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Expression of cytochrome P450 *CYP81A6* in rice: tissue specificity, protein subcellular localization, and response to herbicide application*

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Abstract: The cytochrome P450 gene *CYP81A6* confers tolerance to bentazon and metsulfuron-methyl, two selective herbicides widely used for weed control in rice and wheat fields. Knockout mutants of *CYP81A6* are highly susceptible to both herbicides. The present study aimed to characterize the *CYP81A6* expression in rice. Quantitative real-time polymerase chain reaction (PCR) analyses demonstrated that foliar treatment of bentazon (500 mg/L) greatly induced expression of *CYP81A6* in both wild-type (Jiazhe B) and its knockout mutant (Jiazhe mB): a 10-fold increase at 9 h before returning to basal levels at 24 h in Jiazhe B, while in the mutant the expression level rose to >20-fold at 12 h and maintained at such high level up to 24 h post exposure. In contrast, metsulfuron-methyl (500 mg/L) treatment did not affect the expression of *CYP81A6* in Jiazhe B within 80 h; thereafter the expression peaked at 120 h and returned gradually to basal levels by Day 6. We suggest that a metabolite of metsulfuron-methyl, 1H-2,3-benzothiazin-4-(3H)-one-2,2-dioxide, is likely to be responsible for inducing *CYP81A6* expression, rather than the metsulfuron-methyl itself. Use of a promoter-GUS reporter construct (*CYP81A6Pro::GUS*) demonstrated that *CYP81A6* was constitutively expressed throughout the plant, with the highest expression in the upper surfaces of leaves. Subcellular localization studies in rice protoplasts showed that CYP81A6 was localized in the endoplasmic reticulum. These observations advance our understanding of *CYP81A6* expression in rice, particularly its response to the two herbicides.

Key words: CYP81A6, Bentazon, Metsulfuron-methyl, Expression induction, Xenobiotics
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1 Introduction

Crop production continues to be dependent on herbicide application for weed control across several different cropping systems (Gianessi and Reigner, 2007). Bentazon, which inhibits the electron transfer in photosystem II (PSII), is commonly applied to cereals to selectively remove broad-leaf weeds (Han and Wang, 2002). Other selective weed killers such as the sulfonylurea herbicide, metsulfuron-methyl, inhibit the biosynthesis of branched-chain amino acids by irreversibly binding to the enzyme acetolactate synthase (ALS) (Pang *et al*., 2003), while pendimethalin, widely used in rice cultivation, inhibits weed seed germination by inhibiting mitosis (Glover and Schapaugh, 2002). Unlike the non-selective herbicides such as glyphosate, use of these selective herbicides causes little damage or stress to

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the desired crop. Tolerance in rice to these herbicides is due to its ability to effectively metabolize the compounds with a range of non-target-site resistance systems including P450 mono-oxygenases, glutathione *S*-transferases and glycosyltransferases (Ohkawa *et al*., 1999; Yuan *et al*., 2007). In addition to the presence of these detoxifying enzymes, further selectivity occurs from the absence of binding sites in target proteins due to structural differences between crop plants (Konishi *et al*., 1996; Green, 2009).

Substantial experimental data indicates that cytochrome P450s are involved in the metabolism, tolerance, selectivity, and compatibility of xenobiotics. The gene *CYP81A6* is the key component involved in bentazon detoxification metabolism in rice (Zhang *et al*., 2006). CYP81A6, a member of the cytochrome P450 heme-containing membrane-associated proteins, exhibits the characteristic folding and catalytic mechanism found within this superfamily, namely the ability to catalyze the scission of dioxygen binding to the heme unit. One oxygen is inserted into the substrate and the other oxygen is reduced to H_2O by acquiring two electrons from the nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 oxidoreductase (POR) complex (Hannemann *et al*., 2007). This process is also widely utilized in plants to detoxify xenobiotics by rendering them more hydrophilic and enabling further modification via conjugation with glucose before exclusion from the protoplasm.

The *CYP81A6* gene was previously cloned using a map-based cloning strategy (Pan *et al*., 2006). Related studies indicated that CYP81A6 also plays a role in tolerance to metsulfuron-methyl herbicides (Zhang *et al*., 2002). Therefore, *CYP81A6* has also been engineered into non-gramineous crops to confer tolerance to bentazon, or used as a selective marker in genetic transformation (Liu *et al*., 2012). Furthermore, because *CYP81A6* knockout mutants are sensitive to bentazon, this bentazon lethality (*bel*) trait has been specifically induced in male sterile lines of hybrid rice to effectively eliminate self-pollinated seedlings among F_1 hybrids (Zhang *et al*., 2006; Wang *et al*., 2012). The *bel* trait has also been incorporated into restorer lines to simplify hybrid seed production by selecting against restorer plants after pollination; this enables mixed growing of male sterile and fertility restorer lines and bulk harvest of seeds in fields. Based on the ability of CYP81A6 to detoxify bentazon, the use of RNA interference cassettes, which specifically suppress the expression of this detoxification enzyme, has been proposed as a novel transgenic plant containment strategy to prevent escape of transgenes (Lin *et al*., 2008).

Despite the potential role of CYP81A6 as a breeding tool in modern crop production, particularly in rice, as stated above, knowledge of its expression remains largely unknown. The present study reports on the expression of *CYP81A6* in rice through analysis of its response to herbicides, protein subcellular localization and tissue specificity.

2 Materials and methods

2.1 Plant materials and herbicide treatment

Jiazhe B is a maintainer line for the cytoplasmic male sterile line Jiazhe A, which is the female parent of hybrid rice Jiayou 99 (Fu, 2004). Jiazhe mB is a γ induced mutant of Jiazhe B resulting from a 1-bp knockout deletion mutation of *CYP81A6*; it is susceptible to both bentazon and metsulfuron-methyl herbicides (Wang *et al*., 2012).

For study of response to herbicides, seeds of both Jiazhe B and Jiazhe mB were imbibed in water for 2 d, then germinated on damp filter paper for a further 2 d at 37 °C. The germinated seeds were then transferred to a net floating on the culture solution (Yokosho *et al*., 2011), containing the following macronutrients: $(NH_4)_2SO_4$ (0.18 mmol/L), $MgSO_4$ (0.27 mmol/L), KNO_3 (0.09 mmol/L), $Ca(NO_3)_2$ (0.18 mmol/L), KH_2PO_4 (0.09 mmol/L), and the following micronutrients: Fe-EDTA (20 μmol/L), $MnCl_2$ (0.5 μmol/L), H_3BO_3 (3 μmol/L), $(NH_4)_6Mo_7O_{24}$ (1 μmol/L), $ZnSO_4$ (0.4 μmol/L), $CuSO_4$ (0.2 μmol/L). Seedlings were then grown in an environmentally controlled growth room with a 14 h/26 °C day and a 10 h/22 °C night. Three-week-old seedlings (about 18 cm long with 4 leaves) were sprayed with the herbicides bentazon, metsulfuron-methyl, or pendimethalin at a range of concentrations (0, 250, 500, 1000, and 1500 mg/L). The effect of the protein translation inhibitor cycloheximide (CHX) on *CYP81A6* expression was tested by spraying 20 μmol/L CHX 1 h before bentazon treatment (500 mg/L), as performed in rice bean by Liu *et al*. (2013).

2.2 RNA extraction, reverse transcription and real-time PCR

To investigate the effects of the three herbicides (each with five doses) on the expression of *CYP81A6*, leaf tissues were harvested for RNA extraction at 6 h post-treatment. To study the time course of the effects of bentazon and metsulfuron-methyl (500 mg/L), leaf tissues were collected from treated seedlings at the time points of 2, 4, 6, 9, 12, 15, 18, 21, and 24 h post-treatment. For the herbicide metsulfuron-methyl, transcript levels were also investigated in leaf tissues harvested at 8 h intervals between 64 and 144 h post-treatment.

Total RNA was extracted from leaf tissues in 1000 μl TRIzol reagent according to the manufacturer's instructions. The cDNA was generated from 500 ng RNA using moloney murine leukemia virus (M-MLV) reverse transcript kit (TaKaRa Company, Dalian, China). Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on an Eppendorf MasterCycler® ep RealPlex4 (Wesseling Berzdorf, Germany) using CYP81A6-RT primers in 10 μl aliquots, containing 1 μl cDNA, 0.2 μl of each primer (10 μmol/L), and 5 μl 2× mix buffer (Master mix, TOYOBO), supplemented with sterile distilled water, with the following program: 94 °C 2 min, 40 cycles of 94 °C 30 s and 60 °C 1 min. The relative quantification of gene expression was analyzed using the $2^{-\Delta\Delta C_T}$ method, where the C_T value is the cycle number at which the fluorescent signal rises statistically above the background; all treatments were performed in three biological replicates with *Actin* as an internal standard (Livak and Schmittgen, 2001).

2.3 Generation of transgenic plants expressing the *CYP81A6* promoter-*GUS* fusion gene and histochemical staining

A fragment (about 1.9 kb) in the promoter region of *CYP81A6* was PCR amplified using CYP81A6-Pro primers (Table 1), which were designed according to sequence information available on the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). PCRs were performed in 25 μl with 1.0 μl (50 ng/μl) DNA, 0.2 mmol/L dNTPs, 1.5 mmol/L $MgSO_4$, 0.3 μmol/L each primer, 0.5 μl high-fidelity DNA polymerase, supplemented with sterile distilled water, with the following temperature profile: 94 °C 2 min; 40 cycles of 94 °C 10 s, 57 °C 30 s, 68 °C 60 s; and a final extension at 68 °C for 5 min. The resulting amplicons were cloned into the pMD19-T simple vector after electrophoresis and purification. Sequence verified promoter fragments were subcloned into the multicloning site (MCS) between *Sal*I and *Sma*I of pCAMBIAI1300 and a *CYP81A6Pro::GUS* vector was constructed (Fig. 1a). The *CYP81A6Pro::GUS* construct was then introduced into the *Agrobacterium tumefaciens* strain EHA105 and used for transformation of the rice line Nipponbare according to Hiei and Komari (2008). *CYP81A6Pro::GUS* positive transgenic plants were identified and the root, stem, leaf, sheath, flower, and seed tissue samples were histochemically stained for 24 h at 37 °C with GUS (β-glucuronidase) dye solution containing 100 mmol/L phosphate buffer (pH 7.0), 20% methanol, 0.5% Triton X-100, 0.5 mg/ml X-Gluc. Stained tissues were then alcohol decoloured and photographed by light microscopy.

Table 1 Primers used for RT-PCR and cloning of promoter and coding sequences of CYP81A6

Primers	Forward (F) and reverse (R) sequence (5'→3')	Amplicon size (bp)
Actin	F: ACAGGCGTTATGGTTGGG	185
	R: TGGATGCTCTTCTGGTGCT	
CYP81A6-RT	F: ACGCATCCGTCGGCAACT	420
	R: GCCCGCCACCTTCAGTCATGTCG	
CYP81A6-Pro	F: AAAgtcgacAGACGGACAATCAACGGGTAC	1990
	R: AAAcccgggGGCTAAGCTGAGCTCACGAAT	
CYP81A6-CDS	F: AAAcccgggGCACCAGAGTCACAGAAACAC	1542
	F: AAAggatccGACGAGCTCGCGAAGAAC	

The small letters refer to restriction enzyme recognition sites for *Sal*I (GTCGAC), *Sma*I (CCCGGG) and *Bam*HI (GGATCC) in CYP81A6-Pro or CYP81A6-CDS. Pro: promoter; CDS: coding sequence

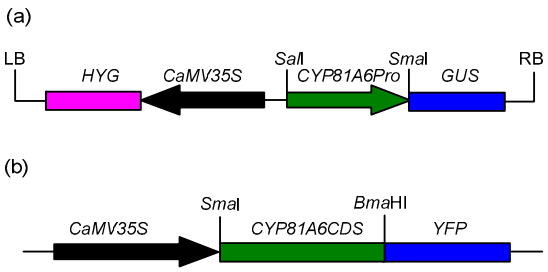

Fig. 1 Schematic diagrams of vectors *CYP81A6Pro::GUS* (a) and *CYP81A6CDS::YFP* (b)
HYG: hygromycin B resistance gene; *CaMV35S*: cauliflower mosaic virus (CaMV) 35S; *Pro*: promoter; *GUS*: glucuronidase gene; *YFP*: yellow fluorescent protein gene; LB: left border; RB: right border

2.4 Subcellular localization

The full-length coding region of *CYP81A6* (except for the stop codon) was amplified by PCR using the primers CYP81A6-CDS (Table 1). PCR products were subcloned into the pMD19-T simple vector for sequence confirmation and further cloned into the vector pTZM28-YFP to produce a fusion gene with yellow fluorescent protein gene (*YFP*) under the control of the cauliflower mosaic virus (CaMV) 35S (*CaMV35S*) promoter (Fig. 1b). Nipponbare seeds were germinated and cultured for 8 d on 1/2 MS culture medium at 28 °C under continuous lighting. For preparation of protoplasts, the tender stems were cut into 2 mm sections with sterile blades and used for isolation of protoplasts (Yoo *et al*., 2007). Ten microgram *35S::OsCYP81A6::YFP* plasmids were mixed with 220 μl 40% (0.4 g/ml) PEG-4000 and used for transformation of 200 μl protoplasts with gentle blending and incubating 16 h in the dark. For co-localization, the endoplasmic reticulum (ER) targeting pBINm-GFP5-ER plasmid was co-transformed. The transformed protoplasts were examined under a light microscope as well as a laser confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

2.5 Data analysis

The mean and standard errors were calculated from three independent replicates. Statistical analyses were performed using the one-way analysis of variance (ANOVA) programme StaView. The differences were considered to be significant when the probability (P) was less than 0.05.

3 Results

3.1 Correlation between *CYP81A6* expression and bentazon exposure

Previous work has shown that the mutant lines used in the present study are sensitive to the herbicides bentazon and metsulfuron-methyl, whereas the wild-type rice is tolerant. A third herbicide, pendimethalin, shows no effect on either the wild-type or the mutant lines (Zhang *et al*., 2002; Wang *et al*., 2012). Expression of *CYP81A6* in wild-type plants following treatment with bentazon showed a dose response effect (Fig. 2a). At a concentration of 250 mg/L, expression levels were greater than 2-fold after 6 h, compared with non-treated plants, increasing to approximately 5-fold in those plants sprayed with the herbicide at 1500 mg/L (Fig. 2a). In contrast, neither metsulfuron-methyl nor pendimethalin caused any changes in *CYP81A6* transcript levels 6 h post-treatment, irrespective of the concentrations used (Fig. 2a).

When treated with bentazon (500 mg/L), expression of *CYP81A6* in the wild-type plants continued to increase for up to 9 h post-application. Gene expression reached a maximum of 10-fold that of controls at this time point (Fig. 2b). However, once maximal gene expression had been achieved, expression then declined until basal expression was regained after 24 h. Expression of *CYP81A6* also increased in the mutant line following bentazon treatment, reaching a maximum 12 h after exposure (Fig. 2b). Expression in the mutant line was both at an increased rate and to a greater level (>20-fold) than that in the wild type. Furthermore, unlike the wild type, expression of *CYP81A6* did not return to the basal level at any time during the 24-h period after exposure, but was maintained at elevated levels slightly below the peak (Fig. 2b). In contrast, no increase of *CYP81A6* transcripts was noted within 24 h post-treatment with either metsulfuron-methyl or pendimethalin.

The results of these gene expression studies were reflected in the phenotypes of the wild-type and mutant plants following exposure to the three herbicides, with only the bentazon-treated mutant showing signs of tissue damage within 24 h, where the symptoms exhibited were similar to those following dehydration (data not shown). Thus, despite the high, and maintained, levels of the transcript, bentazon is toxic to the

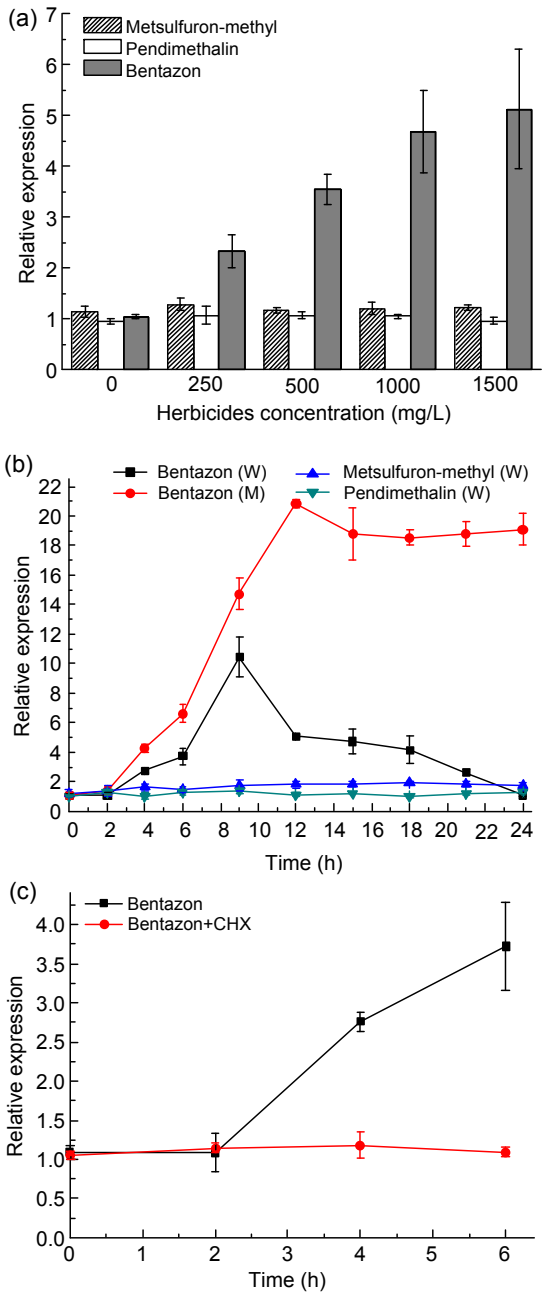

Fig. 2 Expression of *CYP81A6* in wild-type rice cultivar Jiazhe B (W) and its knockout mutant Jiazhe mB (M) through qRT-PCR analyses

(a) *CYP81A6* expression 6 h post-treatment with bentazon, metsulfuron-methyl and pendimethalin (0–1500 mg/L); (b) Time course of gene expression in rice leaves in response to these herbicides (500 mg/L) over a 24-h period; (c) *CYP81A6* expression in rice plants treated with 20 μmol/L CHX prior to bentazon spray (500 mg/L). Data are expressed as mean±standard error (SE) of three independent experiments. Expression levels were normalized to that of the *Actin* gene

mutant line, suggesting either that the mutation in *CYP81A6* results in the non-translation of the product, or that the encoded enzyme is non-functional and unable to detoxify the herbicide.

Treatment of rice seedlings with 20 μmol/L CHX prior to exposure to bentazon resulted in the relative suppression of *CYP81A6* expression. However, in plants not pre-treated with CHX, there was a rapid accumulation of *CYP81A6* transcripts between 2 to 6 h (Fig. 2c). This finding implies that *de novo* protein biosynthesis is required for increased transcription of *CYP81A6* under bentazon stress, and rules out the possible involvement of a repressor protein suppressing the expression of *CYP81A6*.

3.2 Delayed induction of *CYP81A6* caused by metsulfuron-methyl treatment

In contrast to bentazon, which resulted in the rapid onset of tissue necrosis leading to plant death in the mutant line, the other two herbicides did not cause such effects within the same time frame (24 h). However, over a longer period (>4 d), mutants exposed to metsulfuron-methyl (500 mg/L) did display symptoms of tissue death, but from the leaf tips downwards. These symptoms were accompanied by an increase in *CYP81A6* transcript levels. Semi-quantitative RT-PCR analysis, at 8 h intervals, showed that up to 80 h post herbicide exposure, expression of *CYP81A6* did not accumulate above basal levels. However, by 88 h post-treatment, there was a substantial increase in the levels of these transcripts, which continued to increase to a maximum of 13-fold higher than that of the control at 120 h post-exposure. Thereafter, expression declined, finally returning to basal levels by 144 h (Fig. 3).

3.3 Spatial expression of *CYP81A6* in rice

Spatial expression patterns of *CYP81A6* expression in different rice tissues were determined in transgenic plants using a promoter::GUS reporter construct (*CYP81A6Pro::GUS*). Data provided by the rice gene expression database (http://ricexpro.dna.affrc.go.jp/) allowed identification of a 1.9 kb-region upstream of the ATG translation start signal corresponding to the promoter region of *CYP81A6*. The resulting construct was used to generate transgenic rice plants (cv. Nipponbare) and five were selected at T_0 (first generation) using hygromycin screening.

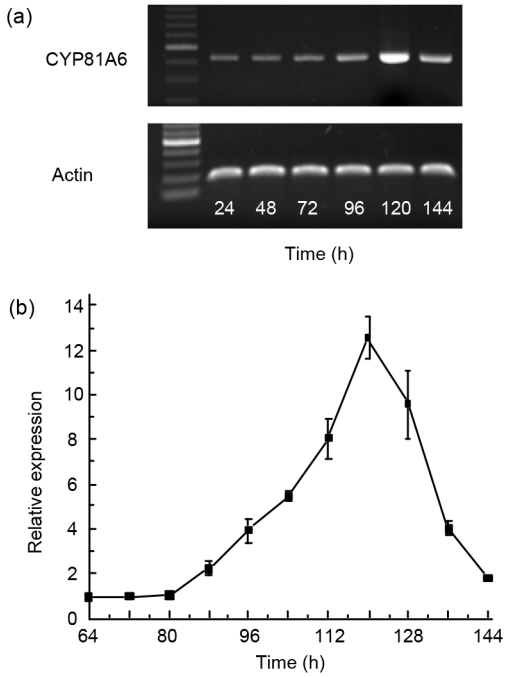

Fig. 3 Analysis of *CYP81A6* expression by RT-PCR in response to metsulfuron-methyl at 500 mg/L over time (up to 144 h)

(a) Semi-quantitative RT-PCR analysis for tissue collected every 24 h; (b) Quantitative RT-PCR analysis of samples collected every 8 h (64–144 h post-treatment). Data are expressed as mean±SE of three independent experiments. Expression levels were normalized to that of the *Actin* gene

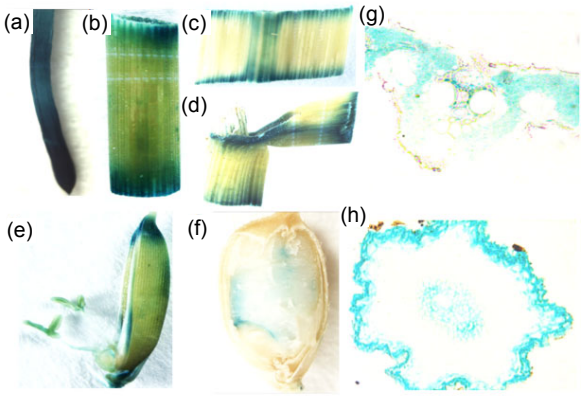

Fig. 4 Histochemical localization of GUS activity in transgenic plants expressing the *CYP81A6* promoter-GUS fusion gene

GUS activity was expressed in the root (a), stem (b), leaf (c), leaf sheath (d), flower (e), and seeds (f). These results indicate that *CYP81A6* is expressed constitutively in rice. Cross-sections of the leaf (g) and root (h) show that GUS activity was predominantly in the upper surface of the leaf and the peripheral layers of the root

Histochemical staining for GUS activity showed that *CYP81A6* was strongly expressed in root, stem, leaf, and sheath tissues (Figs. 4a–4d), but only weakly in flowers and seeds (Figs. 4e and 4f). Staining throughout the leaf cross-sections was not homogeneous, with more staining evident on the upper surface of the leaf than within the tissues of the lower dermal layers (Fig. 4g). Preferential GUS staining was noted throughout the root cross-section, with the actively growing peripheral regions of the roots exhibiting more intense staining. This observation demonstrates that the greater expression of the GUS reporter occurs in these areas and hence the greater *CYP81A6* expression (Fig. 4h).

3.4 CYP81A6 anchors to the ER

CYP81A6 contains the highly conserved domain, characteristic of the eukaryotic cytochrome P450 N-terminal signal peptide: proline-rich domain involved in membrane anchoring. Constitutive expression of a chimeric CYP81A6CDS-yellow fluorescent protein (CYP81A6::YFP) under the control of the *CaMV35S* promoter in rice protoplasts was used to identify the subcellular localization of the protein encoded by *CYP81A6* (Fig. 5b), while a chimeric

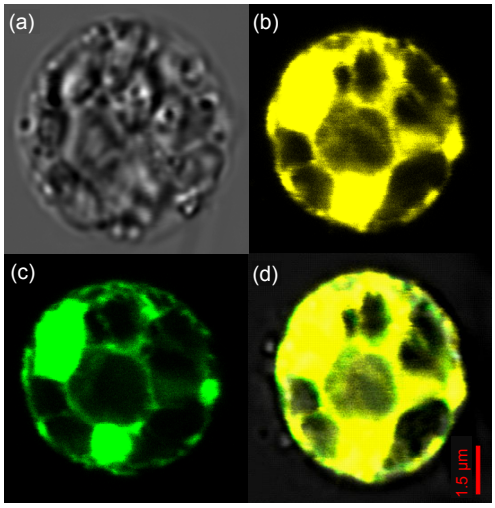

Fig. 5 Subcellular localization of CYP81A6 in rice protoplasts co-transformed with CYP81A6CDS-YFP and pBINm-GFR5-ER (a marker for the ER) using confocal microscopy

(a) Under white light; (b) Yellow fluorescence indicates the presence of CYP81A6; (c) Green fluorescence indicates the presence of ER marker protein; (d) Overlay of these two images (b, c) indicates that the CYP81A6 is localized to the ER (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

marker protein pBINm-GFP5-ER was used to visualize the ER (Fig. 5c). Overlay of these two images demonstrated that the patterns were identical between the CYP81A6 protein and the maker protein, indicating that the CYP81A6 is localized to the ER (Fig. 5d).

4 Discussion

There are currently 5100 sequences annotated for plant CYPs, which account for about 1% of the total protein-coding genes; this is significantly greater than that for any other gene family (Lv *et al*., 2012). *CYP81A6*, a cytochrome P450 gene, encoding a mono-oxygenase, has been previously identified as conferring tolerance to bentazon and metsulfuron-methyl (Pan *et al*., 2006). The mono-oxygenase activity of CYP81A6 is required for the degradation of both these xenobiotics, and the action of newly synthesized enzymes would aid in protection against the action of the herbicide and also mitigate the secondary effects from reactive oxygen species (Narusaka *et al*., 2004; Nelson, 2011). Despite the apparent importance of this mono-oxygenase encoded by *CYP81A6* in rice, little is known either about its mechanisms of degradation or, intriguingly, reasons as to why the gene is expressed in response to exposure to these two herbicides that have very different molecular characteristics and very different modes of action. In order to elucidate the role of CYP81A6 mono-oxygenase in rice, studies were carried out to investigate its tissue specificity and subcellular localization within the plant, as well as its expression in response to herbicide application.

Results from the present study demonstrated that the wild-type plant could respond positively and quickly to the application of bentazon. In the loss-of-function mutant line, *CYP81A6* was also highly expressed after bentazon treatment, with transcript being more abundant than in wild-type plants and little reduction after reaching a maximum level during a 24-h period. While the return of *CYP81A6* expression to basal level in wild-type plants suggests that bentazon is degraded to a level that no longer induces *CYP81A6* expression within 24 h in rice, the small return of expression indicates that unmetabolized bentazon eventually resulted in lethality in the mutant line (Fig. 2c). The slight decrease of *CYP81A6* transcripts might reflect that bentazon already starts damaging plants physiologically 12 h after treatment, although not yet visually observable.

Involvement of exogenous chemical signals in gene regulation is widespread and found in many kingdoms (Ward *et al*., 1991; Courcelle *et al*., 2001; Ahmad *et al*., 2008). These include control of the *lac* operon in bacteria, which results in rapid transcription upon induction with allolactose, and binding of the plant hormone indolyl acetic acid (IAA) directly to the promoter region sequence thus providing self-regulation of IAA biosynthesis (Dvorak *et al*., 2005; Zhao, 2010). Here, the expression of *CYP81A6* was rapidly induced by the treatment of bentazon, but the process was inhibited by pre-application of 20 μmol/L CHX (Fig. 2c). These results indicate that *CYP81A6* expression is up-regulated by a protein (probably a transcription factor) that is translated *de novo* and is directly or indirectly induced by bentazon; they also rule out the possible involvement of a repressor protein suppressing its transcription in the absence of bentazon. A similar phenomenon was reported in rice bean on the Al-induced expression of *VuMATE1* (Liu *et al*., 2013).

Histochemical staining of *CYP81A6Pro::GUS* transgenic seedlings revealed that the gene is constitutively expressed in root, culm, leaf, sheath, floret and seed tissues, with the greatest levels of expression being in the upper leaf surfaces and the outer root layers. Constitutive expression of CYP81A6 mono-oxygenase, with higher concentrations in the upper leaf surfaces and outer root layers, is consistent with a role in detoxification of exogenous pollutants, particularly toxicants such as herbicides that are applied topically, or contaminants in the soil. Once the exogenous toxic molecules enter the plant cells, they are able to respond within a short period of time, up-regulating *CYP81A6* transcription. The herbicide bentazon is an ester, which is able to enter the cell through the membrane composed of a lipid bilayer; thus, for the plant to survive, it is important that all the tissues are able to respond to the stress rapidly. The evidence presented here indicates that this cytochrome P450 gene, *CYP81A6*, may encode a mono-oxygenase with multiple functions that enable the detoxification of a broad spectrum of xenobiotics. Since bentazon and metsulfuron-methyl are fully synthetic chemical compounds with no natural

homologues, the precise role of CYP81A6 *in situ* has yet to be resolved. However, for rice to invest in its maintenance in the genome, *CYP81A6*, like many cytochrome P450s, must play a role in plant homeostasis. It is thus thought that the constitutive low-level background (RT data of the control) expression patterns, which were also shown to be present in this study, may help the plant to detoxify by-products of metabolism, which will be needed in all tissues at all times. Subcellular localization studies of rice protoplasts expressing CYP81A6CDS-YFP suggested that CYP81A6 was localized to the ER, a finding consistent with the consensus that the majority of activities for xenobiotic catabolism are found in this cell structure (Edwards *et al*., 2011).

In the present study, *CYP81A6* transcription was induced in rice seedlings as early as 2 h post-treatment with bentazon at 500 mg/L. Induction with metsulfuron-methyl, on the other hand, was much slower with transcript levels only becoming detectable 88 h after treatment. Previous high-performance liquid chromatography-mass spectrometry (HPLC-MS) isotopic tracing experiments have shown that intermediary products of bentazon degradation can be identified from cultured rice cell suspensions (Fig. 6a) (Sterling and Balke, 1988). The bentazon degradation pathway is straightforward: after hydroxylation at the benzene ring 6 site, the compound couples with a glucose molecule. This two-stage modification of bentazon makes the molecule more hydrophilic, resulting in its becoming more likely to be transported out of the cell. In comparison, the metsulfuron-methyl degradation pathway is more complex, involving a greater number of chemical modifications, resulting in at least six metabolites, i.e., methyl-*a*-(4-hydroxy-6-methoxypyrimidin-2-yl) carbamoylsulfamoyl-*o*-toluate (M1), methyl-*a*-aminosulfonyl-*o*-toluate (M2), 1H-2,3-benzothiazin-4-(3H)-one-2,2-dioxide (M3), *N*-4,6-dimethoxypyrimidin-2-yl urea (M4), 2-amino-4,6-dimethoxypyrimidine (M5), and 2-amino-4-hydroxy-6-methoxypyrimidine (M6) (Deng and Hatzios, 2003). M1 and M4 are the immediate metabolites of the metsulfuron; M4 is further degraded into M5 and to M6; M2 could be degraded from M1 or directly from metsulfuron, which is further processed into M3 (Deng and Hatzios, 2003).

We propose that it is M3 that induces expression of *CYP81A6*, rather than the complete metsulfuron-methyl compound itself, based on the following reasons: (1) Among the metsulfuron metabolites, only M3 has the molecular structure similar to that of bentazon (Fig. 6b); (2) The time-course of the metabolites' emergence in rice is largely in agreement with *CYP81A6* transcription response to metsulfuron-methyl application. Deng and Hatzios (2003) reported that M1, M2, and M4 appeared to be the major metabolites, without detecting any M3, 24 h after treatment. However, M3 was reported to be one of the major metabolites 3 d after treatment (Omokawa *et al*., 1996). Although we observed a significant increase in *CYP81A6* expression in leaves 88 h post-treatment in the present study (Fig. 3b), there is a 16-h difference between the appearance of M3 metabolite and increase of *CYP81A6* expression. The discrepancy might be attributable to the following factors: the different treatment methods deployed (foliar application in the present study versus addition in culture media in previous studies) and the different rice lines tested (i.e., an indica line Jiazhe B (present work) and a tropical japonica line Lemont in other reports). Further studies are needed to identify the metabolite that induces *CYP81A6* expression in rice.

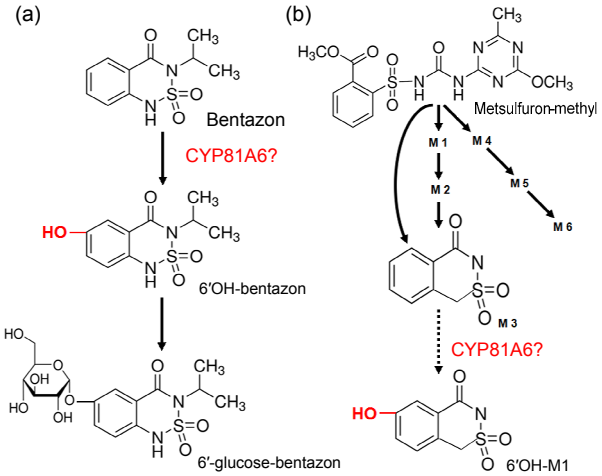

Fig. 6 Schematic diagrams of the degradation pathways of bentazon (a) and metsulfuron-methyl (b), which are drawn based on reports on identification of degraded components using isotope tracer technologies
Bentazon is degraded by a two-step process by hydroxylation at the benzene ring 6 site, followed by coupling to a glucose molecule. In comparison, the metsulfuron-methyl degradation pathway is more complex and many steps are involved. The two steps in which CYP81A6 may play a catalytic role are marked. The molecular structure of M3 is similar to that of bentazon and may act as an inducer for accumulation of CYP81A6 rather than the metsulfuron-methyl itself

5 Conclusions

The present study demonstrates that the CYP81A6 protein is localized in the ER. *CYP81A6* is constitutively expressed in rice plants, with the greatest levels of expression being in the upper leaf surfaces. The expression of *CYP81A6* is very responsive to bentazon treatment, while its reaction to metsulfuron-methyl was not detected until 3 d after application, indicating an intermediate compound degraded from metsulfuron-methyl, rather than itself, is the inducer of *CYP81A6* expression.

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Compliance with ethics guidelines

Hai-ping LU, Martin EDWARDS, Qi-zhao WANG, Hai-jun ZHAO, Hao-wei FU, Jian-zhong HUANG, Angharad GATEHOUSE, and Qing-yao SHU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Ahmad, P., Sarwat, M., Sharma, S., 2008. Reactive oxygen species, antioxidant and signaling in plants. *J. Plant Biol.*, **51**(3):167-173. [doi:10.1007/BF03030694]

Courcelle, J., Khodursky, A., Hanawalt, P.C., 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics*, **158**(1):41-64.

Deng, F., Hatzios, K.K., 2003. Characterization of cytochrome P450-mediated bensulfuron-methyl *O*-demethylation in rice. *Pestic. Biochem. Physiol.*, **74**(2):102-115. [doi:10.1016/S0048-3575(02)00151-7]

Dvorak, Z., Ulrichova, J., Modriansky, M., 2005. Role of microtubules network in *CYP* genes expression. *Curr. Drug Metab.*, **6**(6):545-552. [doi:10.2174/138920005774832623]

Edwards, R., Dixon, D.P., Cummins, I., *et al*., 2011. New perspectives on the metabolism and detoxification of synthetic compounds in plants. *In*: Schröder, P., Collins, C.D. (Eds.), Organic Xenobiotics and Plants, Vol 8. Springer Netherlands, p.125-148. [doi:10.1007/978-90-481-9852-8_7]

Fu, H.W., 2004. Hybrid rice Jiayou 99 with fine quality and super-yield. *J. Anhui Agric. Sci.*, **32**(3):429-432 (in Chinese).

Gianessi, L.P., Reigner, N.P., 2007. The value of herbicides in US crop production. *Weed Technol.*, **21**(2):559-566. [doi:10.1614/WT-06-130.1]

Glover, D.G., Schapaugh, W.T.Jr., 2002. Inheritance of resistance to pendimethalin herbicide induced stem damage in soybean. *Euphytica*, **125(**3):433-437. [doi:10.1023/A:1016078011794]

Green, J.M., 2009. Evolution of glyphosate-resistance crop technology. *Weed Sci.*, **57**(1):108-117. [doi:10.1614/WS-08-030.1]

Han, Y.C., Wang, C.Y., 2002. Physiological basis of bentazon tolerance in rice (*Oryza sativa* L.) lines. *Weed Biol. Manag.*, **2**(4):186-193. [doi:10.1046/j.1445-6664.2002.00067.x]

Hannemann, F., Bichet, A., Ewen, K.M., *et al*., 2007. Cytochrome P450 systems—biological variations of electron transport chains. *Biochim. Biophys. Acta*, **1770**(3):330-344. [doi:10.1016/j.bbagen.2006.07.017]

Hiei, Y., Komari, T., 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.*, **3**(5):824-834. [doi:10.1038/nprot.2008.46]

Konishi, T., Shinohara, K., Yamada, K., *et al*., 1996. Acetyl-CoA carboxylase in higher plants: most plants other than Gramineae have both the prokaryotic and the eukaryotic forms of enzyme. *Plant Cell Physiol.*, **37**(2):117-122. [doi:10.1093/oxfordjournals.pcp.a028920]

Lin, C.Y., Fang, J., Xu, X.L., *et al*., 2008. A built-in strategy for containment of transgenic plants: creation of selectively terminable transgenic rice. *PLoS ONE*, **3**(3):e1818. [doi:10.1371/journal.pone.0001818]

Liu, C., Liu, S.Q., Wang, F., *et al*., 2012. Expression of a rice *CYP81A6* gene confers tolerance to bentazon and sulfonylurea herbicides in both *Arabidopsis* and tobacco. *Plant Cell Tiss. Organ Cult.*, **109**(3):419-428. [doi:10.1007/s11240-011-0106-5]

Liu, M.Y., Chen, W.W., Xu, J.M., *et al*., 2013. The role of *VuMATE1* expression in aluminum-inducible citrate secretion in rice bean (*Vigna umbellata*) roots. *J. Exp. Bot.*, **64**(7):1795-1804. [doi:10.1093/jxb/ert039]

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$. *Methods*, **25**(4):402-408. [doi:10.1006/meth.2001.1262]

Lv, M.Z., Chao, D.Y., Shan, J.X., *et al*., 2012. Rice carotenoid β-ring hydroxylase CYP97A4 is involved in lutein biosynthesis. *Plant Cell Physiol.*, **53**(6):987-1002. [doi:10.1093/pcp/pcs041]

Narusaka, Y., Narusaka, M., Seki, M., *et al*., 2004. Crosstalk in the responses to abiotic and biotic stress in arabidopsis: analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray. *Plant Mol. Biol.*, **55**(3):327-342. [doi:10.1007/s11103-004-0685-1]

Nelson, D.R., 2011. Progress in tracing the evolutionary paths of cytochrome P450. *Biochem. Biophys. Acta*, **1814**(1):14-18. [doi:10.1016/j.bbapap.2010.08.008]

Ohkawa, H., Tsujii, H., Ohkawa, Y., 1999. The use of cytochrome P450 genes to introduce herbicide tolerance in

crops: a review. *Pestic. Sci.*, **55**(9):867-874. [doi:10.1002/(SICI)1096-9063(199909)55:9<867::AID-PS31>3.0.CO;2-S]

Omokawa, H., Wu, J.R., Hatzios, K.K., 1996. Mechanism of safening action of dymuron and its two monomethyl analogues against bensulfuron-methyl injury to rice (*Oryza sativa*). *Pestic. Biochem. Physiol.*, **55**(1):54-63. [doi:10.1006/pest.1996.0035]

Pan, G., Zhang, X.Y., Liu, K.D., *et al*., 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**(6):933-943. [doi:10.1007/s11103-006-0058-z]

Pang, S.S., Guddat, L.W., Duggleby, R.G., 2003. Molecular basis of sulfonylurea herbicide inhibition of acetohydroxyacid synthase. *J. Biol. Chem.*, **278**(9):7639-7644. [doi:10.1074/jbc.M211648200]

Sterling, T.M., Balke, N.E., 1988. Use of soybean (*Glycine max*) and velvetleaf (*Abutilon theopbrasti*) suspension-cultured cells to study bentazon metabolism. *Weed Sci.*, **36**(5):558-568.

Wang, Q.Z., Fu, H.W., Huang, J.Z., *et al*., 2012. Generation and characterization of bentazon susceptible mutants of commercial male sterile lines and evaluation of their utility in hybrid rice production. *Field Crop. Res.*, **137**:12-18. [doi:10.1016/j.fcr.2012.09.001]

Ward, E.R., Uknes, S.J., Williams, S.C., *et al*., 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, **3**(10):1085-1094. [doi:10.1105/tpc.3.10.1085]

Yokosho, K., Yamaji, N., Ma, J.F., 2011. An Al-inducible MATE gene is involved in external detoxification of Al in rice. *Plant J.*, **68**(6):1061-1069. [doi:10.1111/j.1365-313X.2011.04757.x]

Yoo, S.D., Cho, Y.H., Sheen, J., 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.*, **2**(7):1565-1572. [doi:10.1038/nprot.2007.199]

Yuan, J.S., Tranel, P.J., Stewart, C.N.Jr., 2007. Non-target-site herbicide resistance: a family business. *Trends Plant Sci.*, **12**(1):6-13. [doi:10.1016/j.tplants.2006.11.001]

Zhang, J., Xu, Y., Wu, X., *et al*., 2002. A bentazon and sulfonylurea sensitive mutant: breeding, genetics, and potential application in seed production of hybrid rice. *Theor. Appl. Genet.*, **105**(1):16-22. [doi:10.1007/s00122-002-0874-8]

Zhang, L., Lu, Q., Chen, H.G., *et al.*, 2006. Identification of a cytochrome P450 hydroxylase CYP81A6 as the candicate for the bentazon and sulfonylurea herbicide resistance gene, *Bel*, in rice. *Mol. Breed.*, **19**(1):59-68. [doi:10.1007/s11032-006-9044-z]

Zhao, Y.D., 2010. Auxin biosynthesis and its role in plant development. *Annu. Rev. Plant Biol.*, **61**(1):49-64. [doi:10.1146/annurev-arplant-042809-112308]

中文概要

题　目：细胞色素 P450 *CYP81A6* 基因在水稻中的表达：组织特异性、蛋白质亚细胞定位以及对除草剂的响应

目　的：分析 *CYP81A6* 基因在苯达松及甲磺隆处理下的诱导表达模式，解释该基因与两种除草剂代谢相关的可能原因。

创新点：从两种除草剂降解途径中产生的小分子物质的结构相似性出发，通过基因诱导表达的特点分析，解释 CYP81A6 和两种除草剂降解相关的原因。

方　法：通过实时定量聚合酶链反应（PCR）来分析基因表达的特点；利用 *CYP81A6* 启动子与 *GUS* 报告基因构建的载体来分析组织特异性表达；通过亚细胞定位来确定 CYP81A6 发挥功能的场所。

结　论：*CYP81A6* 基因受苯达松及甲磺隆诱导，在不同的时间点开始上调，说明了甲磺隆的降解中间产物可以诱导这个基因的表达；CYP81A6 是组成型表达，在根、茎、叶中均有表达；亚细胞定位结果证明 CYP81A6 是一个内质网上的蛋白。

关键词：CYP81A6；苯达松；甲磺隆；诱导表达；异源物