Cellular and Molecular Life Sciences (2022) 79:205
https://doi.org/10.1007/s00018-022-04171-y

ORIGINAL ARTICLE

Cytochrome P450 CYP709C56 metabolizing mesosulfuron-methyl confers herbicide resistance in *Alopecurus aequalis*

Ning Zhao[1,2] · Yanyan Yan[2] · Weitang Liu[2] · Jinxin Wang[2]

Received: 24 July 2021 / Revised: 21 January 2022 / Accepted: 24 January 2022 / Published online: 25 March 2022

Abstract

Multiple herbicide resistance in diverse weed species endowed by enhanced herbicide detoxification or degradation is rapidly growing into a great threat to herbicide sustainability and global food safety. Although metabolic resistance is frequently documented in the economically damaging arable weed species shortawn foxtail (*Alopecurus aequalis* Sobol.), relevant molecular knowledge has been lacking. Previously, we identified a field population of *A. aequalis* (R) that had evolved metabolic resistance to the commonly used acetolactate synthase (ALS)-inhibiting herbicide mesosulfuron-methyl. RNA sequencing was used to discover potential herbicide metabolism-related genes, and four cytochrome P450s (*CYP709C56*, *CYP71R18*, *CYP94C117*, and *CYP94E14*) were identified with higher expressions in the R vs. susceptible (S) plants. Here the full-length P450 complementary DNA transcripts were each cloned with identical sequences between the S and R plants. Transgenic Arabidopsis overexpressing *CYP709C56* became resistant to the sulfonylurea herbicide mesosulfuron-methyl and the triazolo-pyrimidine herbicide pyroxsulam. This resistance profile generally but does not completely in accordance with what is evident in the R *A. aequalis*. Transgenic lines exhibited enhanced capacity for detoxifying mesosulfuron-methyl into *O*-demethylated metabolite, which is in line with the detection of *O*-demethylated herbicide metabolite in vitro in transformed yeast. Structural modeling predicted that mesosulfuron-methyl binds to CYP709C56 involving amino acid residues Thr-328, Thr-500, Asn-129, Gln-392, Phe-238, and Phe-242 for achieving *O*-demethylation. Constitutive expression of *CYP709C56* was highly correlated with the metabolic mesosulfuron-methyl resistance in *A. aequalis*. These results indicate that CYP709C56 degrades mesosulfuron-methyl and its up-regulated expression in *A. aequalis* confers resistance to mesosulfuron-methyl.

Keywords *Alopecurus aequalis* · Cytochrome P450s · Metabolic herbicide resistance · Mesosulfuron-methyl · Pyroxsulam · Molecular docking

Introduction

In modern agriculture, chemical herbicides play an essential role in efficient weed control. Meanwhile, the evolution of herbicide resistance in weeds has become a global problem seriously implicating the sustainability of arable agriculture [1, 2]. The mechanisms responsible for herbicide resistance can be classed into two categories: target-site-based resistance (TSR) and non-target-site-based resistance (NTSR) [3]. TSR is relatively easily analyzed, as most caused by single nucleotide polymorphisms in the "herbicide" target site encoding genes or overproduction of target site proteins [4–6]. Although the TSR accounts for a large majority of the reported herbicide resistance cases, chemical control can be readily achieved by alternating the use of herbicides with different modes of action (MOAs) [7]. In comparison, NTSR is a much less studied but more threatening mechanism. Most weeds harboring NTSR exhibit altered absorption, enhanced metabolism, or impaired translocation [8–10], counteracting herbicide-imposed toxicity irrespective of their MOAs. More and more studies have suggested the involvement of multiple genes in NTSR [11, 12], hampering

✉ Weitang Liu
liuwt@sdau.edu.cn

✉ Jinxin Wang
wangjx@sdau.edu.cn

[1] Anhui Province Key Laboratory of Integrated Pest Management On Crops, School of Plant Protection, Anhui Agricultural University, Hefei 230036, China

[2] College of Plant Protection, Shandong Agricultural University, Tai'an 271018, China

the identification of genes involved in resistance. Therefore, only a few genes have been shown to be related to NTSR [13–18].

Among the NTSR mechanisms, enhanced herbicide metabolism, also named metabolic resistance, is a powerful route to come into being resistance to graminicides, as the differential rates of detoxification between grasses and cereals represent a key biochemical feature that can be exploited in selective chemical weed control [19]. For grass species, metabolic resistance is often associated with elevated levels of herbicide-detoxifying enzymes, including cytochrome P450 mixed-function oxidases (P450s), UDP-glucose-dependent glycosyltransferases (UGTs), glutathione *S*-transferases (GSTs), and membrane-associated ATP-binding cassette (ABC) drug transporter proteins [12, 20–22]. Of these, P450s are one of the largest superfamilies of enzyme proteins, found in the genomes of virtually all organisms [23]. Plant P450s are a group of heme-thiolate monooxygenases able to catalyze a wide variety of monooxygenation/hydroxylation reactions [24], participating in various biochemical pathways to produce primary and secondary metabolites [25]. A handful of P450s including CYP71, CYP72, CYP73, CYP76, CYP81, and CYP749 have been identified to deliver metabolic resistance to herbicides with different MOAs in field crop species [26]. A notable case reported recently is that CYP81As are demonstrated to be involved in the concomitant resistance of watergrass (*Echinochloa phyllopogon*) to multiple herbicides [14, 27, 28]. In most cases, while overexpression of specific P450s results in herbicide resistance, internal genetic mechanisms such as resistance origin and evolution remain unclear. To date, we are far from fully understanding metabolic herbicide resistance in weedy species due to its complexity of metabolic resistance and the diversity of plant P450s.

Shortawn foxtail (*Alopecurus aequalis* Sobol.) is a diploid ($2n = 14$) and partly cross-pollinated (c. 40%) species of the Poaceae family. This plant severely infests wheat (*Triticum aestivum* L.) and oilseed rape (*Brassica napus* L.) fields, causing significant reduction in grain yields. Mesosulfuron-methyl is a highly efficient acetolactate synthase (ALS)-inhibiting herbicide which has been frequently used for control of this weed in the last decade. In June 2013, herbicide-resistant *A. aequalis* was first found in Jiangsu Province, China [29]. Today, *A. aequalis* plants with TSR or both TSR and NTSR exhibit resistance to at least 10 herbicides from eight different chemical groups: aryloxyphenoxypropionate, cyclohexanedione, phenylpyraxoline, sulfonylurea, imidazolinone, triazolo-pyrimidine, pyrimidinyl-thiobenzoate, and sulfonyl-aminocarbonyl-triazolinone [30]. There seems to be, at least for now, only the photosystem II inhibitor isoproturon and the 4-hydroxyphenylpyruvate dioxygenase inhibitor cypyrafluone left to control this species in China. Investigating the resistance mechanisms in weeds is the base to delay or overcome herbicide resistance and to develop more effective pest management practices. Since the TSR has been well characterized in this species, elucidating the molecular mechanisms of NTSR is urgently required.

Previously, we have identified a field evolved metabolic resistant (R) *A. aequalis* population whose resistance to the ALS inhibitor mesosulfuron-methyl (MM) could be partly reversed by pre-treatment with the P450 inhibitor malathion [31]. In RNA sequencing, four P450s were identified with higher expression in the R vs. susceptible (S) lines [31]. Therefore, we hypothesize that rapid metabolism of MM results from increased activity of P450s, and that this increased activity results from higher expression of novel P450 isoforms with greater affinity towards MM. To date, involvement of P450s in metabolic herbicide resistance of *A. aequalis* has not been investigated in either biochemistry level or molecular level. Which P450 genes are responsible for and how the genes take part in the metabolic resistance still mysteries. Here we report the identification and functional characterization of key P450 genes endowing *A. aequalis* with resistance to ALS inhibitor(s). These results will provide a molecular basis for the understanding of the metabolic herbicide resistance in *A. aequalis* as well as other grass weeds in general.

Results

Metabolism of MM in the S and R lines of *A. aequalis*

According to Aizawa [32], the active body of MM would be primarily inactivated by *O*-demethylation after being absorbed by wheat (Fig. 1A). To determine if the metabolic pathway is the same as in wheat and if the MM metabolism is more active in the R than in the S line, the amounts of MM and its *O*-demethylated metabolite were detected by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) and compared in the S and R lines of *A. aequalis* treated with MM for 24 h. In accordance with our previous results [33], the amount of MM in the R line was about three quarters of that in the S line, while that of *O*-demethylated metabolite was twofold higher (Fig. 1B). This reveals that MM was metabolized more rapidly in the R than in the S line via the same metabolic pathway as in wheat.

Full sequence cloning and analysis of the P450 genes

In previous study, RNA sequencing revealed that the expression of eight P450 genes was significantly higher in the R than in the S plants after MM treatment [31]. Of them, four P450 genes including *CYP94C117* (c39857_g2),

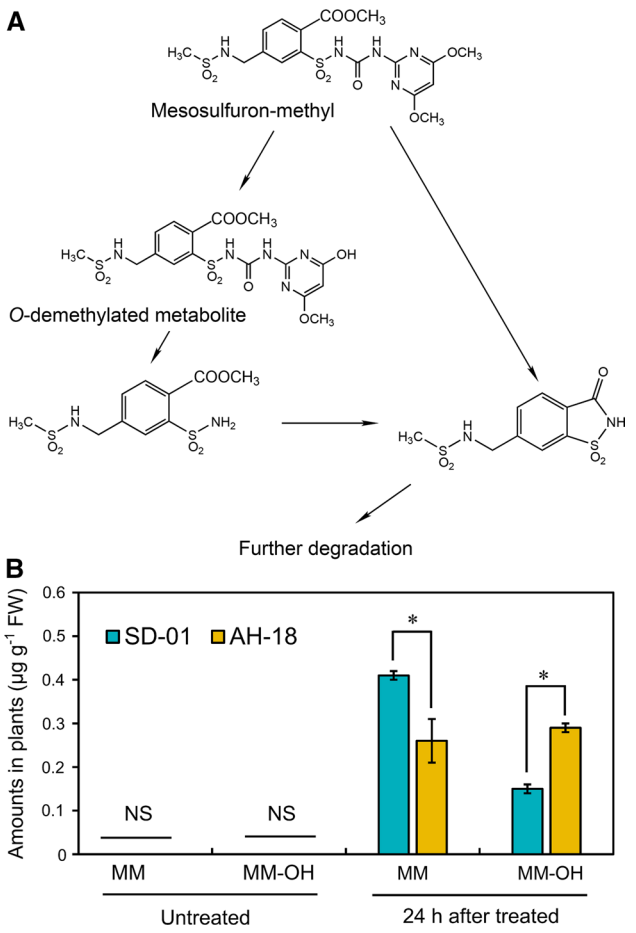


Fig. 1 LC–MS/MS analyses of MM metabolism in S and R lines of *A. aequalis*. **A** Proposed metabolic pathway of MM in wheat. **B** Amounts of MM and its *O*-demethylated metabolite in the S (SD-01) and R (AH-18) lines of *A. aequalis*, respectively. Weed seedlings at the 3–4-leaf stage were treated with MM at the field recommended rate (9 g ai ha^{-1}) for 24 h before LC–MS/MS analysis. *Significant difference at $P < 0.05$. *NS* no significant difference

CYP709C56 (c45454_g1), *CYP94E14* (c21190_g1), and *CYP71R18* (c43350_g3) also showed consistently higher expression in parallel resistant samples than in susceptible samples (up to 32.64-fold) (Table S1). Considering that pretreatment of the P450 inhibitor malathion greatly increased the susceptibility of the R plants to MM (by c. 41%) [31], we presumed the higher expression of these four P450 genes correlates with the enhanced MM metabolism in *A. aequalis*.

Full-length coding sequences of the four P450 genes were each amplified from the S and R plants of *A. aequalis*, and sequence alignments revealed no insertion or single nucleotide polymorphism was present for every gene between the two biotypes (Fig. S1 for *CYP709C56*; data not shown for the other three genes). Gene conserved domains were analyzed by the National Center for Biotechnology Information conserved domain tool (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), confirming the CYP94C117, CYP709C56, CYP94E14, and CYP71R18 all belong to the P450 superfamily. Sequence analysis of the above four *A. aequalis* genes and 219 P450 genes in Arabidopsis showed that *CYP71R18* clustered in CYP71 clan, *CYP709C56* clustered in CYP709 family of CYP72 clan, and *CYP94C117* and *CYP94E14* clustered in CYP94 family of CYP86 clan [34] (Fig. 2A). The nearest-neighbor analysis of characterized P450 protein sequences indicates that *A. aequalis* CYP709C56 has close evolutionary relationships with *T. aestivum* CYP709C1 and *Aegilops tauschii* CYP709B1, forming a sister clade with *Brachypodium distachyon* CYP709B1 (Fig. 2B).

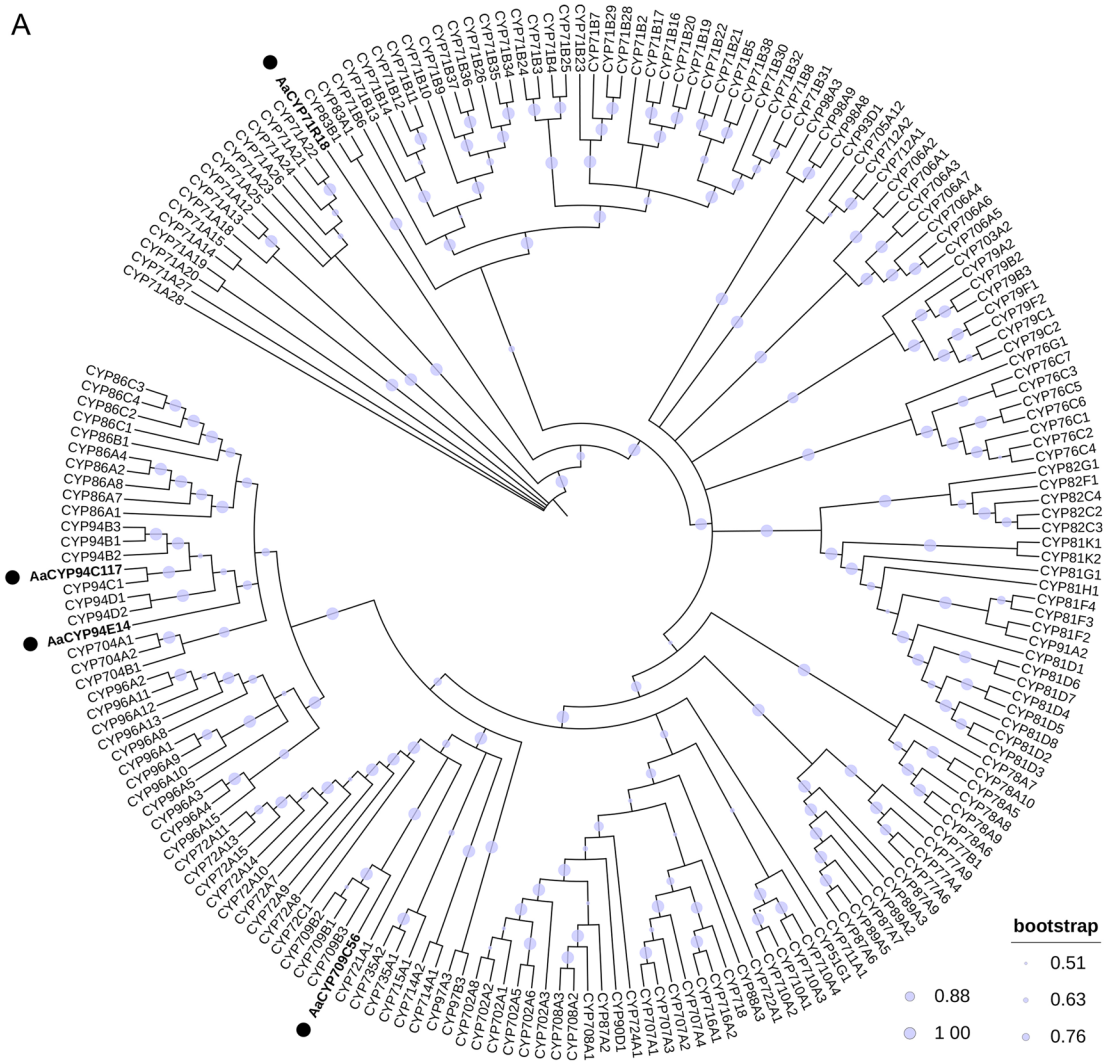
Arabidopsis overexpressing *CYP709C56* became less susceptible to specific ALS inhibitors

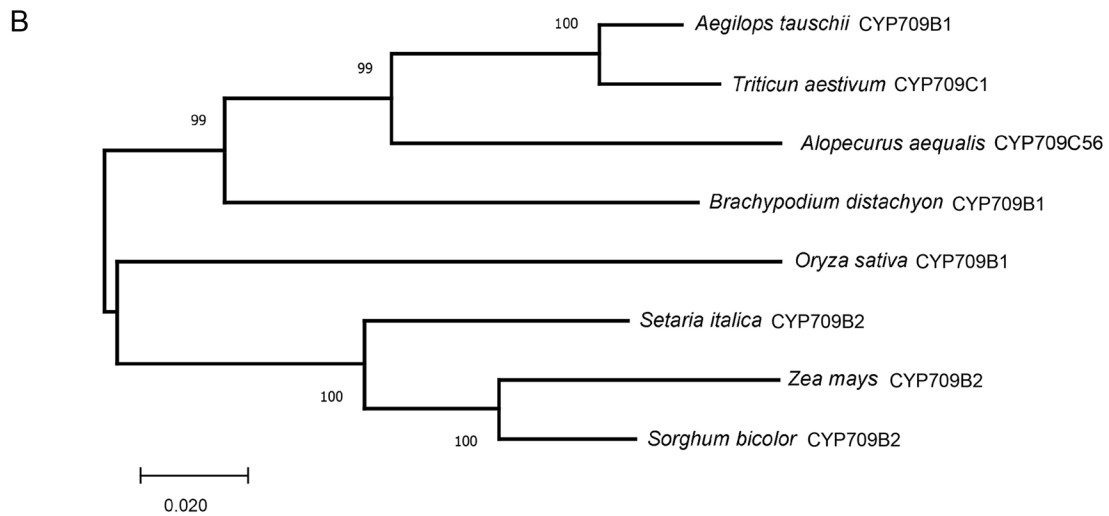
Full-length coding sequences of *A. aequalis CYP94C117*, *CYP709C56*, *CYP94E14*, and *CYP71R18* were each introduced into Arabidopsis (ecotype Columbia-0) under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Table S2). Arabidopsis seedlings overexpressing empty vector (*pPZP211*) were used as the negative control (WT). Ten independent T3 lines were obtained for each transformant, and they were first used for preliminary germination test on medium containing different classes of ALS herbicides.

Unexpectedly, the Arabidopsis lines overexpressing *CYP94C117*, *CYP94E14*, or *CYP71R18* were as susceptible as WT, and their germinations were completely inhibited at 5 nM MM, 5 nM pyroxsulam (PX), or 15 μM flucarbazone–sodium (FS) (Fig. S2). Therefore, no further analysis of these genes was conducted. As expected, the seeds of Arabidopsis overexpressing *CYP709C56* could still germinate at levels at which the WT stopped growing (Fig. 3A), indicating the gene endowed Arabidopsis with tolerance to MM and PX. However, both the WT and the Arabidopsis overexpressing *CYP709C56* did not germinate at 15 μM FS (Fig. 3A), suggesting no tolerance of the transgenic lines occurred to FS. All following experiments were performed based on the *CYP709C56* gene and the Arabidopsis lines overexpressing *CYP709C56*.

Arabidopsis seedlings overexpressing *CYP709C56* became resistant to mesosulfuron-methyl

To determine the relative expression levels of transgene in Arabidopsis, real-time quantitative PCR (RT-qPCR) assay was performed using the *AtF-box protein* as the internal control. The transgenic line exhibiting the lowest relative expression was defined as line CYP-10 with its transcript accumulation level was defined as 1.0. Three lines with higher transcript accumulation levels of transgene were selected for further physiological experiments.


A
AaCYP71R18
AaCYP94C117
AaCYP94E14
AaCYP709C56
bootstrap
0.51
0.63
0.76
0.88
1 00


B
Aegilops tauschii CYP709B1
Triticun aestivum CYP709C1
Alopecurus aequalis CYP709C56
Brachypodium distachyon CYP709B1
Oryza sativa CYP709B1
Setaria italica CYP709B2
Zea mays CYP709B2
Sorghum bicolor CYP709B2
100
99
99
100
100
0.020

◂**Fig. 2** Sequence analysis of *A. aequalis* P450 proteins. (1) Multiple sequence alignment of the *A. aequalis* CYP94C117, CYP709C56, CYP94E14, and CYP71R18 (black circle) and the 219 P450s from Arabidopsis species. (2) Phylogenetic analysis of the *A. aequalis* CYP709C56 and the CYP709s from other plant species including *Brachypodium distachyon* CYP709B1 (XP_003562608), *Aegilops tauschii* CYP709B1 (XP_020184145), *Triticum aestivum* CYP709C1 (AAT68297), *Oryza sativa* CYP709B1 (XP_015645350), *Zea mays* CYP709B2 (PWZ37282), *Setaria italica* CYP709B2 (XP_004958443), and *Sorghum bicolor* CYP709B2 (XP_002463247)

Transcript accumulation levels of *CYP709C56* were different among the three lines, which were separately marked as CYP-1 to CYP-3 based on their mean transcript levels from low to high (Fig. 3B). Whole-plant dose–response experiments were conducted to determine the susceptibility of all *CYP709C56* transformants (Fig. 3C, E). MM susceptibility of each line differed, and the herbicide concentrations resulting in 50% growth reduction (GR_{50} values) of CYP-1 to CYP-3 ranged from 4.20 to 5.96 g active ingredient (ai) ha^{-1}, which were 2.92- to 4.14-fold more resistant to MM than was control (Fig. 3C). The herbicide tolerance was positively related to the transcriptional level of the introduced *CYP709C56* in the transgenic lines (Fig. 3D), suggesting *CYP709C56* endows Arabidopsis with varying degrees of herbicide tolerance depending on its transcript abundance.

Arabidopsis overexpressing *CYP709C56* metabolized mesosulfuron-methyl faster

To test if MM metabolism in the transgenic lines of *CYP709C56* is more active than in the control plants, MM and its metabolites were examined in the T3 Arabidopsis lines overexpressing *CYP709C56* or empty vector. Transgenic line CYP-3 with the highest transgenic expression level was used for the testing (Fig. 3B). LC–MS/MS analysis showed that herbicide content was not prominently different at 2 h (Fig. 4A), indicating the absorption of MM was similar in the WT and transgenic line [14]. As time goes on from 2 to 24 h, the amount of MM decreased rapidly in a similar way in both the WT and the transgenic line, and a significantly lower amount of MM was observed in the transgenic line (Fig. 4A). Similarly, the conversion of MM into non-herbicidal polar metabolite increased with time and peaked at 12 h in both the S and R lines, and the amount of *O*-demethylated MM was always significantly richer in R than that in S ($P < 0.05$) (Fig. 4B). However, the amount of MM metabolite decreased in both lines at 24 h (Fig. 4B), which may owe to their further degradation via enzyme systems in phase II and phase III of xenobiotic metabolism [35]. Clearly, overexpression of *CYP709C56* increased the metabolism of MM to the *O*-demethylated metabolite, therefore, endowing plants with herbicide resistance.

In vitro expressed *CYP709C56* enzyme detoxifies MM via *O*-demethylation

To investigate and validate the function of *CYP709C56*, recombinant CYP709C56 protein was expressed as the N-terminal Flag-Tagged fusion proteins using a yeast (*Saccharomyces cerevisiae*) expression system that carried the Arabidopsis NADPH-cytochrome P450 reductase gene *ATR1* [36]. The immunoblotting showed that CYP709C56 had accumulated in the transgenic yeast (Fig. 5A). The herbicide MM was added to the yeast culture media for the metabolism assay. After shaking for 24 h, the media were analyzed using LC–MS/MS.

Because the *O*-demethylation often occurred to sulfonylurea herbicides [26], we presumed that CYP709C56 could detoxify the herbicide MM via *O*-demethylation (Fig. 5B). Analysis of the reaction system showed that the content of MM detected in the media of yeast expressing CYP709C56 was much less than in the empty-vector control (Fig. 5C). In addition to MM, another peak with a shorter retention time, corresponding to that of the standard of *O*-demethylated MM was detected only in the presence of the CYP709C56 enzyme (Fig. 5D). Clearly, the CYP709C56 could metabolize MM through *O*-demethylation in vitro.

Docking analysis reveals structural interactions of CYP709C56 and MM

As indicated above, CYP709C56 catalyzes a reaction involving the *O*-demethylation of the methoxy of the pyrimidine ring from MM molecule (Fig. 5B). To gain further insights into the potential reaction mechanisms involved, a molecular docking approach was adopted. For this purpose, homology modeling was established for CYP709C56 protein based on the X-ray structure of human CYP4B1 (PDB ID: 6C93) (Fig. S3A). Similar to human P450 4B1 for which structure has been reported, CYP709C56 has multiple main alpha helix regions and two beta strands regions. The Ramachandran plot for CYP709C56 shows that 99% residues are in allowed regions (Fig. S3B), indicating that the three-dimensional structure of the model is reasonable. Structural analysis of the P450 modeling shows the CYP709C56 structure is basically consistent with the template structure (Fig. S3C). The average root-mean-square deviation value of the three-dimensional structure overlap is 1.935 Å, and both have the same alpha helix and beta strands regions.

Based on this model, substrate binding and specificity may involve the amino acid residues Thr-328, Thr-500, Asn-129, Gln-392, Phe-238, and Phe-242 (Fig. 6, Fig. S3D). For the MM molecule specifically, its three oxygen atoms, regarded as hydrogen bond acceptors, form three hydrogen bonds with the backbone nitrogen atom of Phe-242 and the side-chain nitrogen atoms of Asn-129

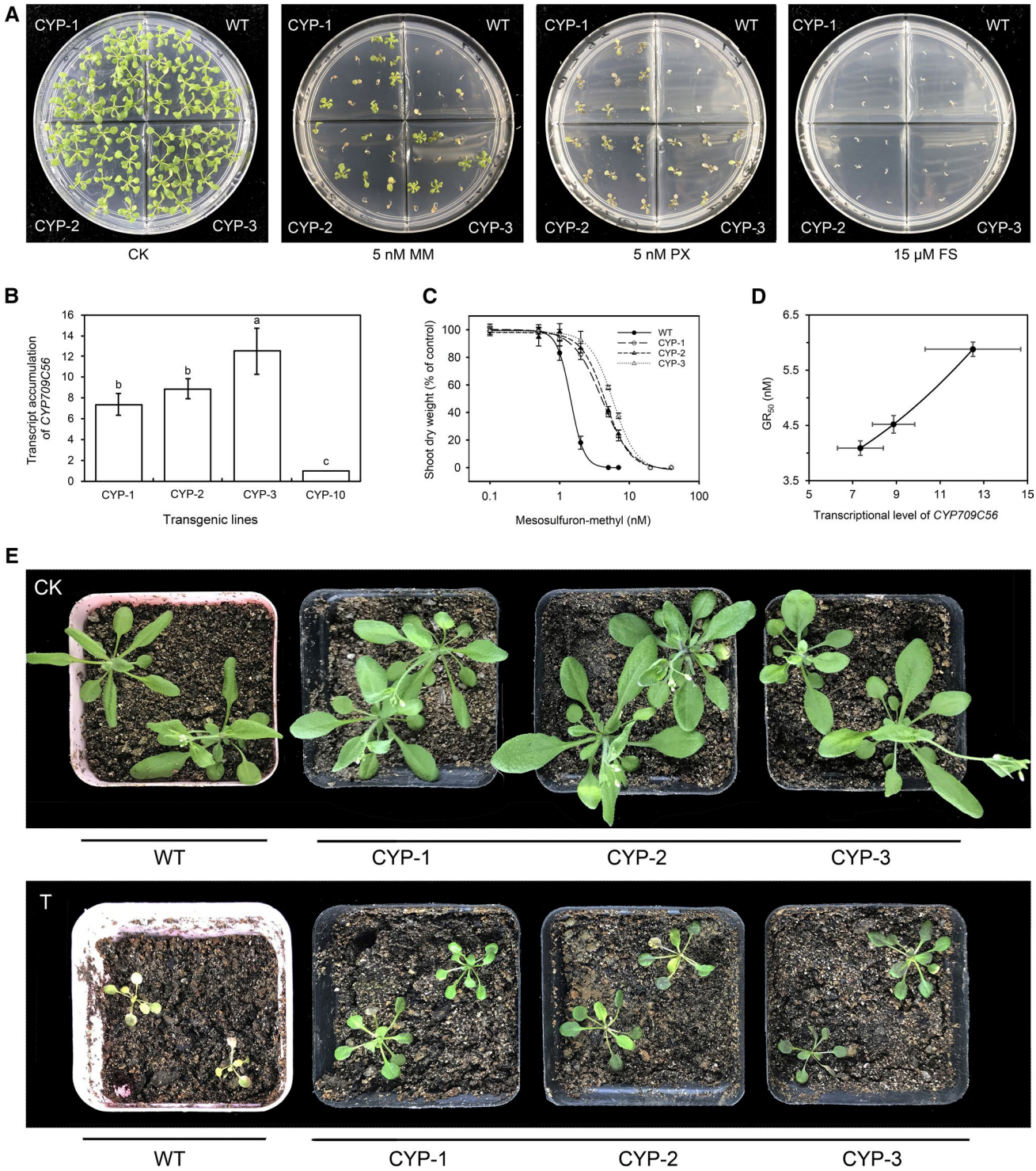


Fig. 3 Susceptibility to ALS inhibitors in Arabidopsis transformed with *CYP709C56*. **A** Seedlings of the transgenic Arabidopsis lines CYP-1, CYP-2, and CYP-3 grown for 12 d on media containing no herbicide (CK), 5 nM mesosulfuron-methyl (MM), 5 nM pinoxaden (PX), or 15 μM flucarbazone–sodium (FS). **B** Transcript accumulation levels of T3 homozygous transgenic Arabidopsis lines CYP-1, CYP-2, and CYP-3. Different letters indicate a significant difference ($P < 0.05$). **C** MM susceptibility of independent transgenic lines was evaluated by whole-plant dose–response experiments. Bars represent SE ($n = 3$). **D** Relationship of transcript level and MM susceptibility in transgenic Arabidopsis overexpressing *CYP709C56*. Transcript level and MM susceptibility of each transgenic line were evaluated by RT-qPCR and GR_{50}, respectively. Bars represent SE ($n = 3$). **E** Growth response to mesosulfuron-methyl of Arabidopsis transformed with the empty vector (WT) or *CYP709C56* (CYP-1, CYP-2, and CYP-3). Growth status was checked at 9 days after treatment at the rate of 0 (CK) or 2.25 g ai ha^{-1} (T)

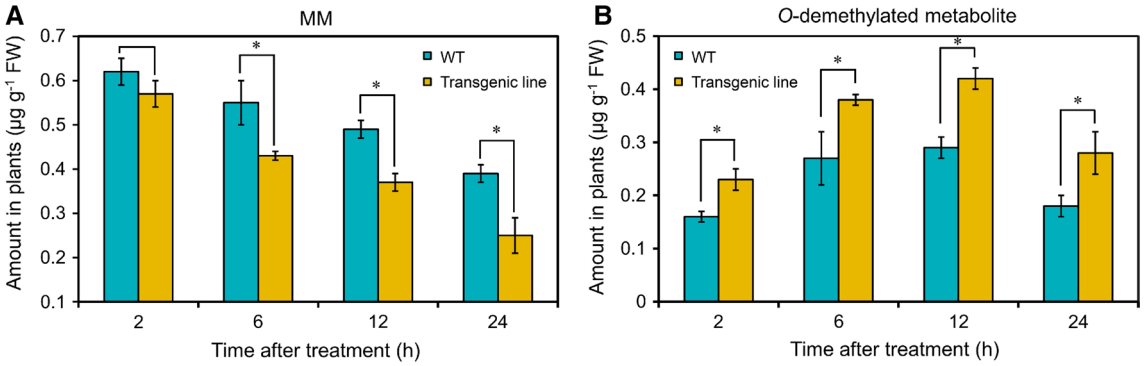


Fig. 4 Time course of MM metabolism in WT and transgenic lines of *A. thaliana*. Plants were foliar-treated with the field-recommended rate of MM at 9 g ai ha^{-1}. The amounts of MM (**A**) and its *O*-demethylated metabolite (**B**) were monitored by LC–MS/MS at *m/z* 502.10 and 488.10, respectively. Bars represent SE ($n=3$). *Significant difference at $P<0.05$

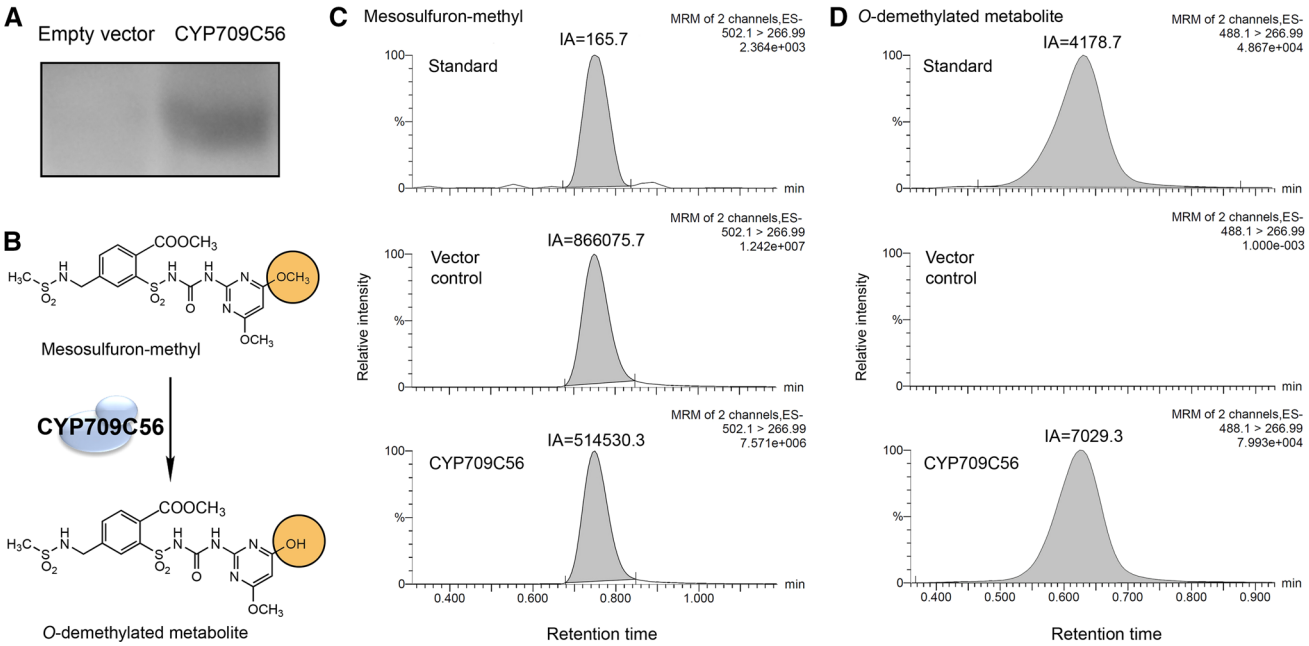


Fig. 5 CYP709C56 detoxifies MM through *O*-demethylation in vitro. **A** Immunoblotting of microsome fractions extracted from yeast expressing CYP709C56. **B** Proposed change in the structure of MM following *O*-demethylation catalyzed by CYP709C56. LC–MS/MS analyses of MM residue (**C**) and its *O*-demethylated metabolite (**D**) in yeast harboring the empty vector or expressing CYP709C56. Standard: analytical grade mesosulfuron-methyl and its *O*-demethylated metabolite. Vector control: mixture of yeast-expressed BSA protein and mesosulfuron-methyl. CYP709C56: mixture of yeast-expressed CYP709C56 enzyme and mesosulfuron-methyl. IA represents integral area

and Gln-392, respectively. The benzene ring of MM forms pi–pi stacking interactions with the benzene rings of Phe-238 and Phe-242, respectively (Fig. 6B). Mesosulfuron-methyl-CYP709C56 interaction energy in this case stabilizes at a level of − 4.76 kcal mol^{-1}. In the current docking model, Thr-328 and Thr-500 are positioned close to the pyrimidine ring and the heme group, which may directly interact with the substrate methoxy for achieving its *O*-demethylation.

Association between metabolic herbicide resistance and basal expression levels of *CYP709C56*

Mesosulfuron-methyl susceptibility in 48 populations of *A. aequalis* was compared in the absence and presence of malathion, a P450 inhibitor, to check whether P450s are involved in the MM tolerance. Seedling growth of seven populations (designated SD-02, SD-06, AH-15, AH-25, AH-29, AH-36, and JS-03) treated with MM in combination with

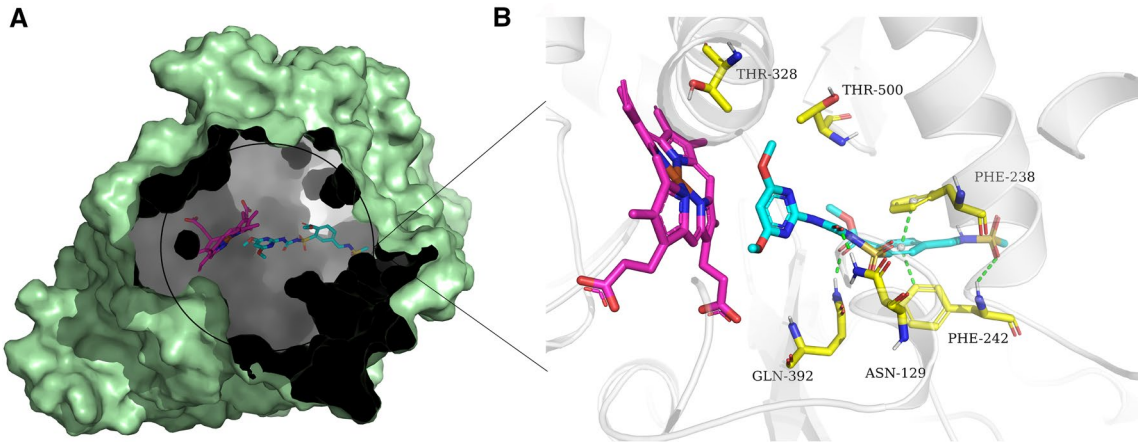


Fig. 6 Molecular docking model for *A. aequalis* CYP709C56 with the molecule of MM. **A** The binding model of MM on the molecular surface of CYP709C56. MM is colored in cyan, heme is colored in purple, and the molecular surface of CYP709C56 is colored in pale green. **B** Close-up view of the substrate binding pocket derived from the CYP709C56 model. MM is colored in cyan, heme is colored in purple, the surrounding residues in the binding pockets are colored in yellow, and the backbone of the receptor is depicted as white cartoons with transparency

1 × malathion was significantly suppressed compared with growth without malathion (Fig. 7). On the contrary, seedling growth of the other 41 populations treated with MM was similar both with and without malathion pre-treatment (Fig. S4), indicating P450s are involved in MM tolerance in the above seven populations.

MM metabolism was examined by LC–MS/MS in different populations of *A. aequalis* at 24 h after treatment. Compared with the S line SD-01, significantly lower levels of MM and correspondingly higher levels of *O*-demethylated metabolite were observed in plants of the above seven populations (Table 1). Therefore, P450s-involved enhanced rates of MM metabolism are present in these seven field-collected *A. aequalis* populations. Relative expression of *CYP709C56* was then measured in the seven *A. aequalis* populations exhibiting metabolic herbicide resistance and compared with the S population SD-01. *CYP709C56* significantly up-regulated (fold change ≥ 2, $P < 0.05$) in five of the seven populations (Fig. 7), exhibiting a high correlation with P450s-inbihitory phenotypes. However, in populations SD-02 and AH-36, constitutive expression of *CYP709C56* was not significantly different with that in SD-01 (Fig. 7), suggesting it may not play a role in their metabolic resistance to MM.

Discussion

Since the late 1990s, P450s have been at the center of herbicide metabolism research because of their ability to endow selectivity in crops and resistance in weeds. However, the identification of genes encoding herbicide-metabolizing P450s is an arduous task in plants, as plants possess hundreds of P450s with varying substrate specificities. Despite the fact that the enhanced herbicide detoxification in weedy species has drawn great interest in recent years [35, 37], the underlying biochemical mechanisms, enzymes, and specific genes are still not well characterized and remain markedly under-explored. Potential candidates are intensively identified based on large-scale omics [38–43], whereas seldom of

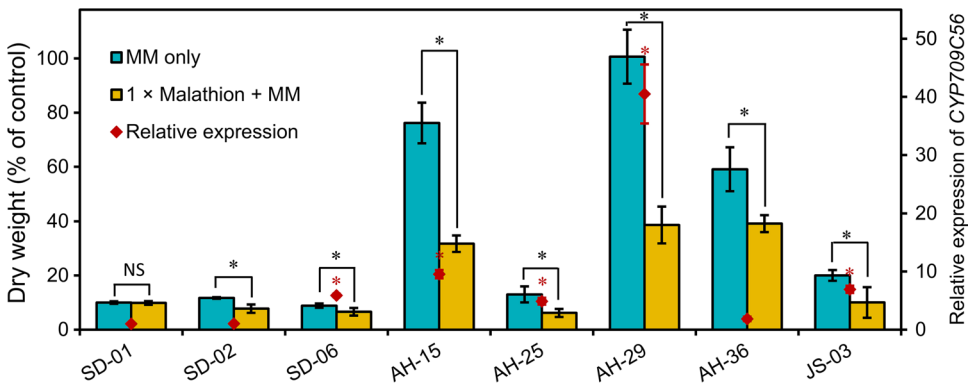


Fig. 7 Effects of the P450 inhibitor malathion on the susceptibility of different populations to MM, along with the relative expressions of *CYP709C56* in different populations. The expression of *CYP709C56* in a specific population relative to that in the SD-01 population is shown by brown diamond. Bars indicate SE ($n = 3$), and *represents significant difference at $P < 0.05$

Table 1 Mesosulfuron-methyl metabolism and target-site mutations in *A. aequalis* plants of field populations with obvious P450s-inhibitory phenotypes in comparison with a known herbicide-susceptible population (SD-01)

Population	Biotype	Target-site ALS mutation	Mesosulfuron-methyl (μg g^{-1})	*O*-demethylated metabolite (μg g^{-1})	Enhanced metabolism (% increase)
SD-01	S	No mutation	0.41 (0.011) a	0.15 (0.006) e	–
SD-02	r	No mutation	0.32 (0.003) c	0.19 (0.009) d	27
SD-06	r	No mutation	0.35 (0.008) b	0.20 (0.013) d	33
AH-15	R	Pro-197-Thr	0.23 (0.025) e	0.31 (0.014) b	107
AH-25	r	No mutation	0.30 (0.002) d	0.19 (0.007) d	27
AH-29	R	Trp-574-Leu/Pro-197-Ser	0.21 (0.010) f	0.34 (0.013) a	127
AH-36	R	Pro-197-Tyr	0.25 (0.008) ef	0.28 (0.010) c	87
JS-03	r	No mutation	0.28 (0.013) d	0.26 (0.008) c	73

S susceptible, *r* tolerant, *R* resistant; Different letters represent significantly difference at $P < 0.05$

them have been demonstrated to be associated with specific herbicide metabolism [18, 44, 45]. In the weedy species *A. aequalis*, while metabolic resistance has been identified at the whole plant level for several years [46], little progress has been made in resistance gene discovery due to great genetic heterogeneity and lack of genome sequences and genetic linkage maps. Our previous studies have shown that the P450s are involved in the metabolic herbicide resistance of *A. aequalis*, and the up-regulated expressions of four P450 genes may be related to the resistance [31]. In this study, we characterize the functions of the candidate P450s and provide clear evidence that a novel P450 gene *CYP709C56* metabolizes MM via *O*-demethylation and thus endows *A. aequalis* with herbicide resistance.

In some crops and grass weeds including *Oryza sativa*, *E. phyllopogon*, and *Lolium rigidum*, CYP81As are demonstrated to be involved in the concomitant resistance to multiple herbicides with different MOAs [13, 14, 45, 47]. Han et al. [45] hence proposed a theory of convergence evolution in P450-mediated NTSR. However, CYP81As seem not to be involved in the resistance of *A. aequalis*, at least in its resistance to MM. In comparison, *CYP709C56* was suggested to play a key role in the resistance/tolerance of *A. aequalis* to specific ALS inhibitors. *CYP709C56* is a member of the CYP72 clan, a quite diverse P450 family widely distributed in plant lineages and has undergone rapid functional divergence [48, 49]. Irmler et al. [50] demonstrated activity of the first member of the family, *CYP72A1*, as secologanin synthase in indole alkaloid biosynthesis. More recently, gibberellin-metabolizing activity was found in some members of CYP72As in the *Brassicaceae* lineage [51]. Notably, Imaishi and Matumoto [52] found that the rice *CYP72A18* can metabolize a nine-carbon fatty acid structure herbicide pelargonic acid by (ω − 1)-hydroxylation, and Saika et al. [15] demonstrated the rice *CYP72A31* can confer multiple resistance to the ALS-inhibiting herbicides bispyribac sodium and bensulfuron-methyl. For the CYP709 family specially, expression of several *CYP709C* genes is reported to be induced by treatment with different herbicides or safeners [53–55]. CYP709C1 expressed in yeast exhibits the potential for hydroxylating different fatty acids with varying chain lengths (C12–C18) and unsaturation [53], suggesting its possible detoxifying function. These previous reports together with our results suggest that *CYP72* genes are widely involved in xenobiotic responses.

In this current study, *CYP709C56* conferred tolerance to both the sulfonylurea herbicide MM and the triazolopyrimidine herbicide PX (Fig. 3A), which may be caused by the substrate specificity and amount of enzyme. It has been suggested that it would be difficult to predict substrate preferences from primary sequences in CYP72A proteins [48]. Here we built the homology modeling for CYP709C56 and characterized its structural interaction with the molecule of MM. Interestingly, the best fitting model structure presented by NCBI BLAST is human CYP4B1, which is a P450 gene functions in both endobiotic and xenobiotic metabolism [56]. P450s are heme-thiolate proteins able to catalyze the activation of molecular oxygen using the electrons from NADPH [24], and the reactions for P450-mediated herbicide metabolism primarily involve alkyl-hydroxylation, *N*-demethylation, *O*-demethylation, and aryl-hydroxylation including NIH-shift [26]. With the aid of critical cofactors of P450 reductase including flavin adenine dinucleotide and flavin mononucleotide, CYP709C56 most likely to utilize the two electrons (one hydride anion, H^-) donated by NADPH to realize the *O*-demethylation of MM.

Transgenic Arabidopsis overexpressing *A. aequalis CYP709C56* was resistant to MM and PX but was susceptible to FS. This resistance profile generally but does not completely in accordance with what is evident in the R *A. aequalis*, since the latter also confers moderate resistance to FS [46]. Besides, the resistance indexes determined in transgenic Arabidopsis for MM were much lower than that determined in R *A. aequalis* [31]. The distinct herbicide responses between the transgenic Arabidopsis and the field evolved *A. aequalis* support the hypothesis that the herbicide

resistance in R *A. aequalis* population is caused by different mechanisms/metabolic genes. Previous RNA sequencing revealed that several other genes including GSTs, GTs, and ABC transporters also exhibited higher basal and/or herbicide-induced expressions in the R population [31]. Of them, GST genes *GSTF1* and *GSTU1* have been shown to be involved in the resistance of *A. myosuroides* and *T. aestivum* to specific herbicides, respectively [16, 57]. This suggests that the enhanced herbicide metabolism in R *A. aequalis* plants most likely to be regulated by multiple genes, at least including both P450s and potential GSTs, which is in accordance with well-studied cases of non-target-site-resistant weeds including *L. rigidum* and *A. myosuroides* [11, 12, 58–60]. The great potential of candidate GSTs in endowing *A. aequalis* plants with multiple herbicide resistance deserves greater research attention.

In this study, we clearly showed the role of *CYP709C56* in metabolic herbicide resistance of the R *A. aequalis* population (AH-18). However, this gene is not consistently manifested among other resistant populations tested (Fig. 7). This is expected especially in genetically diverse, partly cross-pollinated weedy species *A. aequalis* [61], because the multiple metabolic genes/mechanisms will be selected within individuals and among populations [45], sometimes also diverse with the abiotic stresses, such as herbicide application history. This also corroborates the P450 and other genes can flexibly regulate the adaptive evolution of metabolic resistance in *A. aequalis* [45].

Identification of key genes conferring metabolic resistance to specific herbicides makes it possible to develop new biotechnology applications toward improving resistant-weed management. On one hand, basal expression levels of *CYP709C56* may be used as molecular markers for screening putative resistant *A. aequalis* populations to confirm metabolic resistance to MM and/or PX. A similar approach has been utilized in the mosquito *Anopheles funestus* in which the first DNA-based metabolic resistance marker *GSTe2* in mosquitoes was detected and developed as an essential tool to track the evolution of resistance [62]. In addition to improved resistance screening tools, the coding sequence of *CYP709C56* could be utilized to synthesize new chemical inhibitors of herbicide-detoxifying P450s [37]. For instance, silencing of P450 genes in insects by RNA interference has been shown to increase insect susceptibility to insecticides [63, 64]. Besides, the coding sequence of *CYP709C56* could also be used to engineer targeted gene knockout strategies to ultimately overcome the metabolic herbicide resistance. A pioneering work reveals that polynucleotide-based gene knockdown systems are being generated [65], which is especially suitable for overcoming metabolic herbicide resistance in weeds, as this is often under polygenic control.

Although we explicitly identify the *CYP709C56* of considerable effect endowing metabolic herbicide resistance in *A. aequalis*, further study will illustrate how this gene is regulated. In this study, no difference was revealed in comparing sequences of *CYP709C56* between the S and R plants (Fig. S1). Therefore, higher basal expression of *CYP709C56* is not due to the difference in sequences but may be caused by mutations or varying degrees of methylation in the untranslated regions, gene promoters, or transcription factors [26, 66, 67]. Cloning of the promoter sequence and analysis of its methylation status, as well as identification of related transcription factors will likely reveal genetic regulation of the P450 gene [68–70]. While this study reveals the serendipitous role of *CYP709C56* in herbicide detoxification, employing metabolomics will aid in understanding the endogenous function of *CYP709C56* in specialized metabolism in plants [18]. To reveal genetic architecture of P450-driven evolution of metabolic resistance in *A. aequalis*, genome-based work is needed to conduct.

Experimental procedures

Plant materials and growth conditions

The collection, storage, and planting of seeds from the S (SD-01) and R (AH-18) *A. aequalis* populations were the same as described elsewhere [31]. Fresh leaves were collected from *A. aequalis* seedlings at the 3–4-leaf stage, and total RNA was extracted using the *EasyPure*® Plant RNA Kit (*Trans*, Catalog No. ER301). Reverse transcription (RT) was conducted with 5 μg of total RNA as template using the *TransScript*® II One-Step gDNA Removal and cDNA Synthesis SuperMix (*Trans*, Catalog No. AH311).

Arabidopsis (*Arabidopsis thaliana*, ecotype Columbia-0) was used as a model species in this study. All Arabidopsis plants were grown in a controlled-environment growth chambers setting at temperatures of 25/20 °C with a long-day condition (16-h light/8-h dark) for bolting or with a short-day condition (8-h light/16-h dark) for vegetative growth, unless specified otherwise.

Vector construction and plant transformation

The full-length coding region of *CYP709C56* was amplified by RT-PCR with the primer pairs listed in Table S2 using cDNA prepared from fresh shoots of *A. aequalis* at the 3–4-leaf stage. Sequence alignment was performed by Clustal W [71] and ESPript v.3.0 [72]. Phylogenetic analysis was conducted using MEGA-X [73]. Physico-chemical parameters were computed by the Expasy's ProtParam prediction server (http://us.expasy.org/tools/protparam.html).

The amplicons were subcloned into the *pEASY*®-T1 cloning vector (*Trans*, Catalog No. CT111) to yield entry vectors. The plasmids were jointly digested by QuickCut™

*Kpn*I (*TaKaRa*, Catalog No. 1618) and QuickCut™ *Bam*HI (*TaKaRa*, Catalog No. 1605), and then ligated to the binary vector *pPZP211* [74] which carries the CaMV 35S promoter. The binary vector was transformed into chemically competent *Agrobacterium tumefaciens* strain GV3101 through freeze–thaw method [75]. The transformed *Agrobacterium* strains were used to transform the Arabidopsis with the floral dip method [76]. The transformants were selected by kanamycin treatment at 50 mg L^{-1}. Same procedures were conducted to obtain the homozygous Arabidopsis transformants overexpressing *CYP71R18*, *CYP94C117*, and *CYP94E14*, respectively.

Herbicide susceptibility of transgenic Arabidopsis

Herbicide susceptibility of the transgenic lines was evaluated by growth on MS media containing different concentrations of each herbicide. Three classes of ALS inhibitors including the sulfonylurea herbicide MM, the triazolo-pyrimidine herbicide PX, and the sulfonyl-aminocarbonyl-triazolinone herbicide FS were used for the testing. All assays were conducted at 25/20 °C with short-day conditions and a light intensity of 200 μM m^{-2} s^{-1}. After sterilization and washing, the seeds of transgenic Arabidopsis were planted on MS media containing different concentrations of each herbicide in petri dishes and allowed to grow for 14 days [16]. The dishes in the growth chamber were re-arranged every other day. At 14 days after treatment, susceptibility of transgenic Arabidopsis to different herbicides was visually assessed.

The MM susceptibility of independent transgenic lines was determined by spraying whole plants [16]. At 9 days after treatment, the Arabidopsis seedlings were cut at ground level, and their aboveground weights were determined and expressed here as a percent of untreated controls. Each harvest contained three replications, and the GR_{50} was calculated by fitting the data to a four-parameter log-logistic curve using SigmaPlot v.14.0 (Systat Software, San Jose, CA, USA) [77].

RT-qPCR assay

Individual 21-day-old seedlings of T3 homozygous lines were harvested for RNA extraction. RT-qPCR was carried out using the *TransStart*® Tip Green qPCR SuperMix (*Trans*, Catalog No. AQ141) as described recently [78], and the primers used are listed in Table S2. mRNA quantitation was performed by the $2^{-\Delta Ct}$ method [79]. The expression of target genes was normalized to that of the Arabidopsis housekeeping gene *AtF-box protein* (Accession no. At5g15710) [80].

LC–MS/MS analysis of MM metabolism in *A. aequalis* and transgenic Arabidopsis

Seedlings of *A. aequalis* and Arabidopsis at the suitable leaf stages were, respectively, used for MM metabolism analysis following foliar-spray with a commercial formulation of MM at 9 g ai ha^{-1} (30 g L^{-1} oil-miscible flowable, *Sigma*). The aboveground shoots of each line were harvested at different times after herbicide treatment. Each harvest had three replicate samples of at least five plants and these were snap-frozen in liquid N_2 and stored at − 80 °C. The details of MM extraction and LC–MS/MS analysis by Xevo TQ-S (*Waters*) were the same as described previously [33]. MM and its *O*-demethylated metabolite were detected in negative-ion mode with *m/z* 502.10 and *m/z* 488.10, respectively. Statistical analysis of the data was conducted using the Student's *t* test ($P < 0.05$) procedure in SPSS software (IBM, Armonk, NY, USA).

Yeast transformation and CYP709C56 expression

For the expression of *CYP709C56*, the WAT11 yeast strain and the pYeDp60 vector system were used [36]. The full-length coding sequence of *CYP709C56* was amplified and integrated into the *Kpn*I site of the pYeDp60 vector using the *pEASY*®-Uni Seamless Cloning and Assembly Kit (*Trans*, Catalog No. CU101). A triple-Flag epitope sequence was fused just downstream of the ATG through gene synthesis by *Sangon Biotech*. The recombinant plasmid was transformed into WAT11 using a LiAC method according to the manufacturer's instructions (*Takara*, Catalog No. 630439). Yeast cells were induced according to the modified two-stage cultivation method using modified SLI medium [81]. Microsomal fractions were prepared according to Renault et al. [82], after which they were electrophoresed on 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting analysis was performed according to Iwakami et al. [13] using the Anti-Flag-Tag mouse monoclonal antibody (*Sangon Biotech*, Catalog No. D190828) as primary antibody.

Yeast expression of CYP709C56 was performed according to Iwakami et al. [14]. At 14 h after the induction of P450 by galactose, MM was added to the culture media at a final concentration of 60, 100, or 200 μM. After shaking for 24 h, the culture solution was centrifuged at 14,000*g* for 5 min. The supernatant was filtered through a 0.22-μm membrane and analyzed by LC–MS/MS (*Waters*) as described recently [33].

Resistance identification and *CYP709C56* expression in *A. aequalis*

In May 2015 to May 2018, mature seeds of *A. aequalis* were collected from independent wheat fields across the

Provinces of Jiangsu (JS), Anhui (AH), Henan (HN), and Shandong (SD), China. A total of 48 populations of *A. aequalis* were selected and used to test the susceptibility to MM with or without P450 inhibition (Figs. 7, S4). Weed seedlings at the 3–4-leaf stage were treated with malathion, MM, or malathion plus MM [30]. Malathion at 1000 g ai ha^{-1} (1×) and MM at 9 g ai ha^{-1} (1×) were used for the testing. At 21 days after treatment, the aboveground dry weight was determined and expressed as a percentage of untreated control. Plant susceptibility to herbicide was defined according to the following criterion: "R" plants displayed strong regrowth as the untreated control, "r" plants showed visible regrowth but much weaker, and "S" plants showed leaf chlorosis and desiccation. For the populations exhibiting significant P450s-inhibitory phenotypes, herbicide metabolism and relative expression of *CYP709C56* were measured according to the methods described above and compared with the S population (SD-01).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04171-y.

Acknowledgements We would like to thank Dr. David Nelson (Department of Microbiology, Immunology and Biochemistry, University of Tennessee) for naming the P450 genes, Dr. Hailong An (State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University) for providing the expression vector *pPZP211*, and Dr. Xingqi Guo (State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University) for providing Arabidopsis seeds (ecotype Columbia-0).

Author contributions NZ, WL, and JW designed the study. NZ and YY performed the experimental works and statistical analyses. NZ, YY, WL, and JW drafted and critically revised the manuscript.

Funding This work was funded by grants from the National Natural Science Foundation of China (No. 32102237 and 31772181), the Anhui Provincial Natural Science Foundation (No. 2108085QC115), and the Talent Research Project of Anhui Agricultural University (No. rc342004).

Availability of data and materials All data are available in the manuscript and in the Supplementary Information.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

References

1. Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677
2. Bagavathiannan MV, Graham S, Ma Z, Barney JN, Coutts SR, Caicedo AL, De Clerck-Floate R, West NM, Blank L, Metcalf AL, Lacoste M, Moreno CR, Evans JA, Burke I, Beckie H (2019) Considering weed management as a social dilemma bridges individual and collective interests. Nat Plants 5:343–351
3. Yuan JS, Tranel PJ, Stewart CN (2007) Non-target-site herbicide resistance: a family business. Trends Plant Sci 12:6–13
4. Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347
5. Gaines TA, Patterson EL, Neve P (2019) Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance. New Phytol 223:1770–1775
6. Tranel PJ (2017) Herbicide-resistance mechanisms: gene amplification is not just for glyphosate. Pest Manag Sci 73:2225–2226
7. Beckie HJ, Tardif FJ (2012) Herbicide cross resistance in weeds. Crop Prot 35:15–28
8. Délye C (2013) Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Manag Sci 69:176–187
9. Ma R, Kaundun SS, Tranel PJ, Riggins CW, McGinness DL, Hager AG, Hawkes T, Mclndoe E, Riechers DE (2013) Distinct detoxification mechanisms confer resistance to mesotrione and atrazine in a population of waterhemp. Plant Physiol 163:363–377
10. Vila-Aiub MM, Balbi MC, Distéfano AJ, Fernández L, Hopp E, Yu Q, Powles SB (2012) Glyphosate resistance in perennial Sorghum halepense (*Johnsongrass*), endowed by reduced glyphosate translocation and leaf uptake. Pest Manag Sci 68:430–436
11. Petit C, Duhieu B, Boucansaud K, Délye C (2010) Complex genetic control of non-target-site-based resistance to herbicides inhibiting acetyl-coenzyme A carboxylase and acetolactate-synthase in *Alopecurus myosuroides* Huds. Plant Sci 178:501–509
12. Busi R, Vila-Aiub MM, Powles SB (2011) Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in *Lolium rigidum*. Heredity 106:817–824
13. Iwakami S, Endo M, Saika H, Okuno J, Nakamura N, Yokoyama M, Watanabe H, Toki S, Uchino A, Inamura T (2014) Cytochrome P450 CYP81A12 and CYP81A21 are associated with resistance to two acetolactate synthase inhibitors in *Echinochloa phyllopogon*. Plant Physiol 165:618–629
14. Iwakami S, Kamidate Y, Yamaguchi T, Ishizaka M, Endo M, Suda H, Nagai K, Sunohara Y, Toki S, Uchino A, Tominaga T, Matsumoto H (2019) CYP81A P450s are involved in concomitant cross-resistance to ALS and ACCase herbicides in *Echinochloa phyllopogon*. New Phytol 221:2112–2122
15. Saika H, Horita J, Taguchi-Shiobara F, Nonaka S, Nishizawa-Yokoi A, Iwakami S, Hori K, Matsumoto T, Tanaka T, Itoh T, Yano M, Kaku K, Shimizu T, Toki S (2014) A novel rice cytochrome P450 gene, *CYP72A31*, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and Arabidopsis. Plant Physiol 166:1232–1240
16. Cummins I, Wortley DJ, Sabbadin F, He ZS, Coxon CR, Straker HE, Sellars JD, Knight K, Edwards L, Hughes D, Kaundun SS, Hutchings SJ, Steel PG, Edwards R (2013) Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. Proc Natl Acad Sci USA 110:5812–5817
17. Evans AF, O'Brien SR, Ma R, Hager AG, Riggins CW, Lambert KN, Riechers DE (2017) Biochemical characterization of metabolism-based atrazine resistance in *Amaranthus tuberculatus* and identification of an expressed GST associated with resistance. Plant Biotechnol J 15:1238–1249

18. Pan L, Yu Q, Han HP, Mao LF, Nyporko A, Fan LJ, Bai LY, Powles SB (2019) Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. Plant Physiol 181:1519–1534
19. LeBaron HM, McFarland J (1990) Herbicide resistance in weeds and crops. Managing resistance to agrochemicals. American Chemical Society, Washington, pp 336–352
20. Brazier M, Cole DJ, Edwards R (2002) *O*-Glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). Phytochemistry 59:149–156
21. Cummins I, Moss S, Cole DJ, Edwards R (1997) Glutathione transferases in herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). Pestic Sci 51:244–250
22. Conte SS, Lloyd AM (2011) Exploring multiple drug and herbicide resistance in plants—Spotlight on transporter proteins. Plant Sci 180:196–203
23. Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol 1:reviews3003
24. Nelson DR (2009) The cytochrome p450 homepage. Hum Genom 4:59–65
25. Mizutani M, Ohta D (2010) Diversification of P450 genes during land plant evolution. Annu Rev Plant Biol 61:291–315
26. Dimaano NG, Iwakami S (2021) Cytochrome P450-mediated herbicide metabolism in plants: current understanding and prospects. Pest Manag Sci 77:22–32
27. Dimaano NG, Yamaguchi T, Fukunishi K, Tominaga T, Iwakami S (2020) Functional characterization of cytochrome P450 CYP81A subfamily to disclose the pattern of cross-resistance in *Echinochloa phyllopogon*. Plant Mol Biol 102:403–416
28. Guo F, Iwakami S, Yamaguchi T, Uchino A, Sunohara Y, Matsumoto H (2019) Role of CYP81A cytochrome P450s in clomazone metabolism in *Echinochloa phyllopogon*. Plant Sci 283:321–328
29. Guo WL, Liu WT, Li LX, Yuan GH, Du L, Wang JX (2015) Molecular basis for resistance to fenoxaprop in shortawn foxtail (*Alopecurus aequalis*) from China. Weed Sci 63:416–424
30. Zhao N, Yan YY, Ge LA, Zhu BL, Liu WT, Wang JX (2019) Target site mutations and cytochrome P450s confer resistance to fenoxaprop-*P*-ethyl and mesosulfuron-methyl in Alopecurus aequalis. Pest Manag Sci 75:204–214
31. Zhao N, Li W, Bai S, Guo WL, Yuan GH, Wang F, Liu WT, Wang JX (2017) Transcriptome profiling to identify genes involved in mesosulfuron-methyl resistance in *Alopecurus aequalis*. Front Plant Sci 8:1391
32. Aizawa H (2014) Mesosulfuron-methyl. Handbook of metabolic pathways of xenobiotics. Wiley, Oxford, pp 1761–1763
33. Zhao N, Yan YY, Luo YL, Zou N, Liu WT, Wang JX (2019) Unravelling mesosulfuron-methyl phytotoxicity and metabolism-based herbicide resistance in *Alopecurus aequalis*: insight into regulatory mechanisms using proteomics. Sci Total Environ 670:486–497
34. Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S, Werck-Reichhart D (2011) Cytochromes P450. In: The Arabidopsis book. American Society of Plant Biologists, Maryland, pp e0144
35. Ghanizadeh H, Harrington KC (2017) Non-target site mechanisms of resistance to herbicides. Crit Rev Plant Sci 36:24–34
36. Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods in enzymology. Academic Press, Massachusetts, pp 51–64
37. Yu Q, Powles SB (2014) Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. Plant Physiol 166:1106–1118
38. Gaines TA, Lorentz L, Figge A, Herrmann J, Maiwald F, Ott MC, Han HP, Busi R, Yu Q, Powles SB, Beffa R (2014) RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum*. Plant J 78:865–876
39. Duhoux A, Carrere S, Gouzy J, Bonin L, Delye C (2015) RNA-Seq analysis of rye-grass transcriptomic response to an herbicide inhibiting acetolactate-synthase identifies transcripts linked to non-target-site-based resistance. Plant Mol Biol 87:473–487
40. Gardin JA, Gouzy J, Carrere S, Delye C (2015) ALOMYbase, a resource to investigate non-target-site-based resistance to herbicides inhibiting acetolactate-synthase (ALS) in the major grass weed *Alopecurus myosuroides* (black-grass). BMC Genom 16:590
41. Bai S, Zhao YF, Zhou YM, Wang ML, Li YH, Luo XY, Li LX (2020) Identification and expression of main genes involved in non-target site resistance mechanisms to fenoxaprop-p-ethyl in *Beckmannia syzigachne*. Pest Manag Sci 76:2619–2626
42. Liu WT, Bai S, Zhao N, Jia SS, Li W, Zhang LL, Wang JX (2018) Non-target site-based resistance to tribenuron-methyl and essential involved genes in *Myosoton aquaticum* (L.). BMC Plant Biol 18:225
43. Yan BJ, Zhang YH, Li J, Fang JP, Liu TT, Dong LY (2019) Transcriptome profiling to identify cytochrome P450 genes involved in penoxsulam resistance in *Echinochloa glabrescens*. Pestic Biochem Physiol 158:112–120
44. Pan L, Yu Q, Wang JZ, Han HP, Mao LF, Nyporko A, Maguza A, Fan LJ, Bai LY, Powles SB (2021) An ABCC-type transporter endowing glyphosate resistance in plants. Proc Natl Acad Sci USA 118:e2100136118
45. Han HP, Yu Q, Beffa R, González S, Maiwald F, Wang J, Powles SB (2020) Cytochrome P450 CYP81A10v7 in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. Plant J 105:79–92
46. Guo WL, Lv LL, Zhang LL, Li Q, Wu CX, Lu XT, Liu WT, Wang JX (2016) Herbicides cross resistance of a multiple resistant short-awn foxtail (*Alopecurus aequalis* Sobol.) population in wheat field. Chil J Agric Res 76:163–169
47. Pan G, Zhang XY, Liu KD, Zhang JW, Wu XZ, Zhu J, Tu JM (2006) Map-based cloning of a novel rice cytochrome P450 gene *CYP81A6* that confers resistance to two different classes of herbicides. Plant Mol Biol 61:933–943
48. Nelson D, Werck-Reichhart D (2011) A P450-centric view of plant evolution. Plant J 66:194–211
49. Hamberger B, Bak S (2013) Plant P450s as versatile drivers for evolution of species-specific chemical diversity. Philos Trans R Soc B 368:20120426
50. Irmler S, Schröder G, St-Pierre B, Crouch NP, Hotze M, Schmidt J, Strack D, Matern U, Schröder J (2000) Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J 24:797–804
51. He J, Chen QW, Xin PY, Yuan J, Ma YH, Wang XM, Xu MM, Chu JF, Peters RJ, Wang GD (2019) CYP72A enzymes catalyse 13-hydrolyzation of gibberellins. Nat Plants 5:1057–1065
52. Imaishi H, Matumoto S (2007) Isolation and functional characterization in yeast of CYP72A18, a rice cytochrome P450 that catalyzes (omega-1)-hydroxylation of the herbicide pelargonic acid. Pestic Biochem Physiol 88:71–77
53. Kandel S, Morant M, Benveniste I, Blee E, Werck-Reichhart D, Pinot F (2005) Cloning, functional expression, and characterization of CYP709C1, the first sub-terminal hydroxylase of long chain fatty acid in plants induction by chemicals and methyl jasmonate. J Biol Chem 280:35881–35889
54. Xu WY, Di C, Zhou SX, Liu J, Li L, Liu FX, Yang XL, Ling Y, Su Z (2015) Rice transcriptome analysis to identify possible herbicide quinclorac detoxification genes. Front Genet 6:306

55. Yu XZ, Lu CJ, Tang S, Zhang Q (2020) Transcriptomic analysis of cytochrome P450 genes and pathways involved in chromium toxicity in *Oryza sativa*. Ecotoxicology 29:503–513
56. Jennings GK, Hsu MH, Shock LS, Johnson EF, Hackett JC (2018) Noncovalent interactions dominate dynamic heme distortion in cytochrome P450 4B1. J Biol Chem 293:11433–11446
57. Thom R, Cummins I, Dixon DP, Edwards R, Cole DJ, Lapthorn AJ (2002) Structure of a Tau class glutathione S-transferase from wheat active in herbicide detoxification. Biochemistry 41:7008–7020
58. Neve P, Powles S (2005) High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. Heredity 95:485–492
59. Neve P, Powles S (2005) Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*. Theor Appl Genet 110:1154–1166
60. Busi R, Neve P, Powles S (2013) Evolved polygenic herbicide resistance in *Lolium rigidum* by low-dose herbicide selection within standing genetic variation. Evol Appl 6:231–242
61. Zhao N, Yan Y, Du L, Zhang X, Liu W, Wang J (2020) Unravelling the effect of two herbicide resistance mutations on acetolactate synthase kinetics and growth traits. J Exp Bot 71:3535–3542
62. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, Ismail HM, Hemingway J, Ranson H, Albert A, Wondji CS (2014) A single mutation in the *GSTe2* gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol 15:R27
63. Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, Huang YP, Chen XY (2014) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol 25:1307–1313
64. Bautista MAM, Miyata T, Miura K, Tanaka T (2009) RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, *Plutella xylostella*, reduces larval resistance to permethrin. Insect Biochem Mol Biol 39:38–46
65. Sammons RD, Ivashuta S, Liu H, Wang D, Feng PC, Kouranov AY, Andersen SE (2011) Method for controlling herbicide-resistant plants. US Patent Application No. US2011/0296556 A1
66. Yu DQ, Chen CH, Chen ZX (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. Plant Cell 13:1527–1539
67. Mahmood K, Mathiassen SK, Kristensen M, Kudsk P (2016) Multiple herbicide resistance in *Lolium multiflorum* and identification of conserved regulatory elements of herbicide resistance genes. Front Plant Sci 7:1160
68. Li CB, Qiao ZY, Qi WW, Wang Q, Yuan Y, Yang X, Tang YP, Mei B, Lv YD, Zhao H, Xiao H, Song RT (2015) Genome-wide characterization of cis-Acting DNA targets reveals the transcriptional regulatory framework of *opaque2* in maize. Plant Cell 27:532–545
69. Shimono M, Sugano S, Nakayama A, Jiang CJ, Ono K, Toki S, Takatsuji H (2007) Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. Plant Cell 19:2064–2076
70. Yang X, Deng S, Wei XG, Yang J, Zhao QN, Yin C, Du TH, Guo ZJ, Xia JX, Yang ZZ, Xie W, Wang SL, Wu QJ, Yang FS, Zhou XG, Nauen R, Bass C, Zhang YJ (2020) MAPK-directed activation of the whitefly transcription factor CREB leads to P450-mediated imidacloprid resistance. Proc Natl Acad Sci USA 117:10246–10253
71. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
72. Robert X, Gouet P (2014) Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res 42:W320–W324
73. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549
74. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994
75. Weigel D, Glazebrook J (2006) Transformation of agrobacterium using the freeze-thaw method. Cold Spring Harb Protoc 2006:1031–1036
76. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
77. Seefeldt SS, Jensen JE, Fuerst EP (1995) Log-logistic analysis of herbicide dose-response relationships. Weed Technol 9:218–227
78. Zhao N, Yan YY, Wang HZ, Bai S, Wang Q, Liu WT, Wang JX (2018) Acetolactate synthase overexpression in mesosulfuron-methyl-resistant shortawn foxtail (*Alopecurus aequalis* Sobol.): reference gene selection and herbicide target gene expression analysis. J Agric Food Chem 66:9624–9634
79. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408
80. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139:5–17
81. Jiang HX, Morgan JA (2004) Optimization of an in vivo plant P450 monooxygenase system in *Saccharomyces cerevisiae*. Biotechnol Bioeng 85:130–137
82. Renault H, Alber A, Horst NA, Lopes AB, Fich EA, Kriegshauser L, Wiedemann G, Ullmann P, Herrgott L, Erhardt M, Pineau E, Ehlting J, Schmitt M, Rose JKC, Reski R (2017) A phenol-enriched cuticle is ancestral to lignin evolution in land plants. Nat Commun 8:14713