclofop-methyl is a pro-herbicide that is activated
103 by endogenous esterase as is the case for most aryloxyphenoxy-propanoate herbicides. [11] Following
104 the de-esterification, the resultant active body diclofop-acid is inactivated by P450-mediated
105 hydroxylation followed by further glycosylation (Fig. 1A). [12–14] In the metabolism scheme,

106 diclofop-acid hydroxylation is the rate-limiting step for diclofop-methyl inactivation in plants. [15] Our
107 previous study showed that both the MHR and S lines converted the majority of diclofop-methyl into
108 diclofop-acid by 3 h after treatment for 30 min. [8] In this study, we further analyzed the dynamics of
109 putative OH metabolites of diclofop-acid in the extract of plants prepared as before (Fig. 1B). Two
110 peaks with retention times of 11.3 and 12.4 min (M1 and M2, respectively), which correspond to
111 putative OH metabolites, were detected by liquid chromatography-tandem mass spectrometry
112 (LC-MS/MS) analysis using a multiple reaction monitoring (MRM) mode (Fig. 1C). The signal intensity
113 of M2 was higher than that of M1 under this condition. In line with our study, a variety of hydroxylated
114 diclofop-acid metabolites were reported in wheat, [16,17] suggesting the presence of at least partially
115 overlapping metabolic pathway for diclofop-methyl between wheat and *E. phyllopogon*.
116 We next compared the amount of each hydroxylated metabolite between the MHR and S lines.
117 To accurately evaluate the hydroxylation activity, possible glycosylated metabolites were converted
118 into hydroxylated diclofop-acid by treating β-glycosidase to the extract of the diclofop-methyl treated
119 plants (Fig. 1A), which was then subjected to comparative quantification between the lines. As a
120 result, the amounts of both hydroxylated metabolites were higher in MHR plants at 0 and 1 HAT
121 although no difference was observed at 3 and 6 HAT (Fig. 1D).
122 To investigate whether the metabolites are produced by CYP81A12 and CYP81A21, the
123 P450s responsible for the resistance to the majority of herbicides in the MHR line, [4] we analyzed the
124 *in vivo* diclofop-methyl metabolism of yeast carrying *CYP81A12/21*. In the yeast system, another
125 peak (M3), which was not clearly separated from M1, was observed in addition to M1 and M2.
126 Furthermore, the chromatographic profile of M1 and M2 differed between *E. phyllopogon* and yeast.
127 The intensity of M2 was higher in *E. phyllopogon*, while that of M1 was prominently higher in yeast
128 (Fig. 1E). The result suggests that an additional enzyme that catalyzes hydroxylation to form M2 is
129 involved in diclofop-methyl resistance in the MHR line.
130
131 **Candidate genes for high-level diclofop-methyl resistance were identified by gene expression**
132 **study**
133 To identify the additional diclofop-hydroxylating enzyme, we performed RNA-seq analysis using the
134 recently released draft genome of *E. phyllopogon* with 66,521 gene models. [18] Among the gene
135 models, we identified 549 putative P450 genes. A transcriptome comparison detected some P450
136 genes highly expressed in the MHR line. A closer look at the sequences of these P450 genes found

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

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

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

159

160 **Identification of novel diclofop-methyl metabolizing P450**

161 For the evaluation of the diclofop-metabolizing activity of each gene, we used the rice transformation
162 system as employed previously. [8] We expressed the six candidate genes in rice calli with the
163 pCAMBIA1390 vector, where the transgene was placed under the control of Cauliflower mosaic virus
164 35S (CaMV35S) promoter. The calli were tested for growth on the media supplemented with
165 diclofop-methyl. Among the six genes, only *CYP709C69* conferred marked resistance at the lethal
166 dose of diclofop-methyl (0.4 μM) to rice calli (Fig. S2A). No difference was observed in the coding
167 sequence of CYP709C69 between the S and MHR lines. When 12 independent transformed lines

168 were subjected to diclofop-methyl, all the lines with *CYP709C69* stopped growing at 6 μM (Fig. 3A).
169 The diclofop-methyl resistance level was equivalent to the cases of *CYP81A12* and *CYP81A21*.
170 We also tested diclofop-methyl sensitivity of *Arabidopsis thaliana* transformed with
171 *CYP709C69* although the major plastidic form of ACCase is insensitive to ACCase inhibitors. Using
172 the same vector, we transformed *A. thaliana* and selected the highest expressing line among the
173 three T3 homozygous lines (Fig. 3B). The highest expressing line (#31-5) was tested for
174 diclofop-methyl sensitivity together with previously described *A. thaliana* lines with high-level
175 expression of *CYP81A12* or *CYP81A21*, which exhibit high-level resistance to many herbicides. [4,7]
176 Although diclofop-methyl is an ACCase inhibitor specifically effective to Poaceae plants, a high dose
177 of diclofop-acid inhibits the growth of *A. thaliana*. [19] In the current study, the similar growth inhibition in
178 wild-type *A. thaliana* was observed after the treatment with diclofop-methyl at 3 to 30 μM. On the
179 other hand, the lines with the P450 genes were less affected although only marginal resistance was
180 observed in the *CYP81A12* line. Thus, we confirmed that CYP709C69 protects the species where the
181 dominant form of the herbicide target-site is insensitive to it.
182 We then transformed *CYP709C69* in yeast and investigated diclofop-methyl metabolism *in*
183 *vivo* as performed previously. [8] LC-MS/MS analysis revealed that M2 was produced as the main peak
184 (Fig. 3C). To determine the hydroxylation position in the OH-metabolites of diclofop-acid, we purified
185 M1 and M2 from yeast culture applied with diclofop-methyl using high-performance liquid
186 chromatography (HPLC), which were then subjected to nuclear magnetic resonance (NMR) analysis.
187 Since the NMR structural analysis of three OH-metabolites of diclofop-methyl has been reported
188 previously, [20] we compared the chemical shift patterns in the aromatic regions of M1 and M2 with the
189 reported data. The results showed that M1 has a similar pattern with the metabolite containing a
190 2,5-dichloro-4-hydroxyphenoxy moiety and M2 with that containing a 2,4-dichloro-5-hydroxyphenoxy
191 moiety (Fig. S3). The position of one of the chlorine atoms in M1 is different from the original position,
192 which is thought to be formed by intramolecular migration during enzymatic hydroxylation of the
193 aromatic ring (so-called NIH-shift) as previously reported (Fig. 3D). [20] This implies that CYP81A12
194 and CYP81A21 mediate the NIH-shift reaction in diclofop metabolism. In this study, we could not
195 determine the structure of M3 due to the limited amount available from CYP81A expressing yeast (Fig.
196 1E, 3C). Considering that wheat produces the hydroxylated diclofop with a
197 2,3-dichloro-4-hydroxyphenoxy moiety in addition to the metabolites shown above, M3 may carry this
198 moiety (Fig. 3D) although further experiments are required.

199 We further tested the diclofop-methyl metabolizing activity of other CYP709Cs in *E.*
200 *phyllopogon* although no association with resistance was observed in the gene expression analyses
201 (Fig. 2G). We isolated five more genes, i.e., *CYP709C67* (Contig12_pilon.29), *CYP709C68*
202 (Contig817_pilon.380.a), *CYP709C70* (Contig960_pilon.352), *CYP709C71* (Contig817_pilon.378),
203 and *CYP709C* (Contig960_pilon.355), and transformed them in rice calli. In stark contrast to
204 CYP709C69, only a marginal activity was observed in CYP709C68/70/71, where some callus lines
205 slightly grew only at 0.4 μM (Fig. S2B). The genome holds six more putative *CYP709C* genes
206 (Contig1380_pilon.11, Contig12_pilon.1, Contig291_pilon.387, Contig1182_pilon.1,
207 Contig291_pilon.396, and Contig120_pilon.313) although the annotated protein sequences other
208 than Contig1380_pilon.11 and Contig291_pilon.387 were too short (<331) for a canonical P450
209 sequence. Together, CYP709C69 is likely the major contributor to the production of M2 metabolite in
210 *E. phyllopogon*.
211
212 **CYP709C69 has a narrow substrate specificity**
213 To investigate the role of CYP709C69 in the metabolism of other herbicides, we tested the herbicide
214 sensitivity of *CYP709C69*-expressing *A. thaliana*. We also used *CYP709C69*-expressing rice calli for
215 the evaluation of its metabolizing activity to ACCase inhibitors (Fig. 4A). Among a total of 46
216 herbicides from at least 15 modes of action, a DXS inhibitor clomazone was the only herbicide where
217 a marked alteration in the sensitivity of the transgenic plants was observed (Fig. S4). The *A. thaliana*
218 lines expressing *CYP709C69* became more vulnerable to clomazone in inverse proportion to the
219 expression level (Fig. 4B, Fig. 3B), in contrast to the *A. thaliana* transformed with *CYP81A*s. [9]
220 Clomazone is a pro-herbicide where P450 was proposed as the enzyme to convert 5-OH clomazone,
221 which is then further converted to the active form 5-keto clomazone (Fig. 4C). [6] Thus, CYP709C69
222 may be involved in this sequential bio-activation reaction.
223
224 **Distantly located herbicide-metabolizing genes are simultaneously overexpressed**
225 Finally, to gain insights into the mechanism of overexpression of the three herbicide-metabolizing
226 P450 genes (*CYP81A12*, *CYP81A21*, and *CYP709C69*), we estimated the genomic loci of the three
227 genes. Since the current draft genome of *E. phyllopogon* is contig-based, the physical relationships of
228 the three P450 genes in the chromosome are unclear. Thus, we allocated the loci of the three P450
229 genes in the chromosome-scale genome of diploid *Echinochloa* sp. (*E. haploclada*). [18] The synteny

230 around the *CYP81A* regions (Contig168 and Contig1257) and *CYP709C* regions (Contig817 and
231 Contig960) is highly conserved in the *E. haploclada* genome (Fig. 5A). The syntenic dot plot also
232 shows that the two contig sets are highly conserved (Fig. 5B), providing strong evidence that each set
233 represents a part of the homeologous chromosome. The *CYP81A* and *CYP709C* loci respectively
234 correspond to chromosomes 1 and 3 in the *E. haploclada* genome (Fig. 5C). The data strongly
235 suggest that the three P450 genes are physically unlinked. Considering that the MHR including
236 diclofop-methyl resistance is inherited as a Mendelian trait [7,8] and that the overexpression of the three
237 genes is genetically linked (Fig. 2), a single genetic element is behind the MHR of the Californian
238 population of *E. phyllopogon*. The causal element may directly or indirectly regulate the expression of
239 the P450s (Fig. 5D).
240
241 **Discussion**
242 **Molecular mechanism of multiple-herbicide resistance**
243 The expression of MHR in metabolism-based resistance is one of the biggest mysteries in herbicide
244 resistance in weeds. Previous studies have uncovered that *E. phyllopogon* carries enzymes with low
245 substrate specificity, namely CYP81A12 and CYP81A21 that when overexpressed, lead to resistance
246 to many herbicides ranging from multiple modes of action. [8] On the other hand, MHR *E. phyllopogon*
247 exhibits resistance even to some herbicides to which the CYP81As have little or no activity, making
248 the overexpression of CYP81As not the prime explanation to the expression of MHR trait. In this study,
249 by focusing on the discrepancy between the resistance level of diclofop-methyl in the Californian
250 MHR line and the metabolic activity of CYP81A12/21 to diclofop-methyl, we revealed that the
251 overexpression of a novel P450 *CYP709C69* is genetically linked with MHR in the crossed progeny,
252 where the overexpression of *CYP81A12* and *CYP81A21* has also been observed. Although the
253 diclofop-methyl metabolizing activity of CYP709C69 inferred from the rice transformation experiments
254 is not distinctly different from those of CYP81As, the simultaneous actions of these P450s would
255 result in high-level resistance to diclofop-methyl. The findings add another layer in the understanding
256 of metabolic resistance that herbicide-metabolizing enzymes can act simultaneously on herbicides.
257 Our findings show that MHR *E. phyllopogon* neutralizes many herbicides by simultaneous
258 overexpression of several herbicide-detoxifying genes with different enzymatic properties, expanding
259 the resistance window. This simultaneous overexpression of the multiple genes is strongly suggested
260 to be under a single switch in this MHR line, which differs from a mere stepwise accumulation of

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

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

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

292 *phyllopogon* may have some physiological or ecological function in *E. phyllopogon*. As CYP81As in
293 maize are involved in the biosynthesis of zealexins, antimicrobial substances,[26] CYP81As in *E.*
294 *phyllopogon* may have a similar function. Elucidation of the regulatory system and the endogenous
295 function of each P450 may provide insight into the fitness costs associated with resistance
296 expression and hints for the control of MHR plants that are hardly controlled by herbicide chemistries.
297 In addition, the deeper understanding may also provide insight into the generality and the likelihood of
298 this agronomically-devastating evolutionary mechanism.
299
300 **New aspects on the modes of herbicide metabolism in plants**
301 The current study enlightens new aspects of the modes of P450-mediated herbicide-metabolism in
302 plants. Firstly, our results show that a single chemistry is subjected to independent hydroxylation
303 reactions by multiple P450s *in planta*: i.e., the active body of diclofop-methyl, diclofop-acid, is
304 hydroxylated at the different positions of its aromatic ring by two types of P450 enzymes. Similarly,
305 the involvement of more than one P450 for a single herbicide metabolism was suggested in other
306 species although their influence on herbicide metabolism *in planta* remains untapped: e.g., Jerusalem
307 artichoke (*Helianthus tuberosus*) for phenylurea herbicides by CYP76B1, CYP73A1, and CYP81B1
308 [27–29] and *indica* rice for bensulfuron-methyl by CYP81A6 and CYP72A31. [30–32] In addition to providing
309 evidence of simultaneous inactivating-actions to diclofop-methyl by multiple enzymes (CYP81A12/21
310 and CYP709C69), our results also suggest a possibility that these enzymes also act on clomazone
311 both in activating (CYP709C69) and inactivating (CYP81A12/21) ways in *E. phyllopogon*. Clomazone
312 is one of the herbicides that undergoes complexed multiple oxidative pathways in plants. [33] Our
313 previous studies showed that *A. thaliana* expressing *CYP81A12* or *CYP81A21* became resistant to
314 clomazone [9] and that they oxidize clomazone to an unidentified metabolite. [4] Although the detailed
315 role *in planta* remains to be explored, the three P450s likely take part in clomazone metabolism in *E.*
316 *phyllopogon*. Note that the Californian MHR *E. phyllopogon* exhibits resistance to clomazone, [34]
317 suggesting that CYP81A12 and CYP81A21 are the greater influence in the balance of the activating
318 and inactivating forces by CYP709C69 and CYP81As, respectively. The higher transcript levels of
319 CYP81A12/21 in the MHR line (Fig. 2) may play a role in the expression of resistance phenotype to
320 clomazone. While some herbicides are metabolized through a single major metabolic pathway, many
321 herbicides follow multiple metabolic pathways in plants. Connecting the dynamics of herbicide
322 metabolism in plants with the factors involved in each metabolic step is important for better

323 understanding of not only the evolution of weed resistance but also the basis of herbicide selectivity
324 and mode of action.
325 In addition, we disclosed that CYP81As mediate chlorine migration reaction in the process of
326 diclofop hydroxylation. The hydroxylation of the aromatic ring can cause the migration of the original
327 functional group such as halogen, alkyl group, and carboxyl group, to the adjacent carbon, which is
328 called NIH-shift. [35] P450s are among the major enzymes involved in NIH-shift with the detailed
329 molecular mechanisms of the migration reaction not fully understood. [36] 2,4-D and diclofop-methyl are
330 the two major herbicides involving NIH-shift in their metabolism *in planta*. [37] It will be interesting to see
331 whether CYP81A also induces NIH-shift for 2,4-D.
332
333 **Negative cross-resistance by P450 genes**
334 The negative aspect of CYP709C69 that we found in this study is of particular interest. When a
335 mechanism that confers resistance to one herbicide increases susceptibility to another herbicide, the
336 phenomenon is called negative cross-resistance. While negative cross-resistance has been
337 documented in the case of TSR mechanisms such as photosystem II inhibitors, [38] phytoene
338 desaturase inhibitors, [39] and tubulin assembly inhibitors, [40] it is rarely associated with NTSR. A similar
339 phenomenon has been observed for *CYP81A12/21*, which increases sensitivity to ALS inhibiting
340 herbicide pyriftalid when expressed in *A. thaliana* [4]. The results suggest that the P450 genes act as
341 the Achilles' heel of metabolic resistance. The MHR line from California is resistant to both clomazone
342 and pyriftalid, [4] which is likely due to the influence of other genes as discussed above in the case of
343 clomazone. Simpler resistance mechanisms such as gene amplification in one of the three P450
344 genes would be interesting to further investigate the sensitivity to these herbicides.
345 The insights from this study may offer potential novel targets for the generation of genetically
346 modified herbicide-resistant crops. The introduction of herbicide-resistant crops has been
347 instrumental in efficient weed management by enabling new uses of existing herbicide technologies.
348 These herbicide-resistant crops have been produced mainly by transforming a herbicide-resistance
349 gene from other species or introducing a point mutation into the TSR genes through random
350 mutagenesis [41]. If the herbicide-activation functions observed in CYP709C and CYP81A of *E.*
351 *phyllopogon* are conserved in crops, knocking out the corresponding crop genes may result in the
352 generation of herbicide-resistant cultivars, providing an additional approach to genetically modified
353 herbicide-resistant crop technology.

354

355 **Conclusion**

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

368

369 **METHODS**

370 **Origin of plant material**

371 S and MHR lines of *E. phyllopogon* were originated from Sacramento Valley in California. [43] The F6
372 progeny of the two lines was derived from a single seed descent method. [7] The herbicide responses
373 of F6 lines were evaluated previously. [7–9,44]

374

375 **Analysis of diclofop metabolites in plants**

376 *E. phyllopogon* were cultured hydroponically to around 2.5 leaf-stage at 25°C with a 12-h photoperiod
377 (approximately 300 μmol $m^{-2}s^{-1}$) as described previously. [8] The shoots of hydroponically cultured *E.*
378 *phyllopogon* (2.5 leaf stage) were dipped in 30 μM diclofop-methyl solution supplemented with Tween
379 20 (0.01%) for 30 min. The shoots of 10 plants (approximately 300 mg) were collected at 0, 1, 3, and
380 6 h after application. On collection, they were rinsed in distilled water containing 20% methanol and
381 0.2% Triton X-100, and snap-frozen in liquid nitrogen. The plant tissue was ground into powder in
382 liquid nitrogen with a mortar and pestle and extracted with 6 ml of 80% cold methanol, followed by
383 centrifugation at 9,000 g for 10 min at 4°C. The pellet was further extracted twice with 2 ml of 80%
384 cold methanol. The extract was combined, evaporated to dryness, and dissolved in 1 ml of 0.1 M

385 sodium acetate buffer (pH 5.0). The solution was treated with 0.3 mg of almond β-glucosidase
386 (BGH-101, Toyobo, Osaka, Japan) and incubated for 24 h at 37°C. After the addition of 1 ml of
387 acetonitrile, the solution was vortexed, left for 15 min at 4°C, and then centrifuged at 1,700 g for 15
388 min. The supernatant was collected and evaporated to remove acetonitrile. The sample was loaded
389 onto a Sep-Pak C18 cartridge (Waters, Tokyo, Japan) and washed with 10 ml of water containing
390 0.1% formic acid, followed by elution with 4 ml of acetonitrile. The fraction eluted with acetonitrile was
391 evaporated to dryness and redissolved in acetonitrile (0.5 ml/300 mg of fresh weight of plant tissue),
392 which was subjected to mass spectrometric analysis.
393
394 **Mass spectrometric analysis**
395 Hydroxylated metabolites of diclofop-acid were analyzed using a liquid chromatography-tandem
396 mass spectrometer (Shimadzu LCMS-8030, Kyoto, Japan) equipped with an electrospray ionization
397 source. The MS parameters used were as follows: interface voltage of 4.5 kV, desolvation line
398 temperature of 250°C, heat block temperature of 400°C, nebulizing gas (N_2) of 2.0 l/min, and drying
399 gas at 15 l/min. MRM was used to quantitate the hydroxylated metabolites of diclofop-acid under the
400 following conditions: negative ion mode with MRM transition of *m/z* 341 to *m/z* 269 (CE= 15 V, Q1 Pre
401 Bias= 15 V, Q3 Pre Bias= 19 V). Separation of analytes was carried out using a reversed-phase
402 column, TSK gel ODS-100V (2 mm ID x 150 mm, 3 μm, Tosoh, Tokyo, Japan). The column was
403 eluted with a linear gradient from 30 to 90% mobile phase B (0.1% formic acid in acetonitrile) in
404 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
405 injection volume was 5 μL.
406
407 **RNA extraction and cDNA synthesis**
408 Plants were cultivated as described above. Total RNA was extracted using an RNeasy Plant Mini Kit
409 (Qiagen, Tokyo, Japan) followed by the application of TURBO DNA-*free* kit (Thermo Fisher Scientific,
410 Tokyo, Japan) to eliminate genomic DNA. Total RNA (1 μg) was reverse-transcribed using ReverTra
411 Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions.
412
413 **RNA-seq analysis**
414 RNAs extracted from the shoot of 2.5-leaf stage plants of S and MHR lines were used for RNA-seq
415 analysis with four biological replications. RNA-Seq libraries were prepared with TruSeq RNA Library

416 Preparation Kit v2 (Illumina, USA), followed by pair-end sequencing on HiSeq 2500 sequencer
417 (Illumina). Reads were filtered using TRIMMOMATIC (ver 0.36) [45] with the following options:
418 TRAILING:25, SLIDINGWINDOW:4, MINLEN:80. The reads were mapped against coding sequences
419 of the draft genome of *E. phyllopogon* [18] using bowtie2 (ver 2.3.5.1) with the following options: '-X
420 900–very-sensitive–no-discordant–no-mixed–dpad 0–gbar 99999999'. The mapped reads were
421 counted using RSEM. [46] Differential expression analysis was performed using edgeR (ver 3.28.0)
422 with glmQLFTest [47] in R.[48] Contigs were annotated by BLASTX (Blast+ v.2.9.0) [48] against rice protein
423 sequences (*Oryza sativa* MSU release 7) and rice P450 protein sequences in Cytochrome P450
424 Homepage (https://drnelson.uthsc.edu/).
425
426 **Real-time PCR**
427 Real-time PCR was performed as described previously [49] using primers listed in Table S1. Some of
428 the primer sets were developed elsewhere [49–51]. Eukaryotic translation initiation factor 4B (*EIF4B*)
429 was used as an internal control gene and data was analyzed by the ΔΔCt method. [52]
430
431 **Isolation of P450 genes**
432 The full-length sequence of each P450 gene was amplified from the cDNA of *E. phyllopogon* (line
433 511) using primers listed in Table S1 with KOD FX Neo (TOYOBO) or PrimeSTAR GXL DNA
434 Polymerase (Takara). The amplicons were subcloned using pGEM-T Easy Vector Systems Kit
435 (Promega, Tokyo, Japan) or Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific, Tokyo, Japan)
436 according to the manufacturer's instructions.
437
438 **Plant transformation**
439 The coding region of each P450 gene was cloned into pCAMBIA1390 vector with the In-Fusion DH
440 Cloning Kit (TaKaRa, Kusatsu, Japan) or SLiCE reaction [53] using primers listed in Table S1. The
441 binary construct was introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze and
442 thaw method. [54] Rice calli (*Oryza sativa* cv. Nipponbare) were transformed as previously described.
443 [8,55] *A. thaliana* (ecotype Columbia-0) was transformed by floral dip method [56] with the selection of
444 hygromycin B (20 mg l^{-1}). T3 homozygous lines were selected based on the segregation ratio.
445
446 **Herbicide sensitivity assay**

447 Independently transformed calli were placed on N6D solid media supplemented with a herbicide as
448 described previously [8]. The calli were cultured at 30°C for 3 weeks. For the *A. thaliana* sensitivity
449 assay, the surface-sterilized seeds were placed on Murashige and Skoog solid media. [57] After three
450 days of stratification at 4°C, the media were placed at a climate chamber with 22°C, 12-h photoperiod,
451 and 70 μE m^{-2} s^{-1}light intensity for 12 days. In the case of the diclofop-methyl assay, the plates were
452 kept in the chamber for 14 days. Chlorophyll was extracted and measured as described previously. [58]
453 The herbicides and the doses were summarized in Table S2.
454
455 **Heterologous expression of P450s in yeast and whole-cell herbicide metabolism assay**
456 Expression in yeast (*Saccharomyces cerevisiae*) was conducted using WAT11 strain and pYeDP60
457 vector system. [59] The coding regions of other P450s were cloned into the pYeDP60 vector with the
458 yeast Kozak sequence inserted as described before. [8] WAT11 was transformed using the lithium
459 acetate method. [60] A whole-cell assay for diclofop-methyl metabolism was performed according to
460 Iwakami et al. [8] with the modification of diclofop-methyl concentration to 300 μM. After incubation for
461 24 h, yeast cells were centrifuged at 13,500 *g* for 10 min. The supernatant was cleaned up using a
462 Sep-Pak C18 cartridge as described above and subjected to LC-MS/MS analysis.
463
464 **Structure determination of diclofop metabolites**
465 The yeast expressing *CYP81A12* or *CYP709C69* in whole-cell assay buffer (350 ml) was incubated
466 with diclofop-methyl (300 μM) for 24 h. The culture was centrifuged at 1,500 *g* for 5 min to collect the
467 supernatant. The yeast cells were resuspended in 1/10 volume of the buffer without diclofop-methyl.
468 After incubation for an additional 12 h, the supernatant was collected by centrifugation, which was
469 repeated twice. The whole procedure was repeated 30 times. After combining all the supernatants,
470 the pH of the solution was adjusted to 1 by hydrochloric acid. The solution was extracted four times
471 with diethyl ether (0.7 volume). After evaporating diethyl ether *in vacuo*, the resultant residue was
472 dissolved in acetonitrile. HPLC (LC-10ADvp and SPD-M10Avp, Shimadzu) separation was carried
473 out on a reversed-phase column (TSK gel ODS-100, 4.6 mm ID x 250 mm, 5 μm, Tosoh, Tokyo,
474 Japan) with the following conditions: linear gradient from 30 to 72% mobile phase B (0.1% formic acid
475 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
476 40°C. The elution was monitored by UV absorbance at 215 and 280 nm. The fractions containing
477 each hydroxylated metabolite of diclofop-acid were evaporated to dryness and dissolved in

478 deuterated chloroform. ^{1}H-NMR spectra of each metabolite were recorded on a Bruker Ascend 400
479 MHz spectrometer (Billerica, MA, USA) with tetramethylsilane as an internal standard.
480
481 **Synteny analysis**
482 The genome of *E. haploclada* [18] was used for the synteny analysis. The local synteny around the
483 *CYP81A*s and *CYP709C*s was visualized with clinker (ver 0.0.21). [61] The synteny of putative
484 homeologous contigs was analyzed with D-GENIES. [62]
485
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496
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498 Conceptualization, S.I.; Research design, S.I., H.S., M.M., Y.Y., T.Y., K.T., S.T., T.T.; RNA-sequencing,
499 K.T.; Bioinformatics, S.I., H.S., T.K.; LC-MS/MS and NMR analysis, H.S., Y.Y., M.M.; Gene
500 characterization, H.S., S.I.; Writing, S.I., H.S., M.M.; Visualization, S.I., H.S., T.K.; Supervision, S.I.,
501 T.T.; Funding acquisition, S.I.
502
503 **DATA ACCESSIBILITY:**
504 The mRNA-Seq data have been deposited in the DDBJ Sequence Read Archive (DRA) database
505 (accession number DRA013092).
506
507 **DECLARATION OF INTERESTS**
508 The authors declare no competing interests.

509

510 **REFERENCES**

511 1. Powles, S.B., and Yu, Q. (2010). Evolution in action: plants resistant to herbicides. Annu. Rev.
512 Plant Biol. *61*, 317–347.

513 2. Gaines, T.A., Duke, S.O., Morran, S., Rigon, C.A.G., Tranel, P.J., Kupper, A., and Dayan, F.E.
514 (2020). Mechanisms of evolved herbicide resistance. J. Biol. Chem. *295*, 10307–10330.

515 3. Yu, Q., and Powles, S. (2014). Metabolism-based herbicide resistance and cross-resistance in
516 crop weeds: a threat to herbicide sustainability and global crop production. Plant Physiol. *166*,
517 1106–1118.

518 4. Dimaano, N.G., Yamaguchi, T., Fukunishi, K., Tominaga, T., and Iwakami, S. (2020). Functional
519 characterization of cytochrome P450 CYP81A subfamily to disclose the pattern of
520 cross-resistance in *Echinochloa phyllopogon*. Plant Mol. Biol. *102*, 403–416.

521 5. Yamasue, Y. (2001). Strategy of *Echinochloa oryzicola* Vasing. for survival in flooded rice. Weed
522 Biol. Manag. *1*, 28–36.

523 6. Nandula, V.K., Riechers, D.E., Ferhatoglu, Y., Barrett, M., Duke, S.O., Dayan, F.E.,
524 Goldberg-Cavalleri, A., Tétard-Jones, C., Wortley, D.J., Onkokesung, N., et al. (2019). Herbicide
525 metabolism: crop selectivity, bioactivation, weed resistance, and regulation. Weed Sci. *67*,
526 149–175.

527 7. Iwakami, S., Endo, M., Saika, H., Okuno, J., Nakamura, N., Yokoyama, M., Watanabe, H., Toki,
528 S., Uchino, A., and Inamura, T. (2014). Cytochrome P450 CYP81A12 and CYP81A21 are
529 associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*.
530 Plant Physiol. *165*, 618–629.

531 8. Iwakami, S., Kamidate, Y., Yamaguchi, T., Ishizaka, M., Endo, M., Suda, H., Nagai, K., Sunohara,
532 Y., Toki, S., Uchino, A., et al. (2019). CYP81A P450s are involved in concomitant
533 cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in
534 *Echinochloa phyllopogon*. New Phytol. *221*, 2112–2122.

535 9. Guo, F., Iwakami, S., Yamaguchi, T., Uchino, A., Sunohara, Y., and Matsumoto, H. (2019). Role
536 of CYP81A cytochrome P450s in clomazone metabolism in *Echinochloa phyllopogon*. Plant Sci.
537 *283*, 321–328.

538 10. Délye, C. (2005). Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update.
539 Weed Sci. *53*, 728–746.

540 11. Wenger, J., Niderman, T., and Mathew, C. (2012). Acetyl-CoA carboxylase inhibitors. In Modern
541 Crop Protection Compounds, Second, Revised and Enlarged Edition, W. Krämer, U. Schirmer, P.
542 Jeschke, and W. Matthias, eds. (Wiley-VCH), pp. 447–477.

543 12. Shimabukuro, R.H., Walsh, W.C., and Hoerauf, R.A. (1979). Metabolism and selectivity of
544 diclofop-methyl in wild oat and wheat. J. Agric. Food Chem. *27*, 615–623.

545 13. Shimabukuro, R.H., Walsh, W.C., and Jacobson, A. (1987). Aryl-*O*-glucoside of diclofop: a
546 detoxication product in wheat shoots and wild oat cell suspension culture. J. Agric. Food Chem.
547 *35*, 393–397.

548 14. McFadden, J.J., Frear, D.S., and Mansager, E.R. (1989). Aryl hydroxylation of diclofop by a
549 cytochrome P450 dependent monooxygenase from wheat. Pestic. Biochem. Physiol. *34*,
550 92–100.

551 15. Owen, W.J. (2000). Herbicide metabolism as a basis for selectivity. In Metabolsim of
552 Agrachemicals in Plants, T. Roberts, ed. (John Wiley & Sons Ltd.), pp. 211–258.

553 16. Tanaka, F.S., Wien, R.G., Zaylskie, R.G., and Hoffer, B.L. (1990). Synthesis of possible
554 ring-hydroxylated metabolites of diclofop-methyl. J. Agric. Food Chem. *38*, 553–559.

555 17. Zimmerlin, A., and Durst, F. (1992). Aryl hydroxylation of the herbicide diclofop by a wheat
556 cytochrome p-450 monooxygenase: substrate specificity and physiological activity. Plant Physiol.
557 *100*, 874–881.

558 18. Ye, C.Y., Wu, D., Mao, L., Jia, L., Qiu, J., Lao, S., Chen, M., Jiang, B., Tang, W., Peng, Q., et al.
559 (2020). The genomes of the allohexaploid *Echinochloa crus-galli* and its progenitors provide
560 insights into polyploidization-driven adaptation. Mol. Plant *13*, 1298–1310.

561 19. Zhang, Q., Zhao, M., Qian, H., Lu, T., Zhang, Q., and Liu, W. (2012). Enantioselective damage
562 of diclofop acid mediated by oxidative stress and acetyl-CoA carboxylase in nontarget plant
563 *Arabidopsis thaliana*. Environ. Sci. Technol. *46*, 8405–8412.

564 20. Tanaka, F.S., Hoffer, B.L., Shimabukuro, R.H., Wien, R.G., and Walsh, W.C. (1990).
565 Identification of the isomeric hydroxylated metabolites of methyl 2-[4-(2, 4-dichlorophenoxy)
566 phenoxy] propanoate (diclofop-methyl) in wheat. J. Agric. Food Chem. *38*, 559–565.

567 21. Preston, C., Tardif, F.J., Christopher, J.T., and Powles, S.B. (1996). Multiple resistance to
568 dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of
569 several herbicide degrading enzymes. Pestic. Biochem. Physiol. *54*, 123–134.

570 22. Ma, R., Kaundun, S.S., Tranel, P.J., Riggins, C.W., McGinness, D.L., Hager, A.G., Hawkes, T.R.,
571 McIndoe, E., and Riechers, D.E. (2013). Distinct detoxification mechanisms confer resistance to
572 mesotrione and atrazine in a population of waterhemp (*Amaranthus tuberculatus*). Plant Physiol.
573 *163*, 363–377.

574 23. Nauen, R., Bass, C., Feyereisen, R., and Vontas, J. (2021). The role of cytochrome P450s in
575 insect toxicology and resistance. Annu. Rev. Entomol.,
576 https://doi.org/10.1146/annurev-ento-070621-061328.

577 24. Singh, K.S., Troczka, B.J., Duarte, A., Balabanidou, V., Trissi, N., Carabajal Paladino, L.Z.,
578 Nguyen, P., Zimmer, C.T., Papapostolou, K.M., Randall, E., et al. (2020). The genetic
579 architecture of a host shift: An adaptive walk protected an aphid and its endosymbiont from plant
580 chemical defenses. Sci Adv *6*, eaba1070.

581 25. Singh, K.S., Cordeiro, E.M.G., Troczka, B.J., Pym, A., Mackisack, J., Mathers, T.C., Duarte, A.,
582 Legeai, F., Robin, S., Bielza, P., et al. (2021). Global patterns in genomic diversity underpinning
583 the evolution of insecticide resistance in the aphid crop pest *Myzus persicae*. Commun Biol *4*,
584 847.

585 26. Ding, Y., Weckwerth, P.R., Poretsky, E., Murphy, K.M., Sims, J., Saldivar, E., Christensen, S.A.,
586 Char, S.N., Yang, B., Tong, A.D., et al. (2020). Genetic elucidation of interconnected antibiotic
587 pathways mediating maize innate immunity. Nat Plants *6*, 1375–1388.

588 27. Pierrel, M.A., Batard, Y., Kazmaier, M., Mignotte-Vieux, C., Durst, F., and Werck-Reichhart, D.
589 (1994). Catalytic properties of the plant cytochrome P450 CYP73 expressed in yeast. Substrate
590 specificity of a cinnamate hydroxylase. Eur. J. Biochem. *224*, 835–844.

591 28. Robineau, T., Batard, Y., Nedelkina, S., Cabello-Hurtado, F., LeRet, M., Sorokine, O., Didierjean,
592 L., and Werck-Reichhart, D. (1998). The chemically inducible plant cytochrome P450 CYP76B1
593 actively metabolizes phenylureas and other xenobiotics. Plant Physiol. *118*, 1049–1056.

594 29. Cabello-Hurtado, F., Batard, Y., Salaun, J.P., Durst, F., Pinot, F., and Werck-Reichhart, D. (1998).
595 Cloning, expression in yeast, and functional characterization of CYP81B1, a plant cytochrome
596 P450 that catalyzes in-chain hydroxylation of fatty acids. J. Biol. Chem. *273*, 7260–7267.

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

600 31. Saika, H., Horita, J., Taguchi-Shiobara, F., Nonaka, S., Nishizawa-Yokoi, A., Iwakami, S., Hori,
601 K., Matsumoto, T., Tanaka, T., Itoh, T., et al. (2014). A novel rice cytochrome P450 gene,
602 *CYP72A31*, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and
603 Arabidopsis. Plant Physiol. *166*, 1232–1240.

604 32. Yamaguchi, T., Matsumura, K., Iwakami, S., Sunohara, Y., and Matsumoto, H. (2021).
605 Heterologous expression of CYP81A6 from rice (*Oryza sativa*) in *Escherichia coli* and structural
606 analyses of bensulfuron-methyl metabolites. Weed Biol. Manag. *21*, 164–171.

607 33. TenBrook, P.L., and Tjeerdema, R.S. (2006). Biotransformation of clomazone in rice (*Oryza*
608 *sativa*) and early watergrass (*Echinochloa oryzoides*). Pestic. Biochem. Physiol. *85*, 38–45.

609 34. Yasuor, H., Tenbrook, P.L., Tjeerdema, R.S., and Fischer, A.J. (2008). Responses to clomazone
610 and 5-ketoclomazone by *Echinochloa phyllopogon* resistant to multiple herbicides in Californian
611 rice fields. Pest Manag. Sci. *64*, 1031–1039.

612 35. Zhao, H., Xu, Y., Lin, S., Spain, J.C., and Zhou, N.-Y. (2018). The molecular basis for the
613 intramolecular migration (NIH shift) of the carboxyl group during para-hydroxybenzoate

614 catabolism. Mol. Microbiol. *110*, 411–424.

615 36. Meunier, B., de Visser, S.P., and Shaik, S. (2004). Mechanism of oxidation reactions catalyzed
616 by cytochrome p450 enzymes. Chem. Rev. *104*, 3947–3980.

617 37. Dimaano, N.G., and Iwakami, S. (2021). Cytochrome P450-mediated herbicide metabolism in
618 plants: current understanding and prospects. Pest Manag. Sci. *77*, 22–32.

619 38. Oettmeier, W. (1999). Herbicide resistance and supersensitivity in photosystem II. Cell. Mol. Life
620 Sci. *55*, 1255–1277.

621 39. Arias, R.S., Dayan, F.E., Michel, A., Howell, J., and Scheffler, B.E. (2006). Characterization of a
622 higher plant herbicide-resistant phytoene desaturase and its use as a selectable marker. Plant
623 Biotechnol. J. *4*, 263–273.

624 40. Délye, C., Menchari, Y., Michel, S., and Darmency, H. (2004). Molecular bases for sensitivity to
625 tubulin-binding herbicides in green foxtail. Plant Physiol. *136*, 3920–3932.

626 41. Green, J.M. (2014). Current state of herbicides in herbicide-resistant crops. Pest Manag. Sci. *70*,
627 1351–1357.

628 42. Bakkali, Y., Ruiz-Santaella, J.P., Osuna, Wagner, H., Fischer, A.J., and DePrado, R. (2007). Late
629 watergrass (*Echinochloa phyllopogon*): mechanisms involved in the resistance to
630 fenoxaprop-*p*-ethyl. J. Agric. Food Chem. *55*, 4052–4058.

631 43. Fischer, A.J., Ateh, C.M., Bayer, D.E., and Hill, J.E. (2000). Herbicide-resistant *Echinochloa*
632 *oryzoides* and *E. phyllopogon* in California *Oryza sativa* fields. Weed Sci. *48*, 225–230.

633 44. Chayapakdee, P., Sunohara, Y., Endo, M., Yamaguchi, T., Fan, L., Uchino, A., Matsumoto, H.,
634 and Iwakami, S. (2020). Quinclorac resistance in *Echinochloa phyllopogon* is associated with
635 reduced ethylene synthesis rather than enhanced cyanide detoxification by beta-cyanoalanine
636 synthase. Pest Manag. Sci. *76*, 1195–1204.

637 45. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina
638 sequence data. Bioinformatics *30*, 2114–2120.

639 46. Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data
640 with or without a reference genome. BMC Bioinformatics *12*, 323.

641 47. Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for
642 differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140.

643 48. R Core Team, (2016). R: A language and environment for statistical computing. R Foundation for
644 Statistical Computing, Vienna, Austria.

645 49. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden,
646 T.L. (2009). BLAST+: architecture and applications. BMC Bioinformatics *10*, 421.

647 50. Tanigaki, S., Uchino, A., Okawa, S., Miura, C., Hamamura, K., Matsuo, M., Yoshino, N., Ueno,
648 N., Toyama, Y., Fukumi, N., et al. (2021). Gene expression shapes the patterns of parallel
649 evolution of herbicide resistance in the agricultural weed Monochoria vaginalis. New Phytol.

650 51. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide
651 identification and testing of superior reference genes for transcript normalization in Arabidopsis.
652 Plant Physiol. *139*, 5–17.

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

656 53. Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T)
657 method. Nat. Protoc. *3*, 1101–1108.

658 54. Motohashi, K. (2015). A simple and efficient seamless DNA cloning method using SLiCE from
659 *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis. BMC
660 Biotechnol. *15*, 47.

661 55. Hofgen, R., and Willmitzer, L. (1988). Storage of competent cells for *Agrobacterium*
662 *transformation*. Nucleic Acids Res. *16*, 9877.

663 56. Toki, S. (1997). Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol.

664 Biol. Rep. *15*, 16–21.

665 57. Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated
666 transformation of *Arabidopsis thaliana*. Plant J. *16*, 735–743.

667 58. Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with
668 tobacco tissue cultures. Physiol. Plant. *15*, 473–497.

669 59. Endo, M., Iwakami, S., and Toki, S. (2021). Precision genome editing in plants via gene targeting
670 and subsequent break-induced single-strand annealing. Plant Biotechnol. J. *19*, 563–574.

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

673 61. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells
674 treated with alkali cations. J. Bacteriol. *153*, 163–168.

675 62. Gilchrist, C.L.M., and Chooi, Y.-H. (2021). Clinker & clustermap.js: Automatic generation of gene
676 cluster comparison figures. Bioinformatics.

677 63. Cabanettes, F., and Klopp, C. (2018). D-GENIES: dot plot large genomes in an interactive,
678 efficient and simple way. PeerJ *6*, e4958.

679

680

681 **Figure legends**

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

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

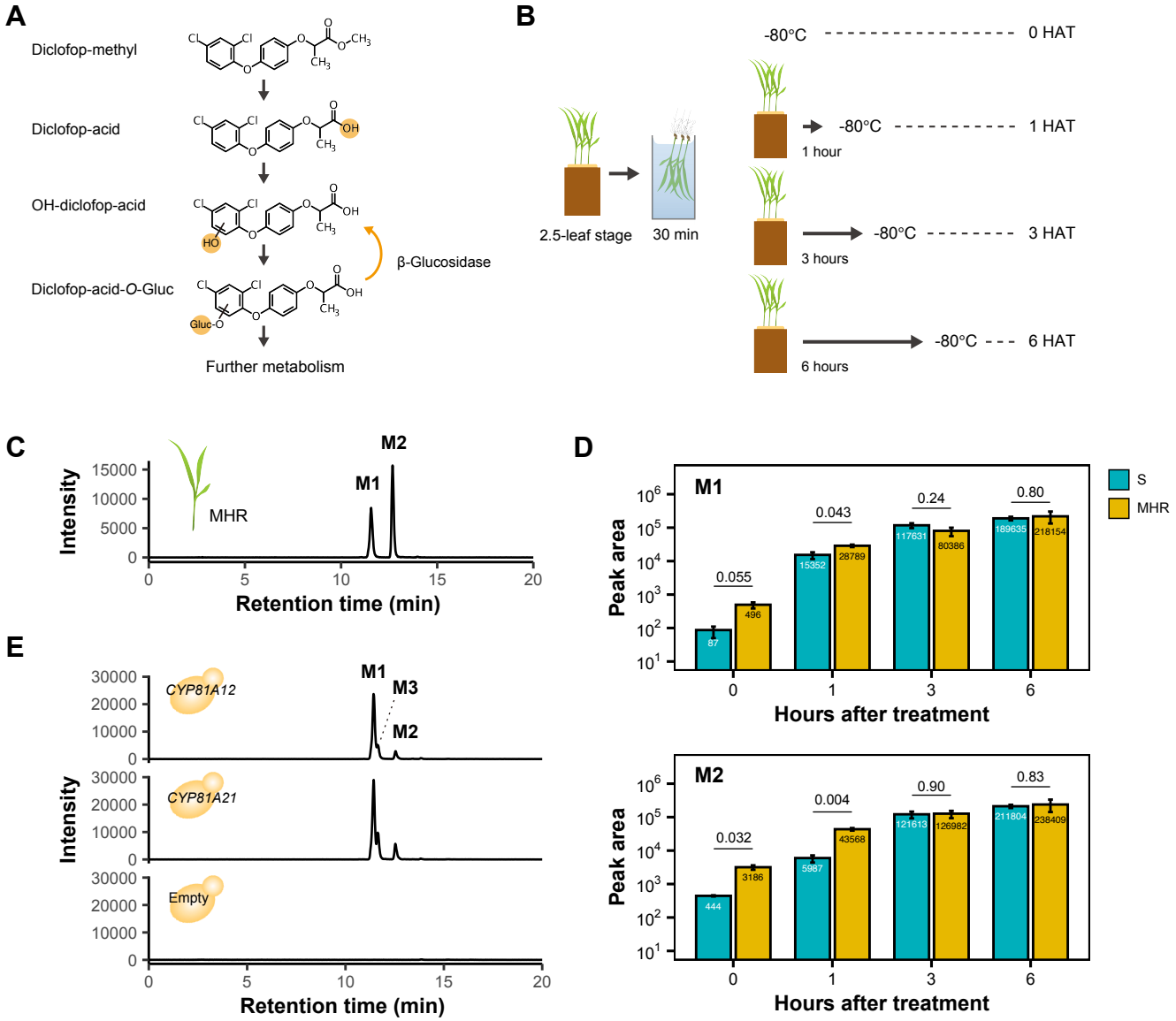
697

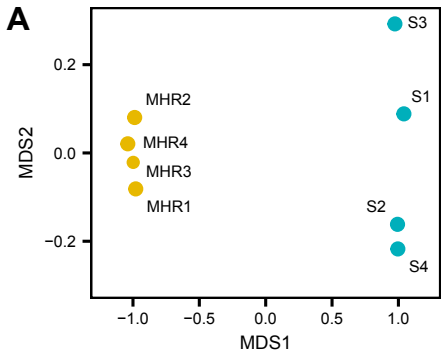
698 **Figure 2. Candidate genes for high-level diclofop-methyl resistance in *Echinochloa***
699 ***phyllopogon* were identified by gene expression study**

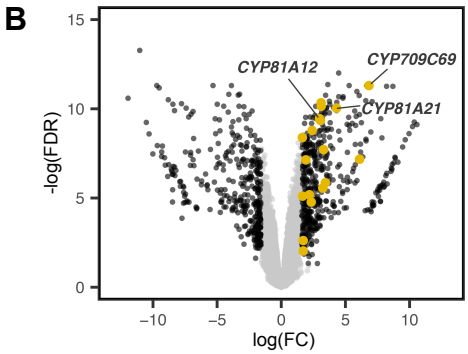
700 (A) Multi-dimensional scaling (MDS) plot of the RNA-seq libraries of the sensitive (S) and
701 multiple-herbicide resistant (MHR) lines. (B) Volcano plot of the S and MHR lines. Differentially
702 expressed genes (fold-change>3, FDR<0.05) are shown in dark gray. Upregulated P450 genes are
703 shown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

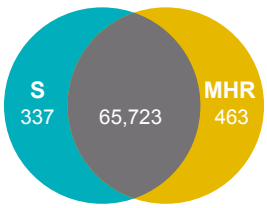
712 expression was not observed in the progeny. Highlight in gray indicates that the gene passed the
713 tests. *, calculated in edgeR. [a], Iwakami et al.[7] [b], Iwakami et al.[8] (G) Progeny association of the mRNA
714 levels of the candidate P450 genes. mRNA levels were quantified by real-time PCR in 16 F6 lines.
715 The signal for CYP71C35 was not detected in the S line. Thus, the values were set as 40 cycles for
716 visualization. F6-S, sensitive F6 lines; F6-R, resistant F6 lines.
717
718 **Figure 3. Identification of novel diclofop-methyl metabolizing P450**
719 (A) Diclofop-methyl sensitivity of rice calli transformed with P450 genes of *Echinochloa phyllopogon.*
720 Twelve independent calli were grown for three weeks. (B) Diclofop-methyl sensitivity of *Arabidopsis*
721 *thaliana* transformed with P450 genes of *E. phyllopogon*. Transcript levels of *CYP709C69* in the
722 transgenic *A. thaliana* were evaluated by real-time PCR. Bars represent SEM. 81A21#12-10 and
723 81A21#6-1 were the lines previously established. [7] (C) *In vivo* diclofop-methyl metabolism assay
724 using yeast harboring empty vector (pYeDP60), *CYP81A12,* or *CYP709C69*. (D) The role of P450s in
725 diclofop-acid hydroxylation. The structures of M1 and M2 were determined by nuclear magnetic
726 resonance analysis. M1 metabolite was produced by the NIH-shift reaction. The arrows indicate
727 migration of chlorine. The structure of M3 metabolite was estimated to have a
728 2,3-dichloro-4-hydroxyphenoxy moiety as reported in wheat. [20]
729
730 **Figure 4. CYP709C69 has a narrow substrate specificity**
731 (A) Rice calli and *Arabidopsis thaliana* transformed with *CYP709C69* were tested for their sensitivity
732 to 46 herbicides from at least 15 modes of action. The tested herbicides were listed in Table S2. (B)
733 Clomazone sensitivity of *A. thaliana* transformed with *CYP709C69*. (C) The pro-herbicide clomazone
734 converted to the active form 5-keto clomazone by the reaction of plant P450s. CYP709C69 may be
735 involved in this reaction.
736
737 **Figure 5. The genetic model of multiple-herbicide resistance in *Echinochloa phyllopogon***
738 (A) The local synteny of *CYP81A* and *CYP709C* regions in the draft genome of *E. phyllopogon*
739 (2n=4X=36) and *E. haploclada* (2n=2X=18). [18] (B) The genomic synteny of the CYP81A and
740 CYP709C contigs in *E. phyllopogon.* The two sets of contigs in *E. phyllopogon* likely forms
741 homeologous pairs. (C) The loci of *CYP81A12*, *CYP81A21*, and *CYP709C69* orthologs in diploid *E.*
742 *haploclada*. (D) A working model for multiple-herbicide resistance in *E. phyllopogon*. A causal

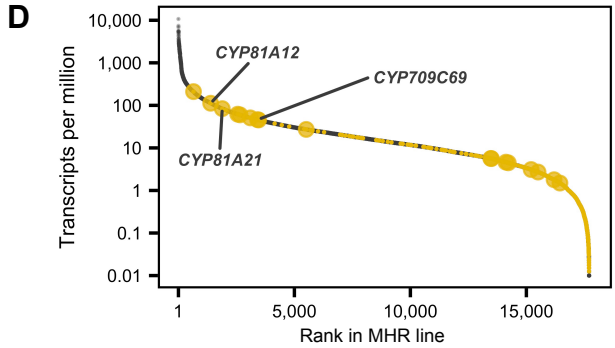
743 mutation activates the expression of at least three herbicide-metabolizing P450s through unknown
744 gene-regulating systems in *E. phyllopogon*. CYP81A12/21 promiscuously metabolize many
745 herbicides at least from five modes of action. CYP709C69 is the more specific enzyme, which
746 simultaneously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

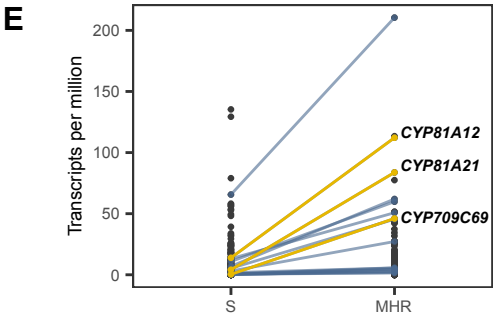

A
Diclofop-methyl
Diclofop-acid
OH-diclofop-acid
Diclofop-acid-O-Gluc
β-Glucosidase
Further metabolism
B
-80°C
0 HAT
2.5-leaf stage
30 min
1 hour
1 HAT
3 hours
3 HAT
6 hours
6 HAT
C
Intensity
MHR
M1
M2
Retention time (min)
D
M1
M2
S
MHR
Peak area
Hours after treatment
0.055
0.043
0.24
0.80
0.032
0.004
0.90
0.83
E
CYP81A12
CYP81A21
Empty
M1
M2
M3
Intensity
Retention time (min)





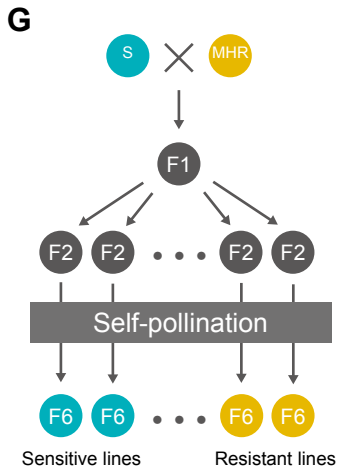


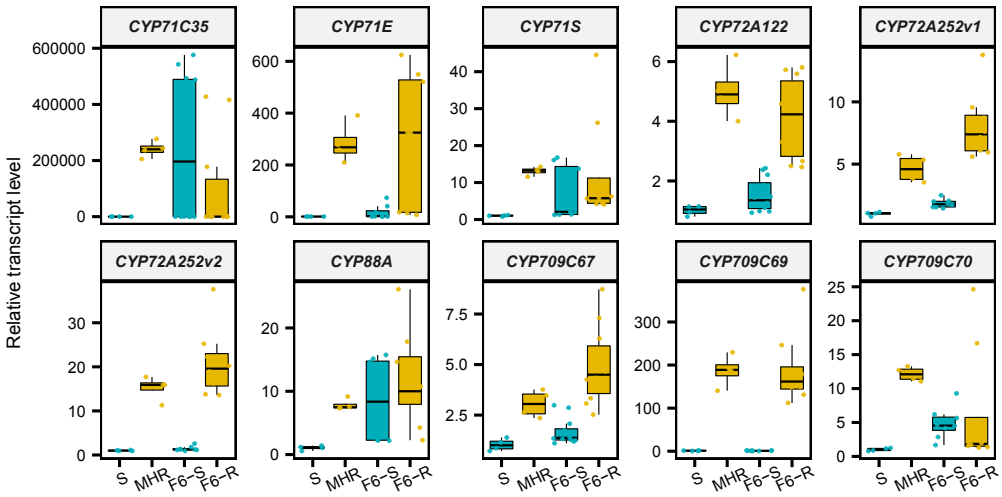


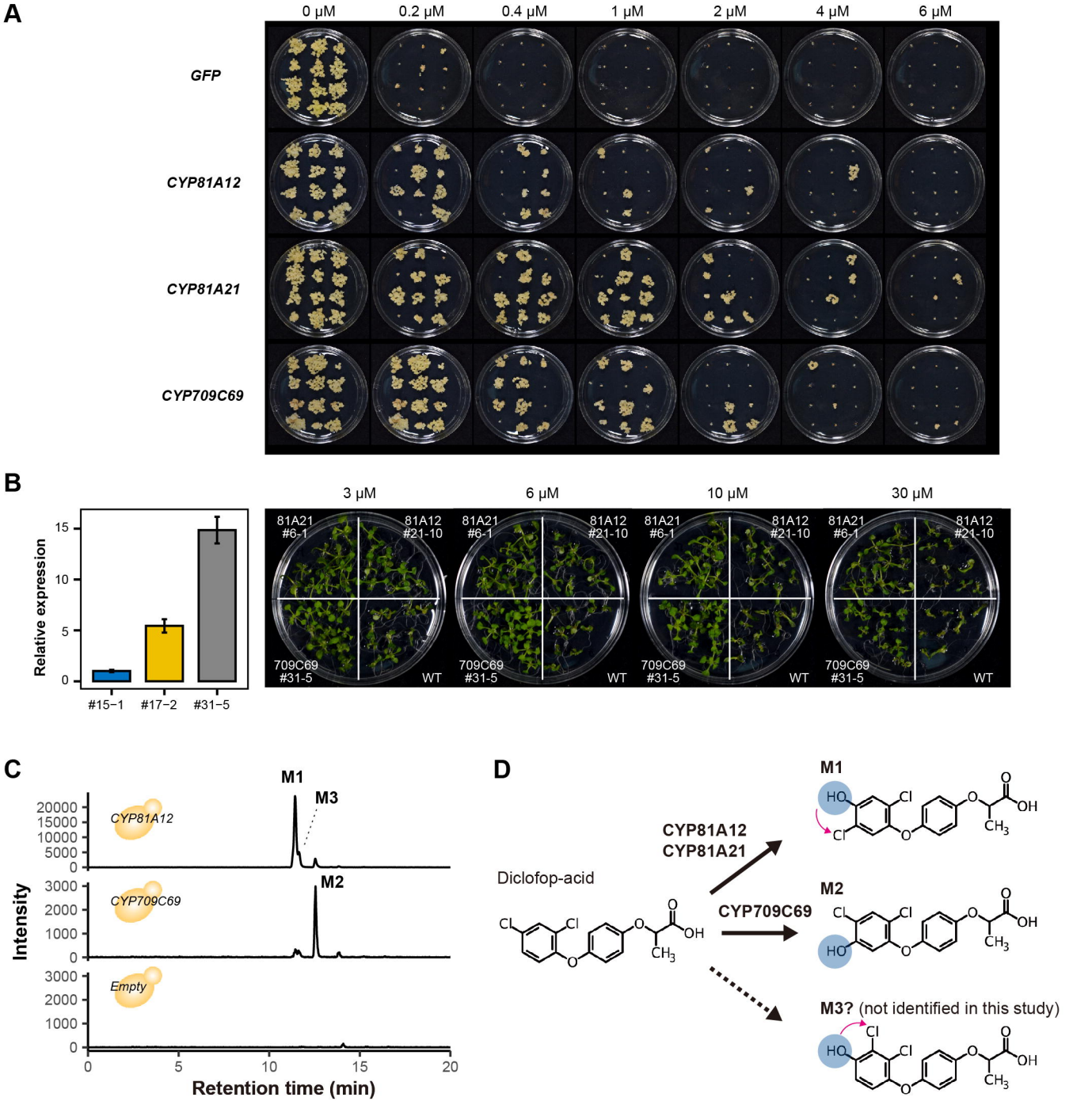


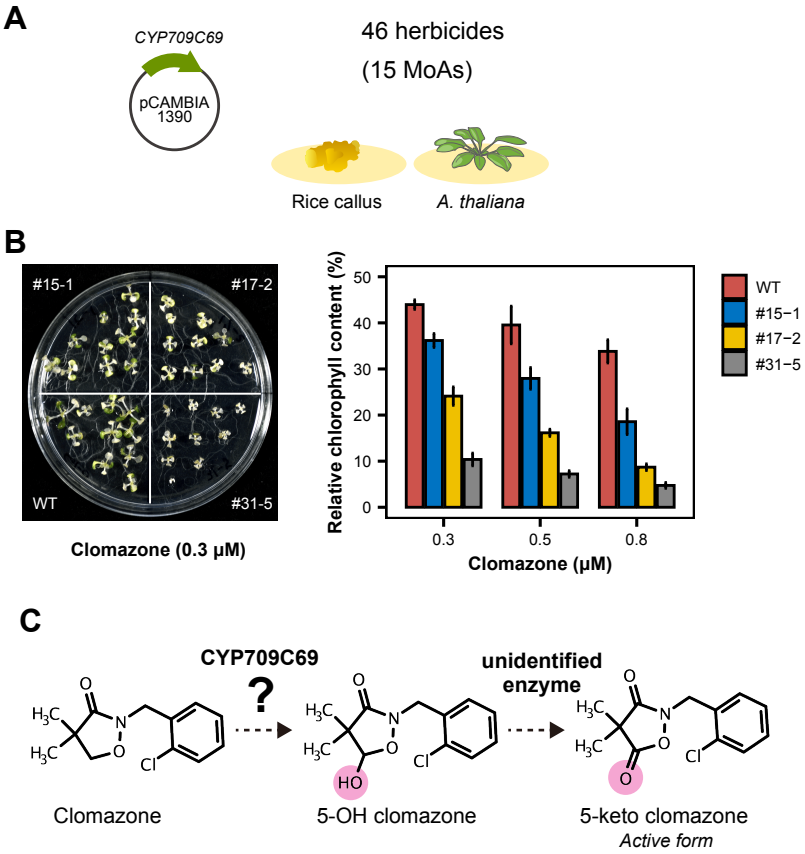
F

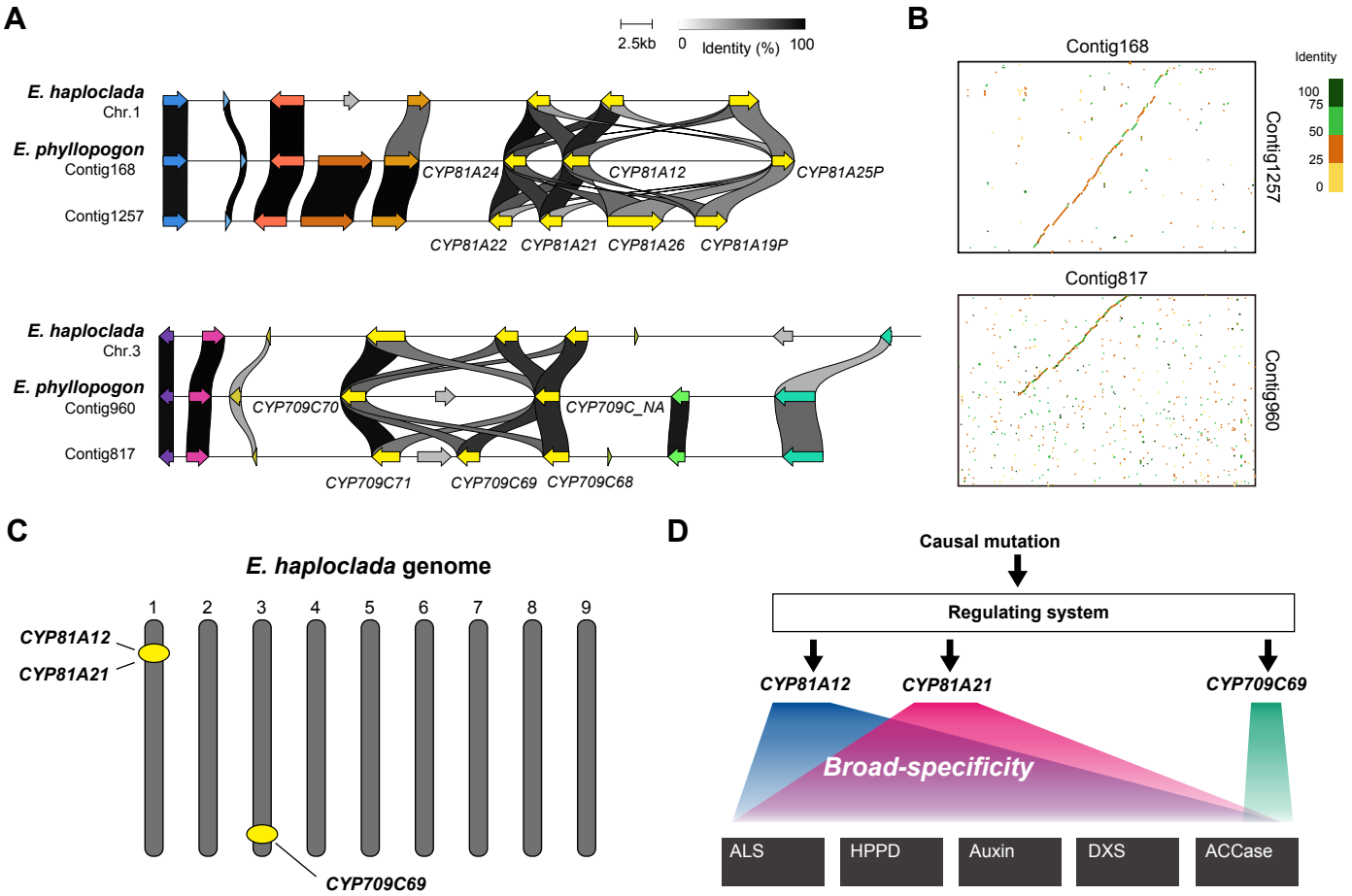
Gene ID	Annotation	logFC*	*q* value*	TPM		Rank in transcriptome		Rank in CYPome		Protein sequence	Progeny association
				S	MHR	S	MHR	S	MHR		
Contig363_pilon.90	CYP72A122	1.64	4.11E-09	65.64	210.46	2407	651	4	1	✓	✓
Contig168_pilon.232	CYP81A12	2.98	3.58E-10	13.88	112.18	8983	1390	42	4	✓	✓[a,b]
Contig1257_pilon.113	CYP81A21	4.30	9.18E-11	4.13	83.76	14348	1890	111	5	✓	✓[a,b]
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	✓	✓
Contig817_pilon.380.b	CYP709C69	6.83	5.00E-12	0.38	46.06	17366	3413	256	12	✓	✓
Contig90_pilon.215	CYP72A252v2	3.05	4.51E-10	5.29	45.46	13611	3457	98	13	✓	✓
Contig1299_pilon.29	CYP71C35	3.10	4.62E-11	3.08	27.21	15169	5510	126	25	✓	×
Contig12_pilon.29	CYP709C67	1.66	7.85E-06	0.55	5.65	17207	13492	233	93	✓	×
Contig10_pilon.131	CYP71S	3.29	1.94E-08	0.55	5.62	17207	13492	233	93	✓	×
Contig960_pilon.352	CYP709C70	3.43	1.40E-06	0.39	4.63	17351	14119	253	107	✓	×
Contig1257_pilon.112	CYP81A22	2.19	6.57E-06	0.92	4.47	16869	14220	204	108	✓	×[a]
Contig222-pillon21.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	×






A
0 μM
0.2 μM
0.4 μM
1 μM
2 μM
4 μM
6 μM
GFP
CYP81A12
CYP81A21
CYP709C69
B
Relative expression
15
10
5
0
#15−1
#17−2
#31−5
3 μM
6 μM
10 μM
30 μM
81A21 #6-1
81A12 #21-10
709C69 #31-5
WT
C
M1
M3
M2
CYP81A12
CYP709C69
Empty
Intensity
20000
15000
10000
5000
3000
2000
1000
0
5
10
15
20
Retention time (min)
D
Diclofop-acid
CYP81A12
CYP81A21
CYP709C69
M1
M2
M3? (not identified in this study)
HO
Cl
O
OH
CH3


A
CYP709C69
pCAMBIA 1390
46 herbicides
(15 MoAs)
Rice callus
A. thaliana
B
#15-1
#17-2
WT
#31-5
Clomazone (0.3 μM)
Relative chlorophyll content (%)
50
40
30
20
10
0
0.3
0.5
0.8
Clomazone (μM)
WT
#15−1
#17−2
#31−5
C
CYP709C69
?
unidentified enzyme
Clomazone
5-OH clomazone
5-keto clomazone
Active form


A
2.5kb
0
Identity (%)
100
E. haploclada
Chr.1
E. phyllopogon
Contig168
Contig1257
CYP81A24
CYP81A12
CYP81A25P
CYP81A22
CYP81A21
CYP81A26
CYP81A19P
E. haploclada
Chr.3
E. phyllopogon
Contig960
Contig817
CYP709C70
CYP709C_NA
CYP709C71
CYP709C69
CYP709C68
B
Contig168
Contig1257
Identity
100
75
50
25
0
Contig817
Contig960
C
E. haploclada genome
1
2
3
4
5
6
7
8
9
CYP81A12
CYP81A21
CYP709C69
D
Causal mutation
Regulating system
CYP81A12
CYP81A21
CYP709C69
Broad-specificity
ALS
HPPD
Auxin
DXS
ACCase