the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2020) doi: 10.1111/tpj.15040

Cytochrome P450 CYP81A10v7 in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action

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Received 15 April 2020; revised 6 October 2020; accepted 12 October 2020.

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SUMMARY

Rapid and widespread evolution of multiple herbicide resistance in global weed species endowed by increased capacity to metabolize (degrade) herbicides (metabolic resistance) is a great threat to herbicide sustainability and global food production. Metabolic resistance in the economically damaging crop weed species *Lolium rigidum* is well known but a molecular understanding has been lacking. We purified a metabolic resistant (R) subset from a field evolved R *L. rigidum* population. The R, the herbicide susceptible (S) and derived F_2 populations were used for candidate herbicide resistance gene discovery by RNA sequencing. A P450 gene *CYP81A10v7* was identified with higher expression in R vs. S plants. Transgenic rice overexpressing this *Lolium CYP81A10v7* gene became highly resistant to acetyl-coenzyme A carboxylase- and acetolactate synthase-inhibiting herbicides (diclofop-methyl, tralkoxydim, chlorsulfuron) and moderately resistant to hydroxyphenylpyruvate dioxygenase-inhibiting herbicide (mesotrione), photosystem II-inhibiting herbicides (atrazine and chlorotoluron) and the tubulin-inhibiting herbicide trifluralin. This wide cross-resistance profile to many dissimilar herbicides in *CYP81A10v7* transgenic rice generally reflects what is evident in the R *L. rigidum*. This report clearly showed that a single P450 gene in a cross-pollinated weed species *L. rigidum* confers resistance to herbicides of at least five modes of action across seven herbicide chemistries.

Keywords: chlorsulfuron, cross-pollinated species, cross-resistance, cytochrome P450, diclofop-methyl, herbicide metabolism, herbicide resistance, *Lolium rigidum*, mesotrione, trifluralin.

INTRODUCTION

Crop-infesting weeds are a major contributor to global crop loss and thus pose a great threat to global grain food production (rice, wheat, soybean, corn, etc.). Herbicides are the technology that has been used for efficient weed control for five decades. Persistent herbicide use, particularly in large areas of the world devoted to field crops, presents very strong selection pressure for the evolution of herbicide-resistant weed populations. Since the reported case of the evolution of 2,4-D resistant weeds (Whitehead and Switzer, 1963), populations of 262 weed species across millions of hectares of the world's best crop land are documented to have evolved herbicide resistance (Heap, 2020). Much research into herbicide resistance endowing mechanisms has been conducted. Resistance mechanisms can be broadly divided into target site resistance (TSR) and non-TSR (NTSR) (Powles and Yu, 2010). TSR involves conformational changes of herbicide target site proteins resulting from amino acid substitution and deletion, or by increased abundance of the target protein due to gene overexpression or amplification. In contrast, NTSR embraces all resistance mechanisms, which are not related to target enzymes. Often, NTSR mechanisms act to minimize a

lethal dose of herbicide reaching a target enzyme and can involve reduced herbicide uptake/translocation, enhanced herbicide metabolism (metabolic resistance) or sequestration. Metabolic herbicide resistance, the most commonly documented NTSR mechanism, is complex and can involve superfamilies of cytochrome P450s (P450s), glucosyltransferases (GTs), glutathione *S*-transferases (GSTs), aldo-keto reductase, transporters and esterases (Yu and Powles, 2014; Pan *et al.*, 2019; Gaines *et al.*, 2020), although definitive data are rare. Importantly, metabolic herbicide resistance can confer cross-resistance to herbicides of different modes of action, including herbicides to which weeds have never been exposed. Metabolic resistance mechanisms can be indicated indirectly by use of P450 or GST inhibitors or directly by enhanced metabolism rates of herbicides (Imaishi and Matumoto, 2007; Ma *et al.*, 2013; Iwakami *et al.*, 2014; Yu and Powles, 2014; Ghanizadeh and Harrington, 2017; Busi *et al.*, 2018; Chen *et al.*, 2018; Iwakami *et al.*, 2019; Maeda *et al.*, 2019; Pan *et al.*, 2019).

Plant P450s, a gene superfamily, make up as much as 1% of the plant genome encoding versatile enzymes catalysing multiple plant metabolism processes, including herbicide detoxification (Mizutani and Sato, 2011; Nelson and Werck-Reichhart, 2011). Recent progress has revealed a handful of P450 genes encoding CYP71, CYP72, CYP73, CYP76, CYP81 and CYP749 delivering metabolic resistance to acetyl-coenzyme A carboxylase (ACCase)-, acetolactate synthase (ALS)-, photosystem II (PSII)-, hydroxyphenylpyruvate dioxygenase (HPPD)-, phytoene desaturase-, deoxy-D-xylulose-phosphate synthase- and protoporphyrinogen oxidase-inhibiting and synthetic auxin herbicides (Table S1) (Pierrel *et al.*, 1994; Cabello-Hurtado *et al.*, 1998; Siminszky *et al.*, 1999; Yamada *et al.*, 2000; Didierjean *et al.*, 2002; Pan *et al.*, 2006; Xiang *et al.*, 2006; Imaishi and Matumoto, 2007; Dam *et al.*, 2008; Iwakami *et al.*, 2014; Saika *et al.*, 2014; Thyssen *et al.*, 2018; Guo *et al.*, 2019; Iwakami *et al.*, 2019; Dimaano *et al.*, 2020). These P450s have mainly been isolated from field crops such as rice (*Oryza sativa*), barley (*Hordeum vulgare*), cotton (*Gossypium hirsutum*), soybean (*Glycine max*), corn (*Zea mays*) and Jerusalem artichoke (*Helianthus tuberosus*) and a weedy species of watergrass (*Echinochloa phyllopogon*). While in most cases overexpression of a specific P450 gene resulted in herbicide resistance, further genetic mechanisms of P450-based resistance remains unclear in weedy species, e.g., whether resistance is due to single nucleotide polymorphisms within the P450 genes, copy number variation, or regulatory changes. Considering the complexity of metabolic herbicide resistance and the diversity and number of plant P450s, we are far from fully understanding metabolic herbicide resistance in weedy species.

Lolium species including obligate out-crossing and genetically diverse grass weeds have evolved widespread metabolic resistance particularly to ACCase- and ALS-inhibiting herbicides in Australia and elsewhere (Christopher *et al.*, 1991; Holtum *et al.*, 1991; Christopher *et al.*, 1992; Cotterman and Saari, 1992; Han *et al.*, 2016). We have long established that resistance is due to enhanced activity of P450 enzymes (Christopher *et al.*, 1991, 1994; Preston *et al.*, 1996). Metabolism pathways of certain herbicides have been documented. For instance, diclofop-methyl metabolism in *L. rigidum* and other cereal species involves P450-catalysed aryl hydroxylation, followed by glucose conjugation (Shimabukuro *et al.*, 1979; Donald and Shimabukuro, 1980; Shimabukuro and Hoffer, 1991). Similarly, a wheat-like P450 metabolism of chlorsulfuron was reported in *L. rigidum* in which chlorsulfuron was hydroxylated at the phenyl ring, followed by glucose conjugation (Sweetser *et al.*, 1982; Christopher *et al.*, 1991; Cotterman and Saari, 1992). In addition, to a much less extent, the cleavage of chlorsulfuron into chlorobenzene sulfonamide and triazine amine was also reported in *L. rigidum* (Cotterman and Saari, 1992). These earlier studies demonstrating involvement of P450 in *Lolium* metabolic herbicide resistance were all conducted at the biochemistry level. However, progress on revealing P450 genes responsible for metabolic resistance in *L. rigidum* has been until now slow (Fischer *et al.*, 2001), and recent RNA-Sequencing (RNA-Seq) analysis implicates involvement of P450 genes in metabolic resistance in *Lolium* (Gaines *et al.*, 2014). Here, building upon our decades of knowledge of metabolic herbicide resistance in the important grass weed *L. rigidum* we report identification, cloning and functional characterization of a *L. rigidum* P450 gene endowing resistance to herbicides across several modes of action and chemistry groups.

RESULTS

Characterization of resistant and susceptible *Lolium* plants for RNA-Seq

Plant vegetative cloning followed by herbicide treatment and target-site gene sequencing was employed to purify a solely metabolism-based resistant (R) subset from a multiple resistant *L. rigidum* population (SLR31) (Heap and Knight, 1990; Christopher *et al.*, 1991) for RNA-Seq. As expected, the R subset displays high-level resistance to the dissimilar herbicides diclofop-methyl (131-fold) and chlorsulfuron (>80-fold), relative to the susceptible (S) population, based on the R/S herbicide rate causing 50% plant mortality (LD_{50}) (Table 1; Figure S1). Correspondingly, the R subset exhibited enhanced diclofop-methyl metabolism, with a 54% increase in diclofop acid metabolites relative to the S control (Figure S2). Although not examined in the R subset, chlorsulfuron metabolism in the original SLR31 population was also found to be greater than the S population (Christopher *et al.*, 1991). It is evident that the R subset is highly resistant to both diclofop-methyl and

Table 1 Estimation of the herbicide rate causing 50% plant mortality (LD_{50}) and rate causing 50% biomass reduction (GR_{50}) values

		Lolium rigidum						Transgenic rice					
		LD_{50} (g ha^{-1})			GR_{50} (g ha^{-1})			LD_{50} (g ha^{-1})			GR_{50} (g ha^{-1})		
Herbicide	Mode of action	R	S	Ratio	R	S	Ratio	CYP81	GFP	Ratio	CYP81	GFP	Ratio
Diclofop-methyl	ACCase	1472	11.2	131	3515	17.9	196	>6000	783	>7.7	>6000	309	>19.4
Chlorsulfuron	ALS	>800	10.0	>80.0	>800	4.2	>190	>400	25.1	>15.9	>400	2.3	>173
Tralkoxydim	ACCase	>800	18.0	>44.4	>800	7.0	>114	>400	44.9	>8.9	233	8.9	26.2
Mesotrione	HPPD	>400	302	-	>400	22.0	>18.2	198	112	1.8	43.2	15.6	2.8
Atrazine	PSII	4311	1391	3.1	756	44.6	17.0	2239	989	2.3	274	71.1	3.9
Chlorotoluron	PSII	7116	1116	6.4	566	42.9	13.2	1686	436	3.9	376	99.7	3.8
Trifluralin	Tubulin	376	51.6	7.3	279	48.8	5.7	289	64.6	4.5	52.1	19.0	2.7

Herbicide dose–responses were conducted in susceptible (S) and metabolic resistant (R) *L. rigidum* and transgenic rice seedlings expressing *CYP81A10v7* (*CYP81*) and *GFP*. Ratio refers to R/S or CYP81/GFP.
ACCase, acetyl-coenzyme A carboxylase; ALS, acetolactate synthase; HPPD, hydroxyphenylpyruvate dioxygenase; PSII, photosystem II.

chlorsulfuron due to increased herbicide metabolism capacity without target-site resistance mutations. In addition, the R subset showed more than 44-fold resistance to the ACCase-inhibiting herbicide tralkoxydim, up to six-fold resistance to PSII-inhibiting herbicides atrazine and chlorotoluron, and seven-fold resistance to the pre-emergence, tubulin-inhibiting herbicide trifluralin, based on R/S ratios of LD_{50}. There is also at least 18-fold resistance to the HPPD-inhibiting herbicide mesotrione based on the R/S rate causing 50% biomass reduction (GR_{50}) (Table 1; Figures S1 and S3). Therefore, this R subset is cross-resistant to herbicides across at least five modes of action, including those never used in the original population (SLR31) before resistance evolution (e.g. chlorsulfuron, mesotrione, atrazine, chlorotoluron), and hence is ideal for metabolic resistance gene discovery. Interestingly, however, the known P450 inhibitor malathion reversed the resistance to chlorsulfuron but not diclofop-methyl (Figure S4).

In this particular SLR31 subset with 50 F_2 individual plants analysed, the segregation ratio of resistance to susceptibility at 750 g ha^{-1} diclofop-methyl and 100 g ha^{-1} chlorsulfuron is 35:15 and 36:14, respectively, fitting 3:1 single gene control model (chi-squared test, $P = 0.41$ for diclofop-methyl and 0.62 for chlorsulfuron). However, at higher herbicide rates (diclofop-methyl 6000 g ha^{-1}, chlorsulfuron 400 and 800 g ha^{-1}) the resistance traits did not fit the single gene control model. Moreover, resistance to diclofop-methyl and chlorsulfuron is linked.

Identification and validation of differentially expressed contigs using RNA-Seq

To reduce and offset genetic variation, the most S and R individuals characterized from both parent and F_2 populations were used for RNA-Seq (Figure S5). In addition, to avoid any complications of differential effects of herbicide treatment, we examined only constitutive differential gene expression.

Owing to the unavailability of a *L. rigidum* reference genome, the *de novo* reference transcriptome was assembled using sequencing data from four S and four R individuals from F_2 populations using Trinity with the total 40 324 180 assembled bases (Grabherr *et al.*, 2011). Transcripts (56 527) were assembled with contig N50 size of 1136 bp and 48 170 putative genes were identified. The transcriptome completeness was assessed to be 40% using BUSCO (Figure S6) (Simão *et al.*, 2015). The genome size of *L. rigidum* is estimated to be about 2.75 Gb, similar to the genetically closely related species *L. perenne* in which there are 28 455 putative genes identified (Śmarda *et al.*, 2008; Stewart *et al.*, 2009; Byrne *et al.*, 2015). This indicates that 68% of *L. rigidum* genes were assembled, even though only leaf tissue was sampled for RNA-Seq. In total, 128 P450s, 96 GSTs and 18 GTs were annotated from our RNA-Seq analysis. To increase the transcriptome coverage, sequence data were mapped to the reference transcriptomes assembled both in this study and by Gaines *et al.* (2014). There were averages of 52 M (92.4%) and 51 M (90.7%) counts per sample that were mapped to the two reference transcriptomes, respectively. Principal components analysis results showed samples from S and R clustered into different groups. However, all eight F_2 samples, including S and R individuals grouped together (Figure S7). Thus, as expected, the genetic background between S vs. R plants within the F_2 population were more similar than between the S vs. R parent plants.

Differential gene expression was evaluated using the DESeq2 R package (1.18.0) at padj < 0.05 between S and R plants from parents and the F_2, respectively. When data aligned with the reference transcriptome assembled in this study, 6631 contigs displayed significantly differential expression between parent S vs. R, much greater than the 544 contigs between F_2 S vs R plants. Only 137 contigs showed differential expression between S and R plants from both parents and the F_2. Similarly, with the data

mapped to the reference transcriptome by Gaines *et al.* (2014), 9551 contigs displayed significantly differential expression between parent S vs. R. Instead, there were 617 contigs showing significant difference in expression between S and R F_2 plants. Only 178 contigs showed differential expression between S and R plants from both parents and the F_2. Clearly, the contigs with differential expression were markedly reduced when the F_2 populations were involved (Figure S8).

Thirteen candidate contigs that showed more than two-fold constitutive upregulation with annotation related to herbicide metabolism (such as P450s, GTs and GSTs) were selected for reverse transcription–quantitative polymerase chain reaction (RT-qPCR) validation using RNA-Seq samples and additional spare ones (Table 2, Figure S9). Eight contigs showed significant upregulation, similar to RNA-Seq, including three P450s, four GTs and one GST. We noticed the large difference in fold-changes measured by RNA-Seq and RT-qPCR for contig 11357. Likely, this is due to the high specificity of the primers designed for RT-qPCR (annealing to the 3′-UTR) and low-level expression of this contig in the S.

Candidate contig validation in other R *Lolium* populations

Eight candidate contigs were used for expression validation using RT-qPCR in eight characterized R survey populations. Two reference genes, CAP binding protein and Ras family GTPase were used as the internal control for gene expression, and the former used for fold-change calculation. The GTPase gene displayed consistent expression among all populations. Three contigs (c14457_g1_i4, c43839_g1_i1 and c14740_g1_i1, with annotations to one CYP72 and two UPDG transferases) were significantly upregulated (up to 31-fold) in seven of eight resistant populations. Contig 11357 (with a CYP81A annotation) was highly (500-fold) upregulated in two resistant populations (Table 3). No single candidate contigs with consistent overexpression across all tested populations could be assigned as transcriptional biomarkers. Therefore, among the three contigs with P450 annotation (CYP81A10v7, CYP704C1, CYP72A71v1), CYP72A71v1 and CYP81A10v7 were prioritized for cloning and functional analysis, based on (i) the above malathion inhibition experiment showing possible involvement of P450s in resistance, and (ii) recent identification of CYP81As (Iwakami *et al.*, 2014; Guo *et al.*, 2019; Iwakami *et al.*, 2019; Dimaano *et al.*, 2020) in *E. phyllopogon*, and CYP72A (Saika *et al.*, 2014) in rice conferring herbicide metabolic resistance.

Herbicide sensitivity of transgenic rice calli overexpressing *CYP72A71v1* and *CYP81A10v7* to ACCase- and ALS-inhibiting herbicides

The full-length cDNAs of *CYP72A71v1* and *CYP81A10v7* were cloned respectively from R plants with the highest expression of either of the two genes, based on their corresponding contig sequences. The 1575-bp *CYP72A71v1* cDNA sequence codes for 525 amino acids were identical to the published *CYP72A71v1* (NCBI accession number AF321870). The 1551-bp *CYP81A10v7* cDNA sequence codes for 517 amino acids (NCBI accession number

Table 2 Identification of differentially expressed contigs in susceptible (S) vs. resistant (R) *Lolium rigidum* using RNA-Sequencing (RNA-Seq)

		Fold-change (R/S)					
		RNA-Seq		RT-qPCR		RT-qPCR	
Contig	Annotation	Parent	F_2	Parent	F_2	Additional parent	Additional F_2
11357	CYP81A10v7	3.3*	4.0*	>500*	34.5*	452*	41.4*
07051	CYP72A1	4.8*	2.9	2.9	2.1	3.1	1.4
15776	CYP704C1	2.9	4.0*	2.5*	6.2*	3.1*	5.8*
c14457_g1_i4	CYP72A71v1	2.3*	1.1	2.2*	3.4*	5.9*	4.2*
47344	Glucosyltransferase	6.3*	4.2*	8.9*	6.0*	19.7*	3.6*
c28089_g1_i1	UPDG transferase	9.7*	3.7*	16.5*	4.6*	1.9	1.4
c43839_g1_i1	UPDG transferase	14.1*	4.2*	15.1*	5.2*	13.0*	4.0*
c13587_g1_i2	Glucosyltransferase	7.1*	3.2*	16.7*	7.1*	26.9*	3.7*
c14740_g1_i1	UDPG transferase	4.2*	5.9*	5.1*	11.7*	22.3*	3.4*
c15780_g1_i1	Glucosyltransferase	3.9	38.3*	0.8	471.4*	1.8	38.1*
c13489_g2_i1	GSTU1	10.3*	15.6*	14.5*	31.2*	3.7	1.8
c15309_g4_i2	GSTF4	7.1*	9.0*	11.1*	10.2*	15.3*	5.6*
c15309_g2_i2	GSTF1	4.6*	3.9	2.6	6.9*	2.3	2.5

Candidate contigs were selected based on the fold-changes calculated DESeq2 R package (1.18.0) at padj < 0.05, and validated using reverse transcription–quantitative polymerase chain reaction (RT-qPCR) with RNA-Seq samples, plus additional spare samples of five parent S, four parent R, six F_2 S and 10 F_2 R.
*Indicates significant difference, padj < 0.05 or $P < 0.05$ (Student's *t*-test).

Table 3 Relative transcript levels of the selected candidate contigs from RNA-Sequencing in the susceptible (S) and metabolic resistant (R) *Lolium rigidum* populations

		Fold-change (R/S)							
		R to diclofop-methyl						R to chlorsulfuron	
Contig	Annotation	H2/3	H4/1	H4/10	M3/47	M2/23	L3/14	H4/13	H4/12
11357	CYP81A10v7	>500*	0.9	479.7*	1.7	2.0	1.0	0.5	1.2
15776	CYP704C1	2.3*	1.8	1.0	4.2*	3.1*	0.4	1.3	0.6
c14457_g1_i4	CYP72A71v1	14.1*	5.3*	3.1*	5.8*	6.3*	19.8*	1.2	4.8*
47344	Glucosyltransferase	0.8	0.5	0.7	1.7	0.2	3.9*	0.2	1.2
c43839_g1_i1	UPDG transferase	4.5*	2.2*	3.5*	11.3*	9.7*	12.2*	1.0	3.3*
c13587_g1_i2	Glucosyltransferase	0.9	4.5*	0.6	1.8	0.1	3.6*	0.1	1.4
c14740_g1_i1	UDPG transferase	14.7*	6.1*	9.2*	4.3*	8.7*	30.6*	2.2	9.7*
c15309_g4_i2	GSTF4	7.3*	1.4	7.3*	2.7*	3.1*	3.6*	1.0	2.2
16303	Ras family GTPase	0.7	0.8	0.7	1.0	1.1	0.9	0.6	0.7

All data contained five and three biological replicates for diclofop-methyl and chlorsulfuron R populations, respectively.
*Indicates significant difference, $P < 0.05$ (Student's *t*-test).

MK629521), with protein sequence identity of 77.4%, 73.2%, 72.5% and 71.8% to CYP81A6 (rice), Nsf1 (corn), CYP81A12 (watergrass) and CYP81A21 (watergrass), respectively. *CYP72A71v1* and *CYP81A10v7* were transformed separately into rice (*Oryza sativa* cv. Nippornbare) calli. The green fluorescent protein (*GFP*) gene was used as the negative control. Ten independent T_0 transgenic rice calli were used for sensitivity test for each ACCase- and ALS-inhibiting herbicide. Unexpectedly, the rice calli overexpressing *CYP72A71v1* showed similar sensitivity with the GFP control to diclofop-methyl, chlorsulfuron or tralkoxydim (Figure S10), and thus did not confer resistance to these herbicides. Therefore, no further analysis of this gene was conducted.

As expected, growth of the rice calli expressing *GFP* was inhibited at 0.4 μM diclofop-methyl, and death occurred at >0.8 μM diclofop-methyl. However, growth of the rice calli overexpressing *CYP81A10v7* was unaffected at 3.2 μM diclofop-methyl. Similarly, the *GFP* transgenic rice calli did not grow at 0.05 μM chlorsulfuron, whereas the calli overexpressing *CYP81A10v7* proliferated at 0.4 μM chlorsulfuron. In addition, growth difference between the calli overexpressing *CYP81A10v7* and *GFP* was only observed at 0.8 μM tralkoxydim, indicating modest resistance to tralkoxydim (Figure 1). No resistance of *CYP81A10v7* transgenic rice calli was observed to other ACCase-inhibiting herbicides clodinafop fenoxaprop, haloxyfop, quizalofop, sethoxydim and pinoxaden, or the ALS-inhibiting herbicides mesosulfuron and iodosulfuron (Figures 1 and S11).

Herbicide sensitivity of transgenic rice plants overexpressing *CYP81A10v7* to herbicides of different modes of action

Seedlings of the rice cultivar Nippornbare are relatively susceptible to diclofop-methyl. Five T_1 rice lines expressing *CYP81A10v7* were treated with high-dose 6000 g diclofop-methyl ha^{-1}, and four of them showed resistance to diclofop-methyl. Two homozygous T_2 *CYP81A10v7* lines were characterized and herbicide dose–response experiments were conducted to determine the level of cross-resistance to diclofop-methyl, chlorsulfuron, tralkoxydim, mesotrione, atrazine, chlorotoluron and trifluralin based on the GR_{50} or LD_{50} ratio (Table 1, Figures 2, S12 and S13). As expected, the growth of *GFP* plants were reduced (by more than 50% in biomass) when treated with 375 g diclofop-methyl ha^{-1}, and all plants were completely controlled at 3000 g ha^{-1}. In contrast, *CYP81A10v7* transgenic plants survived 6000 g diclofop-methyl ha^{-1} treatment (Figure 2), exhibiting up to 19-fold resistance (Table 1). Similarly, the *CYP81A10v7* transgenic plants displayed at least 173-fold resistance to chlorsulfuron with only 24% reduction in biomass at 400 g ha^{-1}. In contrast, *GFP* transgenic plants died at 50 g chlorsulfuron ha^{-1} (Table 1; Figures S12 and S13). Although rice calli expressing *CYP81A10v7* only exhibited modest resistance to tralkoxydim (Figure 1), the T_2 *CYP81A10v7* plants showed more than 26-fold resistance to tralkoxydim (Table 1).

Importantly, overexpressing *CYP81A10v7* in rice endowed moderate levels (up to four-fold) of resistance to post-emergence metabolizable herbicides of other modes of action including mesotrione, atrazine and chlorotoluron (Table 1, Figures 2, S12 and S13). Surprisingly, overexpressing *CYP81A10v7* in rice conferred up to five-fold resistance to the pre-emergence herbicide trifluralin with 80% of plants surviving 120 g trifluralin ha^{-1} compared with 5% of GFP plants, even though plant growth was greatly inhibited in *CYP81A10v7* transgenic lines (Table 1, Figures 2, S12 and S13). Clearly, the *CYP81A10v7* gene confers resistance to herbicides of at least five modes of action across seven herbicide chemistries (Table 1, Figures 1, 2, S12 and

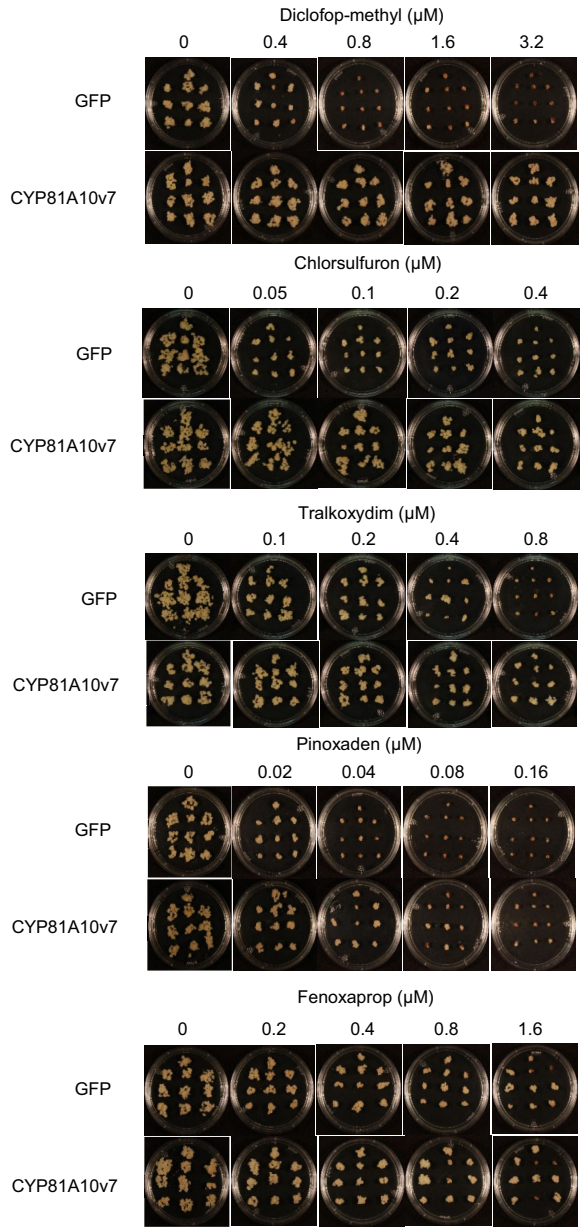

Figure 1. Sensitivity of the rice calli expressing *CYP81A10v7* and green fluorescent protein (*GFP*) control to diclofop-methyl, chlorsulfuron, tralkoxydim, pinoxaden and fenoxaprop.
Ten independent calli expressing *CYP81A10v7* or *GFP* were selected with hygromycin at 50 mg L^{-1} and grown on N6D medium containing the herbicides for 3 weeks. The experiment was conducted twice with similar results.

S13). This cross-resistance profile revealed in *CYP81A10v7* transgenic rice generally correlates with that observed in the purified R *Lolium* population (Table 1).

We noticed that compared with the untransformed and *GFP* transgenic rice plants, all the *CYP81A10v7* transgenic plants are relatively shorter and have more tillers (Figure 2). The phenotype change may be caused by overexpression of the specific P450 gene and/or its pleiotropic effect, and are thus worthy of further study.

CYP81A10v7 variants in *Lolium rigidum*

To determine CYP81A10v7 protein sequence variations, the *CYP81A10v7* cDNA was amplified from plants of S and R parents, F_2 and a survey population (H2/3) with higher *CYP81A10v7* expression. The amplicon was transformed into *Escherichia coli* and three to six clones from each plant sequenced. As an obligate cross-pollinated species, *L. rigidum* showed the expected high genetic diversity, even for a single gene. In total, 12 CYP81A10v7 variants were identified from 10 plants: at least six variants in S plants and six variants in R plants. The CYP81A10v7 variants among S plants showed 96–100% amino acid sequence identity to CYP81A10v7, while they share 98–100% identity in R plants (Figure S14). As identical CYP81A10v7 sequence was identified in the parent S and R, and population H2/3 samples, it is thus the overexpression of this variant that endows herbicide resistance. Nevertheless, it remains to be determined (i) if overexpression of other *CYP81A10v7* variants also confers herbicide metabolic resistance, and (ii) if the amino acid changes in these *CYP81A10v7* variants contribute to resistance.

Metabolism of diclofop-methyl and mesotrione in *CYP81A10v7* transgenic rice

Owing to the availability of ^{14}C-herbicides, metabolism of the very dissimilar herbicides diclofop-methyl and mesotrione was examined in two homozygous T_2 rice lines expressing *CYP81A10v7* or *GFP*. The herbicide diclofop-methyl and its polar metabolites are clearly resolved in our high-performance liquid chromatography (HPLC) conditions with diclofop-methyl retention time at 31 min, diclofop acid at 29 min and major polar metabolites at within 5 min (Figure 3). Once diclofop-methyl is absorbed, esterases efficiently convert it to herbicidally active diclofop acid (Shimabukuro *et al.*, 1979). There was no significant difference observed for diclofop-methyl amount between the plants expressing *CYP81A10v7* and the *GFP* control (Table 4). The conversion of diclofop-methyl into non-herbicidal polar metabolites increased with time. However, the transgenic rice plants expressing *CYP81A10v7* more rapidly metabolized diclofop acid than the *GFP* control. Only 16% diclofop acid remained in *CYP81A10v7* transgenic plants compared with 44% in the transgenic *GFP* control, 72 h after treatment, leading to 57% more metabolites being detected in *CYP81A10v7* than in *GFP* plants. Clearly, overexpression of *CYP81A10v7* increased the metabolism of diclofop-methyl to the polar metabolites, therefore conferring resistance (Table 4; Figure 3).

The comparison of HPLC chromatograms between *CYP81A10v7* transgenic rice and R *Lolium* indicated that both have similar major polar metabolites, with retention

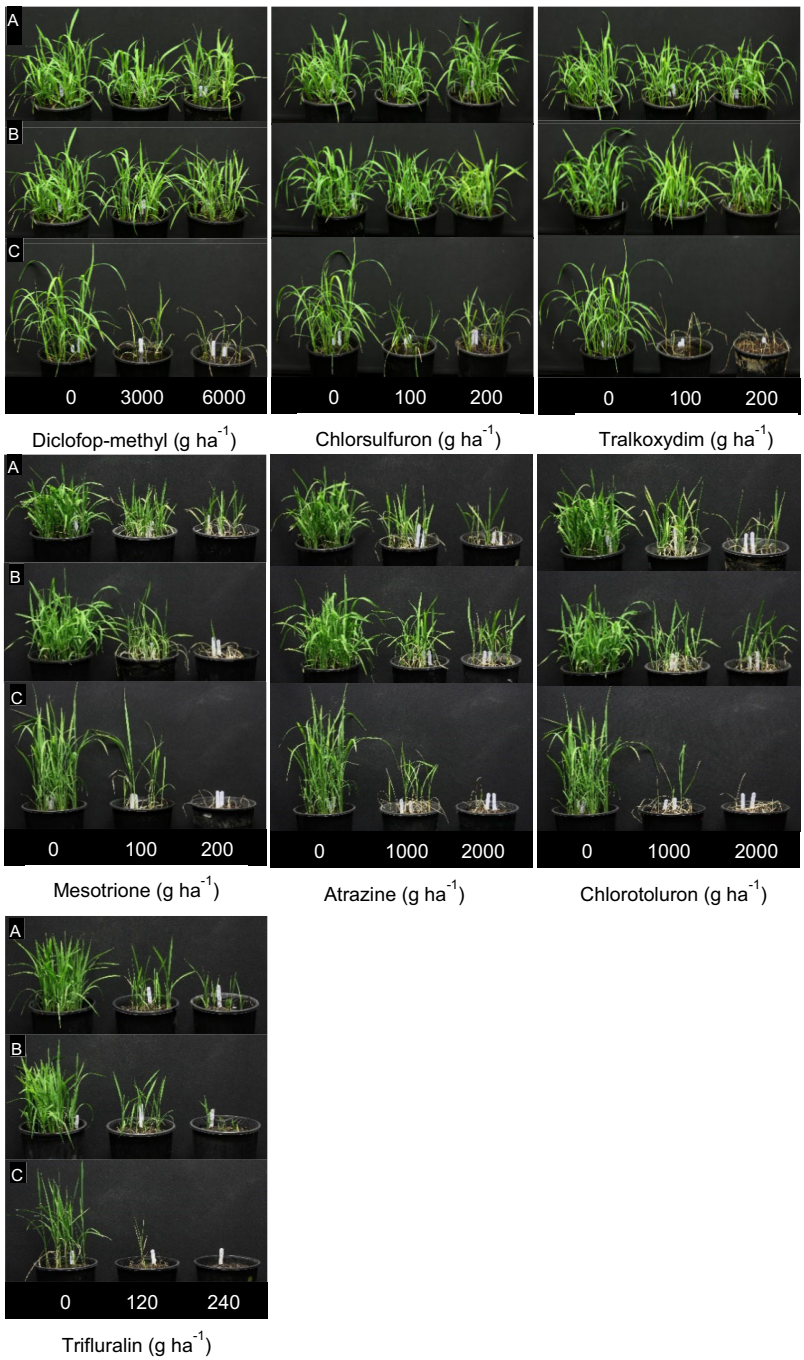

Figure 2. Sensitivity of transgenic rice seedlings expressing *CYP81A10v7* and green fluorescent protein (*GFP*).
The four-leaf stage seedlings of two *CYP81A10v7* and one *GFP* lines were treated with diclofop-methyl, chlorsulfuron, tralkoxydim, mesotrione, atrazine, chlorotoluron or trifluralin. The experiment was conducted twice with two replicates each with five to 10 plants. Photos were taken 3 weeks after treatment. A, CYP81A10v7-6; B, CYP81A10v7-7; C, *GFP*.

times between 2 and 4 min. However, there are minor metabolite peaks with the retention time of 6.7–11 min detected in R *Lolium* but not in rice, showing a somewhat different metabolism pathway in *Lolium* compared with rice (Figures 3 and S2).

For mesotrione metabolism analysis, the parent mesotrione and its two major metabolites were detected with retention time of 22.6, 14.6 and 15.3 min, respectively, in both *CYP81A10v7* and *GFP* lines (Figure 3). The level of remaining parent mesotrione in *CYP81A10v7* lines was

three-fold less than that in GFP lines at 48 h, corresponding to a consistently higher level of major mesotrione metabolite 1 (poorly separated with metabolite 2), 48 and 72 h after treatment (Table 4). Compared with the HPLC chromatograph of mesotrione metabolism in waterhemp (Ma *et al.*, 2013), metabolite 2 (14.6 min) is likely 4-hydroxy-mesotrione (Figure 3).

DISCUSSION

Gene discovery for metabolic herbicide resistance is a daunting task in plants, as plants possess hundreds of P450s with varying substrate specificities (Nelson and Werck-Reichhart, 2011). In certain crop species, some plant P450s can metabolize certain herbicide chemistries and this endows herbicide crop selectivity. For example, wheat CYP71C6v1 (Xiang *et al.*, 2006) and rice CYP81A6 (Pan *et al.*, 2006) can metabolize some ALS- and PSII-inhibiting herbicides. Importantly, a corn P450 (*Nsf1*) metabolizes ALS-, HPPD-, protoporphyrinogen oxidase-, PSII-inhibiting and synthetic auxin herbicides (Dam *et al.*, 2008). In the weedy species *L. rigidum*, while metabolic resistance has been known at the whole plant level for decades, there has been little progress in resistance gene discovery, because of large genome size (2.75 Gb), lack of genome sequences and genetic linkage maps, great genetic heterogeneity and difficulty obtaining true breeding lines due to self-incompatibility. Recently, *CYP81A12* and *CYP81A21* genes, endowing resistance to certain ACCase-, ALS-, PSII-, HPPD-, phytoene desaturase- and deoxy-D-xylulose-phosphate synthase-inhibiting herbicides, have been isolated from a predominantly self-pollinated grass weed *E. phyllopogon* (Iwakami *et al.*, 2014; Guo *et al.*, 2019; Iwakami *et al.*, 2019; Dimaano *et al.*, 2020). Here, in cross-pollinated *L. rigidum*, we also provide clear evidence that a single *CYP81A10v7* gene endows cross-resistance to herbicides of at least five modes of action across seven herbicide chemistries (e.g. ACCase-, ALS-, PSII-, HPPD- and tubulin-inhibiting herbicides) (Table 1; Figures 1, 2, S12 and S13). The R population had never been exposed to many of these herbicides. This ability of P450 to metabolize multiple herbicides is a major threat to both existing and yet-to-be-discovered herbicides (Yu and Powles, 2014).

Our work indicates convergence evolution in CYP450-mediated NTSR. Convergent evolution is the process whereby different organisms independently evolve similar traits because of adaption to similar environments or ecological niches. Molecular convergence has been repeatedly reported in herbicide target-site resistance among many weed species from different taxa, but not yet for NTSR (Powles and Yu, 2010). Higher expression of CYP81A subfamily genes has been independently evolved endowing similar cross resistance to ACCase-, ALS-, PSII- and HPPD-inhibiting herbicides in unrelated weedy species *E. phyllopogon* (Iwakami *et al.*, 2014; Guo *et al.*, 2019; Iwakami *et al.*, 2019; Dimaano *et al.*, 2020) and *L. rigidum* (this current study), indicating molecular convergence at least for the CYP81A subfamily conferring resistance to these four herbicide groups.

However, due to differences in CYP81A protein sequences in *L. rigidum* and *E. phyllopogon* (72–73% identity between CYP81A10v7 and CYP81A12/21), herbicide-metabolizing activities of CYP81As against herbicide chemistry vary in the two species. For example, CYP81A10v7 metabolizes ACCase-inhibiting herbicides diclofop-methyl and tralkoxydim but not clodinafop, fenoxaprop, haloxyfop, quizalofop, sethoxydim and pinoxaden (Table 1, Figures 1, 2 and S11). In contrast, CYP81A12/21 metabolize diclofop-methyl, tralkoxydim and pinoxaden in *E. phyllopogon* (Iwakami *et al.*, 2019). Conversely, CYP81A10v7 in *L. rigidum* but not CYP81A12/21 in *E. phyllopogon* (see Dimaano *et al.*, 2020) confers resistance to the microtubule assembly-inhibiting herbicide trifluralin (Table 1, Figures 2, S12 and S13). CYP81s from *E. phyllopogon* also show different resistance patterns to ACCase-inhibiting herbicides. For example, of nine genes from CYP81A subfamily, only CYP81A12 and CYP81A21 can metabolize diclofop-methyl (Iwakami *et al.*, 2019).

Identification of a single CYP81A10v7 endowing cross-resistance to chemically dissimilar herbicides with a larger

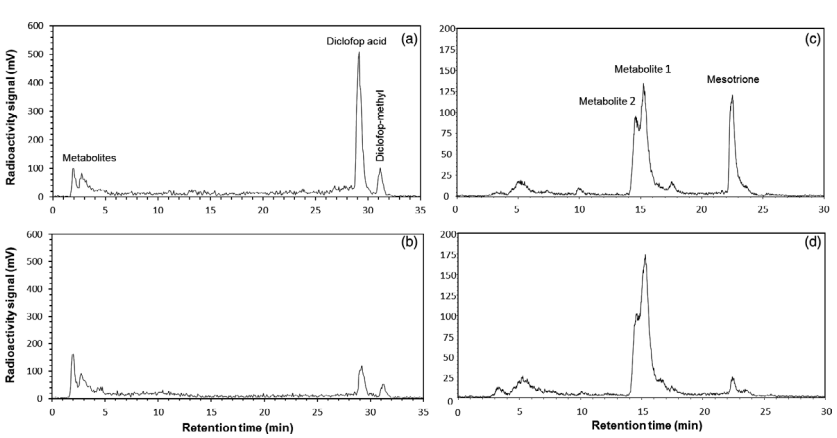

Figure 3. Metabolism of diclofop-methyl and mesotrione in transgenic plants. High-performance liquid chromatography chromatograms of (a, b) ^{14}C-diclofop-methyl and (c, d) ^{14}C-mesotrione metabolism in transgenic rice seedlings expressing (b, d) *CYP81A10v7* and (a, c) *GFP*, 48 h (mesotrione) or 72 h (diclofop-methyl) after ^{14}C treatment. The four-leaf stage seedlings were treated with commercial herbicide formulations (3000 g diclofop-methyl ha^{-1} or 100 g mesotrione ha^{-1}) before the application of ^{14}C-herbicides.

Table 4 Metabolism of ^{14}C-diclofop-methyl and ^{14}C-mesotrione in the homozygous T_2 rice lines

	Diclofop-methyl					
	48 HAT			72 HAT		
Line	Polar metabolites	Diclofop Acid	Diclofop-methyl	Polar metabolites	Diclofop acid	Diclofop-methyl
GFP-38	40.8 ± 2.4^{a}	52.1 ± 1.5^{b}	7.0 ± 0.9^{a}	46.1 ± 0.6^{a}	41.3 ± 4.3^{b}	12.7 ± 3.6^{a}
GFP-40	35.8 ± 4.3^{a}	54.9 ± 3.4^{b}	9.3 ± 1.1^{a}	44.3 ± 3.8^{a}	47.0 ± 3.7^{b}	8.7 ± 0.6^{a}
CYP81A10v7-6	67.3 ± 1.2^{b}	23.4 ± 0.6^{a}	9.3 ± 0.7^{a}	73.3 ± 2.1^{b}	15.3 ± 1.6^{a}	11.4 ± 0.7^{a}
CYP81A10v7-7	62.9 ± 2.7^{b}	29.0 ± 2.8^{a}	8.1 ± 0.2^{a}	68.9 ± 4.3^{b}	16.6 ± 1.1^{a}	14.5 ± 3.5^{a}
	Mesotrione					
	48 HAT			72 HAT		
Line	Metabolite 2	Metabolite 1	Mesotrione	Metabolite 2	Metabolite 1	Mesotrione
GFP-38	23.6 ± 1.6^{a}	40.6 ± 1.1^{ab}	21.9 ± 2.1^{b}	28.0 ± 1.5^{b}	35.8 ± 0.9^{a}	16.1 ± 0.6^{b}
GFP-40	22.4 ± 1.3^{a}	38.4 ± 1.3^{a}	26.7 ± 0.7^{b}	24.8 ± 3.7^{b}	40.2 ± 3.7^{a}	17.5 ± 0.6^{b}
CYP81A10v7-6	21.0 ± 0.5^{a}	52.0 ± 3.7^{c}	6.1 ± 1.3^{a}	15.0 ± 1.9^{a}	58.9 ± 4.9^{b}	6.3 ± 0.5^{a}
CYP81A10v7-7	19.8 ± 2.6^{a}	46.5 ± 0.8^{bc}	10.5 ± 2.8^{a}	14.3 ± 1.8^{a}	48.6 ± 4.6^{ab}	12.4 ± 3.2^{b}

Rice seedlings expressing *CYP81A10v7* (6 and 7) and green fluorescent protein (*GFP*) (38 and 40) were pretreated with commercial diclofop-methyl treatment (3000 g ha^{-1}) and mesotrione (100 g ha^{-1}), followed by the application of ^{14}C-herbicides to the third leaf. Herbicide metabolism was measured 48 and 72 h after treatment (HAT), respectively. There are three biological replicates per treatment each with four to five plants. Data were subjected to Tukey's HSD test. Different letter in the column indicates significant differences ($P < 0.05$).

effect on diclofop-methyl and chlorsulfuron explains why metabolic resistance to chlorsulfuron in *L. rigidum* can be selected by diclofop-methyl and resistance to both herbicides are often linked (Heap and Knight, 1986; Heap and Knight, 1990; Holtum *et al.*, 1991). In this current study with the purified SLR31 subset, resistance to ACCase- and ALS-inhibiting herbicides appeared to be linked in a F_2 population, displaying a single gene-control trait at certain herbicide rates. However, this CYP81A10v7 species may not be the sole gene responsible for metabolic herbicide resistance in the original R or other *Lolium* population. For instance, the P450 inhibitor malathion can reverse resistance to chlorsulfuron rather than diclofop-methyl and trifluralin (Figure S5 this study, Christopher *et al.*, 1994; Tardif and Powles, 1999), and the P450 inhibitor phorate can reverse resistance to both chlorsulfuron and trifluralin (Busi *et al.*, 2017), in the same original R population. Moreover, the level of resistance to mesotrione conferred by *CYP81A10v7* transgenic rice is much lower than that in *L. rigidum* (Table 1). Among other possibilities, these inhibitor and transgenic studies indicate involvement of additional unidentified P450s and/or other metabolic enzymes in resistance at the population level. In fact, diversity of genetic control in metabolic resistance to ACCase- and ALS-inhibiting herbicides has been demonstrated in *L. rigidum* involving from mono- to poly-gene loci, depending on population, pair-cross families and herbicide rates used and even environmental conditions (Busi *et al.*, 2011; Han *et al.*, 2014). Indeed, it has been shown that one herbicide can be metabolized at varying efficacies by several different P450 enzymes. For example, the ALS-inhibiting herbicide bensulfuron-methyl can be metabolized by four P450s (CYP81A12, CYP81A21, CYP71C6v1 and CYP72A31), and the PSII-inhibiting herbicide chlorotoluron by several P450s (CYP71A10, CYP71A11, CYP73A1, CYP76B1, CYP81A10v7, CYP81B1 and CYP81B2) (Table S1), with different catalytic efficiency. In addition, *GSTF*1 in *Alopecurus myosuroides* (Cummins *et al.*, 2013) and *GSTF2* in *Amaranthus tuberculatus* (Evans Jr *et al.*, 2017) have been demonstrated to be responsible for NTSR to atrazine and/or chlorotoluron.

While the role of CYP81A10v7 in herbicide metabolic resistance is clearly identified in the R *L. rigidum* population (SLR31), it is not consistently manifested among other R populations examined (Table 3). This is expected, particularly in genetically diverse, cross-pollinated plant species such as *L. rigidum*. Different metabolic genes/mechanisms can be selected within individuals and among populations. This also reflects flexibility of P450 (and other) genes in regulating adaptive evolution of metabolic resistance in *L. rigidum*.

Although higher expression of *CYP72A* (*CYP72A71v1*) did not endow resistance to diclofop-methyl (Tables 2 and 3; Figure S10), it may be considered as a potential marker for diagnosis of metabolic resistance at least in *L. rigidum,* given that high expression of *CYP72A* is often associated with metabolic resistance in *Lolium* (Gaines *et al.*, 2014; Duhoux *et al.*, 2017). As P450s normally cluster together on

the same chromosome in plants (Nelson and Werck-Reichhart, 2011; Liu *et al*, 2016), the existence of co-regulated gene clusters involving different types of genes (such as P450s, GSTs and UGTs) in a metabolic pathway might occur, so that *CYP72A71v1* could be potentially linked to these genes conferring herbicide resistance. Co-ordinately regulated genes clusters have been reported for biosynthesis of allelochemicals such as DIMBOA and momilactone (see a recent paper by Batada *et al*., 2007; Giacomini *et al*., 2020; Mao *et al*., 2020).

Lolium rigidum is an outcrossing species with higher genetic diversity relative to the self-pollinated *E. phyllopogon*. Cloning of the *CYP81A10* gene from R and S plants across *L. rigidum* populations reveals at least 12 variants in R and S plants (Figure S14). Whereas, there is only one amino acid substitution in CYP81A21 with no mutations in CYP81A12 between R and S *E. phyllopogon* plants (Iwakami *et al*., 2014). Variants of CYP81A9 (Nsf1) have been reported in sweet corn inbred lines, which is responsible for changes in nicosulfuron sensitivity in susceptible lines (Choe and Williams, 2020). In addition to genes from different families or different members from the same family, whether and how these variants affect the level and pattern of herbicide metabolism and thus resistance remain to be determined.

While we explicitly identify a single gene (*CYP81A10v7*) of large effect endowing wide metabolic herbicide resistance in *L. rigidum* (Table 1; Figures 1, 2, S12 and S13), future research will investigate how this gene is regulated. Cloning and analysis of the promoter sequence and its methylation status, as well as transcription factors, will likely reveal genetic and epigenetic regulation of the P450 gene (Tang and Chen, 2015). Genome-based work is needed to reveal genetic architecture of P450-driven evolution of metabolic resistance in *L. rigidum*. We also noticed that overexpression of *CYP81A10v7* in rice led to reduced plant height and increased tillers (Figure 2). Metabolomics studies will help understand the endogenous function of CYP81A10v7 in specialized metabolism in plants, in addition to its significant serendipitous role in herbicide metabolic resistance, as revealed in this study. This identified CYP gene might also have a potential application in generating multiple herbicide resistant crops.

EXPERIMENTAL PROCEDURES

Characterization of resistant and susceptible *Lolium rigidum* plants for RNA-Seq

The herbicide resistant *L. rigidum* population SLR31 originally collected from Bordertown, South Australia (South 36°18′, East 140°46′) exhibits resistance to many different herbicides (Heap and Knight, 1990; Christopher *et al*., 1991, 1992; Tardif and Powles, 1994). This population has TSR mutation and NTS metabolic resistance (Christopher *et al*., 1991; Zhang and Powles, 2006; Han *et al*., 2016). To purify a R subset without any TS mutations, vegetative clones of individual SLR31 plants were made and treated respectively with the ACCase-inhibiting herbicide diclofop-methyl (1500 g ha^{-1}) and the ALS-inhibiting herbicide chlorsulfuron (100 g ha^{-1}). Clone plants surviving both herbicides were selected and their ACCase and ALS genes sequenced using the primers listed in Table S2. The 11 purified R plants did not exhibit TSR mutations for both ACCase and ALS genes, and were bulked as an R subset. The S population is a commercial biotype from Victoria, Australia, which is susceptible to all herbicides used for ryegrass control (Owen *et al*., 2014). F_2 populations were generated from pair crosses of the S and R parent plants.

To characterize the purified R subset for cross-resistance pattern, dose–response experiments were conducted with commercial formulations of the post-emergence herbicides diclofop-methyl, chlorsulfuron, tralkoxydim, mesotrione, atrazine, chlorotoluron and pre-emergence herbicide trifluralin. Seeds were germinated on 0.6% agar-solidified medium in a 20°C growth room. For post-emergence herbicides, germinating seedlings (about 1 cm) were transferred to pots containing potting mixture (50% composted fine pine bark, 30% cocopeat and 20% river sand), and grown in a naturally-lit glasshouse at day/night temperatures of about 21/13°C with regular watering and fertilization. The two-leaf stage seedlings were treated with commercial formulations of post-emergence herbicides in a 117 L ha^{-1} spray volume delivered at 200 kPa with a cabinet sprayer equipped with two flat-fan nozzles. Germinating seeds (about 0.5 cm root) were transplanted to pots with the same potting mix and treated with trifluralin, covered with 1 cm of soil immediately after treatment and lightly watered. Mortality was determined 3 weeks after treatment, and the above-ground plant material was harvested, oven-dried at 70°C for 2 days, and dry biomass weighed. The LD_{50} and GR_{50} were determined using non-linear regression (Han *et al*., 2016). There were three replicates for each treatment and 20 plants for each replicate.

The organophosphate insect malathion can be an *in vivo* plant cytochrome P450 inhibitor (Christopher *et al*., 1994). Dose–response experiments with diclofop-methyl and chlorsulfuron were repeated with and without 1000 g malathion ha^{-1} to indicate involvement of cytochrome P450 in the metabolic resistance to the herbicides.

For RNA-Seq, the S and R plants were characterized individually by vegetative cloning, followed by herbicide treatment. To reduce genetic variation, the S and R individuals were also selected from within one F_2 population derived from an S-R cross.

Seedlings from the S, R and the F_2 populations were grown to the two-tiller stage in the glasshouse as described above, then 25 and 50 uniform plants from each parent and F_2 population were selected, respectively. Leaf samples were harvested individually and snap-frozen in liquid nitrogen for DNA and RNA extraction. To characterize the most S and most R plants for RNA-Seq, the selected plants were grown to the five uniform vegetative tiller stage and cloned. After cloning, the plants were allowed to recover for 7 days before herbicide treatment. The S clones were treated with low discriminating rates of diclofop-methyl (375 and 750 g ha^{-1}) and chlorsulfuron (50 and 100 g ha^{-1}), and the R clones treated with high discriminating rates of diclofop-methyl (6000 and 9000 g ha^{-1}) and chlorsulfuron (400 and 800 g ha^{-1}). At the same time, F_2 plants were treated with 750 and 6000 g diclofop-methyl ha^{-1}, and 100 and 400 g chlorsulfuron ha^{-1}. Mortality was determined 3 weeks after treatment. Parent S plants controlled by 375 g diclofop-methyl ha^{-1} and 50 g chlorsulfuron ha^{-1}, and F_2 plants controlled by 750 diclofop-methyl ha^{-1} and 100 g chlorsulfuron ha^{-1} were regarded as the most S plants. Parent R plants surviving 9000 g diclofop-methyl ha^{-1} and 800 g

chlorsulfuron ha^{-1}, and F_2 plants surviving 6000 g diclofop-methyl ha^{-1} and 400 g chlorsulfuron ha^{-1} were regarded as the most R plants (Figure S5). Four most S and four most R plants from parent and F_2 populations (total 16 plants) were used for RNA-Seq. Additional five parent S and four parent R, six F_2 S and 10 F_2 R plants were also selected for validation of candidate gene expression.

HPLC analysis of diclofop-methyl metabolism in *Lolium* and rice plants

The metabolism of diclofop-methyl and mesotrione in both *L. rigidum* and/or the transgenic rice seedling were measured using ^{14}C-labelled herbicides. The method for foliar application, metabolite extraction and HPLC separation are described in Supporting Information (Method S1).

RNA-Seq

RNA was extracted with ISOLATE II RNA plant kit (Bioline, London, UK). DNA contamination was removed from RNA samples with a TURBO DNA-free kit (Invitrogen, Carlsbad, CA, USA) and checked by PCR with primer pairs of actinF4/actinR1, which is designed to anneal to the intron region of the actin gene and only amplifies a 276-bp actin sequence from genome DNA (Yu *et al.*, 2009). RNA quality was assessed with Agilent 2200 Tape Station system (Agilent, Folsom, CA, USA). RNA sequencing was conducted with an Illumina HiSeq High-Output sequencer on a paired-read of 2× 125 bp (Fasteris Geneva, Swizerland). Raw fastq files were deposited to ENA (project no. PRJEB34839). The RNA-Seq analysis is conducted using the commercial bioinformatics software GeneData expressionist (Genedata AG, Basel, Switzerland). Quality of the raw RNA-Seq data was assessed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adaptor sequences were removed using Trimmomatic (version 0.36) (Bolger *et al*, 2014). To improve sensitivity of the transcriptome reference, merged data of four S and four R plants from the F_2 population were used for *de novo* assembly with Trinity to create the reference transcriptome (Grabherr *et al.*, 2011). Completeness of the *Lolium* transcriptome was assessed using the BUSCO analysis (Simão *et al.*, 2015). Reads were mapped to reference transcriptomes from both this study and that of Gaines *et al.* (2014) using Bowtie 2 (version 2.3.0) (-sensitive, -local and only best alignment reported), and the number of mapped reads for each contig was counted using HTSeq (version 0.6.1). RNA-Seq reads for each contig was normalized to FPKM and low expression contigs were removed. Reproducibility of the biological replicates from each phenotype was checked with principal components analysis. Differential expression analysis was performed using the DESeq2 R package (1.18.0) between S and R samples from parent and the F_2 population with, respectively.

Selection and validation of the candidate contigs from RNA-Seq

Contigs were selected based on the selection criteria: (i) Differential expression between S and R with significance at the adjusted *P*-value (padj)<0.05; (ii) more than two-fold change; (iii) contig sequence length >500 bp; (iv) relevance to herbicide metabolism gene annotations; and (v) upregulation in R vs. S from both parent and F_2 populations. The contig 07051, 15776, c14457_g1_i4, c15780_g1_i1 and c15309_g2_i2 were also selected even though it significantly higher expressed only in either parent R or F_2 plants due to the limited number of interest genes identified. The RNA samples used for RNA-Seq, plus spared additional five parent S, four parent R samples, six F_2 S and 10 F_2 R were used for RT-qPCR validation of candidate contigs. RNA (2 μg) was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen). Primers were designed using the online primer design software provide by Integrated DNA technologies (https://sg.idtdna.com/scitools/Applications/RealTimePCR/). Two reference genes annotated as Ras family GTPase (Gaines *et al.*, 2014) and CAP binding protein (Duhoux and Délye, 2013) served as the internal control, and both demonstrated stable expression in *L. rigidum*. All primers were assessed by both agarose gel electrophoresis and melting curve analysis with 7500 software (version 2.0.6) (Applied Biosystems, Foster City, CA, USA) for expected single product amplification (Table S3). The primer efficiency check was performed with a standard curve by diluting the cDNA in 1:4 ratio from the R F_2 plants. The primers with amplification efficiency between 90% and 110% were selected for RT-qPCR. The qPCR was conducted in 96-well plates with the 7500 Fast Real-Time PCR system (Applied Biosystems). Reactions were conducted in duplicate using SensiFAST SYBR No-Rox kit (Bioline) with 40 ng cDNA as template. The calculation of relative expression level was conducted according to Gaines *et al.* (2014). The fold-changes of contig 11357 (CYP81A10v7) of all the tested parent plants were calculated individually in relative to the plants with the lowest expression level (SVLR1-1). Similar work was done for all the tested F_2 plants in relative to the plant (F_2-9-42). The data were subject to log transformation and presented in a box plot to show the association between the resistance and CYP81A10v7 expression.

Characterization of R plants from survey populations for candidate gene validation

Thirteen diclofop-methyl resistant *L. rigidum* populations displaying high diclofop-methyl metabolism capacity were selected for characterization together with the S (Owen *et al.*, 2014; Han *et al.*, 2016). Seed germination and seedling transplanting were conducted as described above. There were 10 S plants and 25 R plants selected, and three vegetative clones were obtained from each plant. One clone served as control and the other two clones were treated with 1500 g diclofop-methyl ha^{-1} and 100 g chlorsulfuron ha^{-1}. Plants surviving diclofop-methyl and chlorsulfuron treatments containing no ACCase or ALS TSR mutations were selected as being R and used for candidate P450 gene validation (Table S2). Surviving plants from six populations without ACCase TSR mutations and two populations without ALS TSR mutations were confirmed to have metabolic resistance to diclofop-methyl and chlorsulfuron, respectively, and their RNA samples used for candidate gene validation.

Full-length cDNA amplification, vector construction and rice transformation of *CYP72A71V1* and *CYP81A10v7*

A 735-bp P450 candidate gene (contig c14457_g1_i4 sequence) was amplified with the primer pair of C72A71F2/C72A71R2 (Table S4). The PCR conditions were 35 cycles at 98°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec. The 1263 bp 3′-end sequence of the contig was obtained using the 3′-RACE system (Invitrogen) according to the manufacturer's instructions. Based on sequence identity to *CYP72A71v1* (access number: AF321870) (Fischer *et al.*, 2001), full-length cDNA of the P450 candidate gene was amplified with the primer pair of C72A71F-POX/C72A71F-POX (Table S4). The PCR conditions were 35 cycles at 98°C for 10 sec, 55°C for 15 sec and 72°C for 30 sec followed by 72°C for 7 min.

The primer pair of C81A10F1/ C81A10R1 (Table S4) were designed based on the contig (contig11357) sequence to amplify a 593-bp PCR fragment with high fidelity DNA polymerase PrimeSTAR Max DNA polymerase (Takarabio Inc., Shiga, Japan). Primer

C81A10R1 was annealed to the 3′-UTR region to ensure the unique P450 gene was amplified. The PCR conditions were 35 cycles at 98°C for 10 sec, 55°C for 15 sec, and 72°C for 30 sec. The full-length cDNA of the candidate P450 gene was amplified from the same R *Lolium* plant with the primer pair of C81A10F2/C81A10R2 (Table S4), based on sequence similarity to the *CYP81A10v2* (access number: AB159038). The PCR conditions were 35 cycles at 98°C for 10 sec, 55°C for 15 sec and 72°C for 2 min followed by 72°C for 7 min. The PCR fragment was cloned into the T-vector and sequenced with the primer M13F, M13R and C81A10F670 (Table S4).

The full-length cDNAs of *CYP81A10v7*, *CYP72A71v1* and *GFP* were inserted in the binary vector pOX (HA) under the ubiquitin promoter of the linearized pOX (HA) vector using the In-Fusion HD Cloning Kit (Takarabio Inc., Shiga, Japan) according to the manufacturer's instructions. The insert fragment sequence was confirmed by sequencing (Table S4). The resultant binary vector was transferred into an *Agrobacterium tumerficiens* strain LBA4404 (Takarabio Inc.) by electroporation. *A. tumerficiens*-mediated transformation using rice calli derived from immature seeds was performed according to Toki (1997).

Herbicide response of transgenic rice overexpressing *CYP81A10v7 and CYP72A71v1*

The response to the major ACCase- and ALS-inhibiting herbicides was tested in transgenic rice calli, and cross-resistance to other herbicide groups was evaluated using T_2 transgenic seedlings. Ten independent transgenic calli expressing *CYP81A10v7*, *CYP72A71v1* and *GFP* selected by 50 mg L^{-1} hygromycin B were placed on N6D medium containing herbicides and 300 mg L^{-1} carbenicillin. *CYP81A10v7* transgenic calli were tested for sensitivity to diclofop-methyl, chlorsulfuron, tralkoxydim, pinoxaden, fenoxaprop, sethoxydim, mesosulfuron, clodinafop, haloxyfop, quizalofop and iodosulfuron. *CYP72A71v1* transgenic calli were only tested for response to diclofop-methyl, chlorsulfuron and tralkoxydim. Transgenic calli were incubated for 3 weeks at 30°C in the dark before photography. All the experiments were repeated.

Transformation of the *CYP81A10V7*, *CYP72A71v1* and *GFP* genes was confirmed by PCR amplification of the selective hygromycin phosphotransferase gene in at least 40 regenerated T_0 transformants. T_1 seeds from each five T_0 line were germinated on MS medium containing 50 mg L^{-1} hygromycin in a growth cabinet at 30°C. Surviving plants from each line were transplanted into pots. Twenty T_1 plants of each five T_0 lines were grown to maturity and T_2 seeds harvested. The remaining T_1 seedlings surviving hygromycin selection were treated with 6000 g diclofop-methyl ha^{-1} at the four-leaf stage, and mortality was recorded 4 weeks after treatment. Homozygous T_2 lines were selected by seed (T_1) germination test on MS medium containing 50 mg L^{-1} hygromycin. Two homozygous T_2 lines of each gene (nos 38 and 40 for *GFP* and nos 6 and 7 for *CYP81A10v7*) were used for herbicide sensitivity test. Glasshouse herbicide dose–response studies were conducted twice to establish the cross-resistance pattern conferred by *CYP81A10v7*. The four-leaf stage seedlings were treated with commercial herbicide formulations of diclofop-methyl, chlorsulfuron and tralkoxydim with 0.2% (v/v) surfactant BS1000, mesotrione and atrazine with 1% (v/v) surfactant Hasten, and chlorotoluron with 0.5% Hasten. Germinating seeds were used for dose–response to the pre-emergence herbicide trifluralin. There were two replicate pots for each treatment and five to 10 plants per pot. Plant survival and mortality was determined 3 weeks after treatment. Harvested above-ground plant material was oven-dried and dry weight recorded.

ACCESSION NUMBERS

All RNA-Seq data generated in this study have been deposited to ENA (https://www.ebi.ac.uk/ena) with project number PRJEB34839. The identified P450 *CYP81A10v7* sequence in this paper has been deposited in GenBank with accession number of MK629521.

ACKNOWLEDGEMENTS

We thank Dr David Nelson for the nomenclature of cytochrome P450 in ryegrass. The binary vector pOX (HA) were provided by Professor Yaoguang Liu and Dr Zhizhan Chu from South China Agriculture University, China. We also thank Mr Greg Cawthray from the University of Western Australia for assistance with HPLC analysis and Mr Haifei Hu for RNA-Seq analysis. This work was financially supported by the Australian Research Council (ARC) linkage grants (LP140100909 and LP170100903) and by long-term support from the Australian Grains Research and Development Corporation (GRDC).

AUTHOR CONTRIBUTIONS

HH, QY, SBP and RB designed the experiments. HH, SG, FM and JW performed the experiments. HH, QY, SBP and BR wrote the article.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Dose–response of susceptible and metabolic resistant *L. rigidum* to herbicides of different modes of action.

Figure S2. HPCL chromatograms of ^{14}C-diclofop-methyl metabolism in susceptible and resistant *L. rigidum* population.

Figure S3. Photos of susceptible and metabolic resistant *L. rigidum* plants in response to herbicides of different modes of action.

Figure S4. Dose–response of susceptible and metabolic resistant *L. rigidum* to diclofop-methyl and chlorsulfuron with and without the P450 inhibitor malathion.

Figure S5. Characterization of susceptible and resistant individual *L. rigidum* plants from the parent and F_2 populations.

Figure S6. BUSCO analysis of the two reference transcriptomes assembled in this study and by Gaines et al. (2014).

Figure S7. Principal components analysis of the RNA-Seq FPKM data of susceptible and resistant plants from the parent and F_2 populations.

Figure S8. Venn diagram showing differentially expressed contigs identified from susceptible and resistant plants (both the parent and F_2 populations).

Figure S9. Box plot diagram of *CYP81A10v7* gene expression in the parent and F_2 populations.

Figure S10. Sensitivity of rice calli expressing *CYP72A71v1* and *GFP* control to diclofop-methyl, chlorsulfuron and tralkoxydim.

Figure S11. Sensitivity of the rice calli expressing *CYP81A10v7* and *GFP* control to sethoxydim, mesosulfuron, clodinafop, haloxyfop, quizalofop and iodosulfuron.

Figure S12. Mortality response of transgenic rice seedlings expressing *CYP81A10v7* and *GFP* to herbicides of different modes of action.

Figure S13. Growth response of the *CYP81A10v7* transgenic rice to herbicides of different modes of action, compared with the *GFP* control.

Figure S14. Alignment of amino acid sequences of 12 CYP81A10v7 variants.

Table S1. Herbicide-metabolizing P450s identified from plants.

Table S2. Primers for acetolactate synthase (*ALS*) and acetyl CoA carboxylase (*ACCase*) gene sequencing in *L. rigidum*.

Table S3. Primers for RNA-Seq validation.

Table S4. Primers for the binary vector construction overexpressing *CYP81A10v7*, *CYP72A71v1* and enhanced green florescence protein (*GFP*) genes.

Method S1. HPLC analysis of diclofop-methyl metabolism in *Lolium* and rice.

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