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Map-based cloning of a novel rice cytochrome P450 gene *CYP81A6* that confers resistance to two different classes of herbicides

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Abstract Development of hybrid rice has greatly contributed to increased yields during the past three decades. Two bentazon-lethal mutants 8077S and Norin8m are being utilized in developing new hybrid rice systems. When the male sterile lines are developed in such a mutant background, the problem of F1 seed contamination by self-seeds from the sterile lines can be solved by spraying bentazon at seedling stage. We first determined the sensitivity of the mutant plants to bentazon. Both mutants showed symptoms to bentazon starting from 100 mg/l, which was about 60-fold, lower than the sensitivity threshold of their wild-type controls. In addition, both mutants were sensitive to sulfonylurea-type herbicides. The locus for the mutant phenotype is *bel* for 8077S and *bsl* for Norin8m. Tests showed that the two loci are allelic to each other. The two genes were cloned by map-based cloning. Interestingly, both mutant alleles had a single-base deletion, which was confirmed by PCR-RFLP. The two loci are renamed bel^a (for *bel*) and bel^b (for *bsl*). The wild-type *Bel* gene encodes a novel cytochrome P450 monooxgenase, named *CYP81A6*. Analysis of the mutant protein sequence also revealed the reason for bel^a being slightly tolerant than bel^b. Introduction of the wild-type *Bel* gene rescued the bentazon- and sulfonylurea-sensitive phenotype of bel^a mutant. On the other hand, expression of antisense *Bel* in W6154S induced a mutant phenotype. Based on these results we conclude that the novel cytochrome P450 monooxygenase *CYP81A6* encoded by *Bel* confers resistance to two different classes of herbicides.

Keywords bel^a · bel^b · *CYP81A6* · Herbicides resistance · Map-based cloning · *Oryza sativa* L.

Introduction

The development of two-line hybrid rice in China in recent years has the potential of increasing rice yield by another 5–10% as compared with the three-line hybrid rice, which has a yield advantage of 10–15% over inbred varieties (Yuan 2001). The use of photo- or thermo-sensitive genic male sterility (P/TGMS) systems in two-line hybrid rice breeding, however, is affected by the sterility instability of P/TGMS lines caused by temperature fluctuation beyond their critical temperatures for fertility conversion. This sterility instability results in a potential problem for seed production of two-line hybrid rice. The female parent, the P/TGMS line, produces seeds from selfing addition to hybrid seeds, causing a great loss to rice producers once these contaminated hybrid seeds are used in rice production (Zhang et al. 2002). Two bentazon-lethal mutants 8077S and Norin8m obtained from γ-radiated *indica* cultivar W6154S and *japonica* cultivar Norin8 by Zhang and Wu (1999) and Mori (1984), respectively,

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have been used to tag P/TGMS lines for solving this hybrid seed contamination problem, which can be achieved by spraying bentazon at seedling stage. Several lines with such a seed purity-maintaining system have been developed and released to farmers in China since last year.

Previous studies showed that the 8077S and Norin8m mutants had recessive inheritance (Mori 1984; Zhang and Wu 1999). 8077S was governed by a single locus *bel*, while Norin8m by *bsl*. Both *bel* and *bsl* were determined to be on the long arm of chromosome 3 (Zhang et al. 2002; Liu and Lu 2004). *bel* and *bsl* are 7.1 cM and 14.1 cM away, respectively from the closest SSLP markers RM168 (Zhang et al. 2002) and RM3856 (Liu and Lu 2004) residing on the centromere side. There are no linked markers on the side proximal to the telomere. In addition, a previous study also revealed that 8077S was sensitive to several members of sulfonylurea-herbicides, such as metsulfuron-methyl (MSM), tribenuron-ethyl (TBE), bensulfuron-methyl (BSM) and pyrazosulfuron-ethyl (PSM) (Zhang et al. 2002).

Bentazon, known as Basagran commercially, is a benzothiadiazinone contact herbicide for controlling many broadleaf weeds and sedges in most gramineous species including rice and many large seeded leguminous crops. This herbicide disrupts photosynthesis of the target plant by blocking electron transfer in photosystem II (McFadden et al. 1990; Leah et al. 1991; Burton and Maness 1992; Forthoffer et al. 2001). Sulfonylurea, on the other hand, is a different class of herbicides in terms of their translocation ability and mode of action although it has a similar herbicide spectrum to bentazon. After absorption by the sensitive plant, the sulfonylurea molecules bind to acetolactate synthase and inhibit the biosynthesis of branched-chain amino acids leucine, isoleucine and valine. The plant dies subsequently due to the deficiency of these three necessary amino acids. The selectivity of both bentazon and sulfonylurea herbicides between crops and weeds is primarily based on the metabolic detoxification by resistant plants and the oxidative reactions mediated by cytochrome P450 monooxygenases (Brown 1990; Koeppe and Brown 1995; Deng and Hatzios 2003).

Cytochrome P450 monooxygenases are NADPH-dependent heme proteins that cast a large and diverse group of isozymes and mediate a wide arrange of oxidative reactions in plants, animals, and microorganisms (Bolwell et al. 1994; Durst and O'Keffe 1995; Schuler 1996; Chapple 1998). Currently, over 1000 P450 sequences are known in plants, but physiological functions have been assigned only to 30 (O'Keefe 2000). Analyses of genome sequences revealed the presence of 328 full-length P450 genes and 99 designated pseudogenes, but the functions for most of them are presently unknown (Nelson et al. 2004). The isolation and functional identification of plant P450 genes have been difficult due to the sequence complexities, a large number of members, and low abundance of transcripts (Bolwell et al. 1994; Durst and O'Keffe 1995; Chapple 1998).

In plants, cytochrome P450s mediate biosynthesis of lignins, terpenoid, and alkaloids, sterols, fatty acids, and many secondary compounds that act as plant defense agents (Schuler 1996; Chapple 1998; Cou and Kutchan 1998). In addition, certain plant P450s are known to mediate detoxification of natural and synthetic xenobiotics such as herbicides. Several plant P450s involved in herbicide metabolism have been isolated and characterized, including the *CYP71A11* and *CYP81B2* from tobacco (Yamada et al. 2000), *CYP71B1* from *Thlaspi arvensae* (Lamb et al. 1998), *CYP71A10* from soybean (Siminszky et al. 1999a), *CYP73A1* (Pierrel et al. 1994), *CYP76B1* (Didierjean et al. 2002), and *CYP81B1* (Cabello-Hurtado et al. 1998) from *Helianthus tuberosus*. All of the described P450s are for detoxifying chlortoluron of phenylurea herbicide, and there has been no report on the plant P450 genes conferring resistance to two different classes of herbicides. We elucidate report here the nature of mutations responsible for the mutants 8077S and Norin8m, and the cloning of a novel cytochrome P450 gene that confers resistance to both bentazon and sulfonylurea-type herbicides in rice.

Materials and methods

Plant materials and growth conditions

Two bentazon-lethal mutants 8077S and Norin8m, and their original controls W6154S and Norin8 were used in this study. A *bel*-tagged P/TGMS line Pei-Ai64Sm (namely PA64Sm thereafter) and an elite commercial restorer line 93–11 with high level of SSR polymorphism were used to produce an F2 mapping population. All the plants were grown at an experimental station of Hubei Academy of Agricultural Science, Wuhan, China.

Bentazon-sensitivity and allelism test of two mutants

The plants of 8077S and Norin8m mutants and their wild-type controls were treated with the following concentrations of bentazon: 0, 50, 100, 200, 300, 600,

1,250 and 5,800 mg/l, each adding 20 g/l commercially available detergent powder as dispersant. Three plants (15–20 tillers per plant) were used for each treatment at maximum tillering stage and scored in 7 days after the treatment. For allelism test, a cross was made between these two mutants. Thirteen F1 and 800 F2 progeny plants, along with their original parents, were treated with 1,250 mg/l bentazon at maximum tillering stage. Their allelism was determined based on the herbicide sensitivity of the progeny plants.

Fine mapping of *bel* and mutation site detection

For fine mapping of *bel*, an F2 population was developed from the cross between the *bel*-tagged PA64Sm and SSLP marker-rich restore line 93–11, and 987 F2 plants were used for the herbicide sensitivity test. Three leaves of each F2 plants were treated with 1250 mg/l bentazon and were scored 7 days after the treatment. Of 987 F2 plants, 231 plants showed sensitive to bentazon and the healthy leaves were collected from the plants for DNA extraction and SSR analysis. The primer pairs used for the SSR analysis included two existing ones: RM416 and RM3867 (http://www.gramene.org), and four self-designed: 3aF (5′-TGTTTTCTTTTTCGCTGTGTG-3′) and 3aR (5′-GCAAGCCTTTTTGCGTATTC-3′), 7aF (5′-GTCAGAGACAGGTCGGAGAG-3′) and 7aR (5′-TCGGTGATCATTGTCATTTG-3′), 8aF (5′-GGTGAGAAGGATCAGCAGGA-3′) and 8aR (5′-AAGAGAATGGAACGACCTTCTTC-3′), 14aF (5′-GTTCGACGTGGTGCAGGT-3′) and 14aR (5′-CCGGCCATGAGACAGTACAT-3′). To identify the mutation site, the candidate genes from both wild-type and mutant genomes were amplified by PCR using Pyrobest polymerase (Takara) and candidate gene-specific primers: P2-1a (5′-GATCGCATCTGCGTTTCAG-3′), P2-1b (5′-GATGAGCCCCGACATGAG-3′), P2-2a (5′-CCTCATGTCGGGGCTCAT-3′), P2-2b (5′-CGCACCAATGAGAGAATTCAG-3′), P2-3a (5′-AAATCTTAGTTCCACCCTCTTGC-3′), and P2-3b (5′-TCGTCCTGGAGATGCAAAC-3′). The PCR products were purified with Fragment Purification Kit (Takara) and sequenced using ABI Prism Model 3700 sequencer in Jikang Co. Ltd., Shanghai, China.

PCR-RFLP analysis

PCR-RFLP analysis was performed based on the procedure described by Toda et al. (2002). Sample DNAs from plants of the F2 mapping plants were pooled with 46–47 plants per unit to reduce the workload. DNAs from the parental plants were used as controls. The primers 5′-TCTCTTGATGCCGTTCGGCCTG-3′ and 5′-CGCCGCCAAAAAATATTCAGACG-3′ were used for confirming the mutation in 8077S and the primers 5′-CCTCATGTCGGGGCTCAT-3′ and 5′-GACGACCTGCTTAAACTCC-3′ for the mutation in Norin8m. Purified PCR products were digested with restriction enzymes that recognized sequences involving the deleted nucleotides.

Construction of sense and antisense *Bel* plasmids

According to the data from the *indica* genomic sequence (Yu et al. 2002), the entire coding sequence of putative *Bel* was 1,542-bp, encoding a P450 protein with 513 amino acids (BGI-RIS Accession Number Scaffold015925). A 4,311 bp genomic DNA fragment with a native *Bam*HI located at 124 bp from 5′-end and a primer-introduced *Bst*EII at the 3′-end, containing the promoter, coding region and terminator of the candidate P450 gene, was amplified from W6154S using LA *Taq* polymerase (Takara). The primers used were 450-2a (5′-CAAACTTCCAACTTTCCCGTCACCTTCACT-3′) and 450-2b (5′-CCGCGGGTCACCGAGCAGAAAGCCCTTCCTCCCAAGTTAGAA-3′). The purified PCR products were digested with *Bam*HI and *Bst*EII and then ligated into pCAMBIA1301 to replace the 35S promoter and the GUS coding sequence to produce pC450-2 plasmid (Fig. 3A). One positive clone containing the correct insert was introduced into *Agrobacterium tumefaciens* stain EHA105 for transformation of 8077S.

For generating the antisense plasmid of the candidate, a fragment spanning the construct of 10 bp before and 491 bp after ATG of *Bel* was released from the insert of the sense plasmid with *Not*I and *Sac*I and cloned into pBluescript KS(+). The resulting construct was used to transform *E. coli* strain DH10B (Invitrogen). A clone confirmed to contain the correct insert was further digested with *Xba*I and *Sac*I and the released fragment was inserted in the antisense direction into a modified binary vector pCAMBIA1301, generating pAC1. After that, a further cloning step was performed to insert a rice actin I promoter (McElory et al. 1990) with *Hind*III and *Xba*I cohesive ends into the pAC1, generating pAANT1 (Fig. 3B). pAANT1 was then introduced into *Agrobacterium tumefaciens* strain EHA105 for transformation of wild-type W6154S.

Rice transformation

Rice transformation was carried out using the procedures as described by Datta et al. (2000) with modifications. Briefly, embryogenic calli were induced from

mature seeds of the mutant 8077S and wild-type W6154S on MS (Murashige and Skoog 1962) medium supplemented with 2 mg/l of 2,4-dichlorophenoxyacetic (2,4-D). The *Agrobacterium* cells were collected and resuspended with AA medium (Toriyama and Hinata 1985) supplemented with 200 μM acetosyringone (called AA-AS, thereafter). The embryogenic calli were incubated in the bacterial suspension for 20 min, and then transferred onto a sterilized filter paper that covered the co-culture medium CC (Portrykus et al. 1979) supplemented with 200 μM acetosyringone. After incubating on the co-culture medium in the dark at 28°C for 55–60 h, calli were transferred onto 1/2 MN medium [containing MS and N6 (Chu et al. 1975) media, each at the half concentration] supplemented with 500 mg/l cefotaxime to allow calli to recover for 3–4 days in the dark at 28°C. Selection of the transformed cells was carried out on MS medium complemented with 2 mg/l 2,4-D plus 500 mg/l cefotaxime and 50 mg/l hygromycin for the first 3–4 cycles and 4–6 μM BSM for subsequent 1–2 cycles. The resistant calli were regenerated and the plantlets were transplanted in Yoshida's culture solution (Yoshida et al. 1976) and used for molecular analysis and herbicide test.

Analysis of transgenic plants

Transgenic plants were screened first by PCR using primers specific for the hygromycin B phosphotransferase gene (*hph*) present in pCAMBIA1301. These primers were H1 (5′-GCTGTTATGCGGCCATTG TC-3′) and H2 (5′-GACGTCT GTCGAGAAGTTTC-3′). For further detection of the sense transgene in the recipient genome of 8077S, two different pairs of primers that can distinguish the transgene from the endogenous mutant allele were used. These two pairs of primers were: B1 (5′-GAAGTTCATGCCGGA-GAG-3′) on transgene and N1 (5′-ATTGCGGGACT CTAATCATA-3′) on the Nos terminator of the vector for amplifying a 1021-bp fragment from the transformed *Bel*, and B1 and G1 (5′-TTGTGTTGTCTGC TT GTCTG-3′) on genomic DNA for amplifying an 865-bp fragment from the endogenous mutant allele. PCR products were sequenced for further confirmation.

Herbicide test

The herbicide test of transgenic plants harboring the sense or antisense construct of *Bel* was performed using leaf-spreading or root-feeding method at seedling stage. Bentazon and BSM were used at 1250 mg/l and 2.6 mg/l, respectively. Non-transformed 8077S and W6154S were used as controls. The symptoms were scored and photographed 7 days following the treatment.

RNA isolation and RT-PCR analysis

Total RNA was extracted with the TRIzol reagent (Invitragen) from various rice organs of wild-type W6154S, which were grown in the field. RT-PCR was performed with one-step RT-PCR kit (Takara). The primers 5′-GAAACACATCACACATTCGT-3′ and 5′-GATGAGCCCCGACATGAG-3′ was used to amplify the cDNA fragment of *Bel*. The PCR primers 5′-TCAGCAACTGGGATGATATGGAG-3′ and 5′-GCCGTTGTGGTGAATGAGTAAC-3′ were used to amplify the *Actin* fragment as a control.

Results

Characterization of mutant phenotypes and allelism test between *bel* and *bsl*

To characterize the mutant phenotypes, plants of 8077S and Norin8m mutants and their wild-type controls were treated with different concentrations of bentazon. The data confirmed that both 8077S and Norin8m plants started to show symptoms to bentazon at 100 mg/l and the lethal concentration was about 300 mg/l (Table 1). Within this range, 8077S plants were slightly more tolerant than Norin8m plants (Table 1 and Fig. 1A). The threshold concentrations of the bentazon sensitivity for the mutant plants were about 60-fold lower than those for their wild-type controls (Table 1). In addition, both 8077S and Norin8m plants were also sensitive to sulfonylurea-type herbicides (data not shown). Although *bel* for 8077S and *bsl* for Norin8m were shown to be located on chromosome 3, it was not clear whether they represented the same or different genes. To address this question, a cross was made between 8077S and Norin8m, and the resulting F1 and F2 progenies as well as their parents were treated with 1,250 mg/l bentazon. The results showed that in contrast to the healthy plants prior to treatment (Fig. 1B), the parents, 13 F1, and 800 F2 progeny plants all died in 7 days following the treatment (Fig. 1C), suggesting that *bel* and *bsl* were allelic.

Fine mapping of *bel*

To gain information on the exact location of *bel*, an F2 mapping population was developed from a cross between *bel*-tagged line PA64Sm and a SSLP marker-rich line, 93–11. Based on herbicide sensitivity test, a total

Table 1 Bentazon-sensitivities of two mutants. The plants of 8077S and Norin8m mutants as well as the wild-type controls were treated with the bentazon at a concentration indicated. Bentazon was applied at maximum tillering stage. Their sensitivity was scored 7 days after the treatment

Line	Application concentration (mg/l)							
	0	50	100	200	300	600	1250	5800
8077S	√	√	X	XX	XXX^+	XXXX	XXXX	XXXX
W6154S	√	√	√	√	√	√	√	X
Norin8m	√	√	X^+	XX^+	XXXX	XXXX	XXXX	XXXX
Norin8	√	√	√	√	√	√	√	X

√, Plants not affected and survive; X, leaves affected and wilted; XX, leaves and sheaths affected and wilted; XXX, leaves, sheaths, and upper parts of stems affected and wilted; XXXX, whole plant wilted; X^+, XX^+, XXX^+ mean the symptoms are slightly severer than those of X, XX, XXX

of 231 recessive susceptible individuals were selected for fine mapping. The detected polymorphic markers 3a, 7a, 8a, 14a in the genomic region from RM416 to RM3867 were used to survey these 231 recessive susceptible individuals. The data confirmed that the *bel* locus was located between the markers RM416-8a-3a and 7a-14a-RM3867 (Fig. 2A). The map distances between *bel* and two closely linked markers of 3a and 7a were 0.1 cM and 0.4 cM, respectively (Fig. 2A). The markers 3a and 7a were found to be on the same BAC clone (GenBank Accession No. AC084282) and the physical distance between the markers is 110 kb (Fig. 2A).

Bel encodes a novel cytochrome P450

Further analysis revealed that this 110-kb sequence contains 18 putative genes (Fig. 2A), including a cluster of four cytochrome P450 genes, namely *CYP81A5*, *CYP81A6*, *CYP81A7* and *CYP81A8* (GenBank Accession Numbers OSJNBb0048A17.17, OSJNBb0048A17.18, OSJNBb0048A17.19 and OSJNBb0048A17.4, individually). Considering that cytochrome P450s are ubiquitous heme proteins and known to play an important role in detoxification of natural and synthetic xenobiotics such as herbicides, we therefore considered those four cytochrome P450 genes as the primary candidates for the wild *Bel* gene.

To address which of the four candidate genes corresponds to the *bel/bsl* locus present in 8077S and Norin8m, we compared the sequences of the four genes based on PCR products amplified from 8077S and Norin8m, and their respective wild-type progenitors W6154S and Norin8. Comparing 8077S to W6154S, it was observed that among the four genes, only *CYP81A6* from 8077S had a single-base deletion of G at the 2058th nucleotide in its coding sequence (Fig. 2B). In order to verify this deletion mutation, we also amplified and sequenced the fragments containing the mutation site from two *bel*-tagged *indica* sterile lines 03B198S and 03B199S, as well as two *indica* normal restorer lines Minghui63 and 93–11. Identical results were obtained. Surprisingly, a sole single-base deletion of C was also detected in the coding region of *CYP81A6* in Norin8m when compared to Norin8 (Fig. 2B). These results suggested that the *CYP81A6* gene might be the *Bel* locus. Since the two mutant loci are allelic to each other, we rename *bel* as bel^a and *bsl* as bel^b.

To exclude the possibility that the single-base deletions detected in bel^a and bel^b might be caused by sequencing errors, a PCR-RFLP analysis was established. There was no restriction recognition site in bel^a and the wild-type alleles. To detect the single base G deletion in the bel^a mutant, PCR-RFLP analysis was carried out by creating a new *Bgl*I recognition site in the fragment amplified from bel^a by adding two-mismatched Cs in the forward primer. The 11 bp of this new *Bgl*I site cover the mutated nucleotide with 4 bp extending over from the mutated nucleotide. The *Bgl*I site would not be present in the fragment amplified from the wild-type W6154S because its corresponding region contained an undeleted G. When subject to *Bgl*I digest, the PCR product amplified from the wild-type W6154S remained intact, while the PCR product from the mutant 8077S was cut into two fragments of 24 bp and 226 bp (Fig. 2C-i). Furthermore, co-segregation of the polymorphism with recessive mutant phenotype was observed when the 231 recessive F2 individuals and the parents were subjected to the same PCR-RFLP analysis (Fig. 2C-ii).

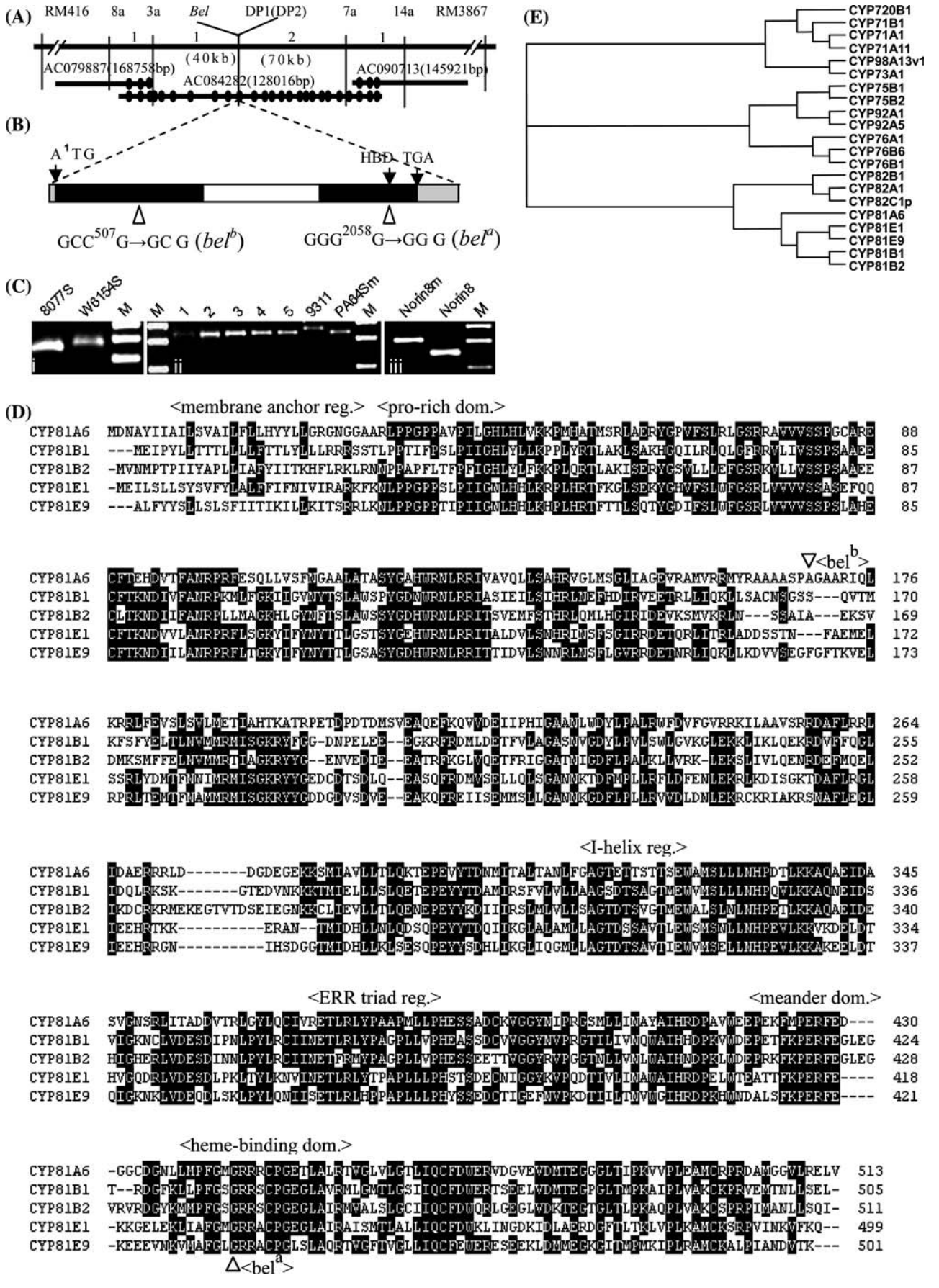
For the bel^b mutant, since the single-base deletion removed a *Nae*I restriction site, PCR-RFLP analysis was performed directly by cutting the PCR products containing the mutated site with *Nae*I. Polymorphism was detected between the fragments amplified from the wild-type Norin8 and the mutant Norin8m. The PCR fragment from Norin8m was uncut after digestion with *Nae*I, while the PCR fragment from Norin8 was cut into two fragments of 50 bp and 151 bp

Fig. 1 Bentazon-sensitivity and allelism test of two mutants. (**A**) Response of mutants 8077S and Norin8m to different concentrations of bentazon. The plants in the front are Norin8m and those in back row are 8077S. Rice plants are individually treated with 300 mg/l (A), 200 mg/l (B), 100 mg/l (C), 50 mg/l (D) of bentazon, and water (E) at the maximum tillering stage. (**B**) Healthy plants of Norin8m (left), F1 hybrid (middle), and 8077S (right) used for allelism test. (**C**) Norin8m (left), 8077S (right), and their F1 hybrid (middle) plants all died after treating with 1250 mg/l bentazon. Only one plant for each type was shown

(Fig. 2C-iii). Taking together, these results strongly demonstrate that a single-base deletion is present in bel^a and bel^b. The polymorphism marker for bel^a allele and its wild counterpart was named DP1 (Fig. 2A), and the marker for the bel^b allele and its wild counterpart was named DP2 (Fig. 2A).

The genomic sequence of the wild-type *CYP81A6* gene was isolated through PCR amplification based on above results. BLAST searches revealed that the deduced amino acid sequence of CYP81A6 was highly similar to that of the previously reported chortoluron hydroxylase CYP81B1 from *Helianthus tuberrosus* (45% identity and 63% similarity) (Cabello-Hurtado et al. 1998) and CYP81B2 from *Nicotiana tabacum* (44% identity and 59% similarity) (Yamada et al. 2000) and isoflavone hydroxylase CYP81E1 from *Glycyrrhiza echinata* (42% identity and 63% similarity) (Akashi et al. 1998) and CYP81E9 from *Medicago truncatula* (42% identity and 63% similarity) (Liu et al. 2003) nearly over the entire region (Fig. 2D). The CYP81A6 sequence also showed similarity to the other hydroxylase proteins (Fig. 2E) such as CYP71A1 (Christoffersen et al. 1995), CYP75B1/B2 (Brugliera et al. 1999; Rupasinghe et al. 2003), CYP76A1/B6 (Batard et al. 1998; Collu et al. 2001), CYP82A1/B1

Fig. 2 Physical map of *bel*, structure of the wild-type *Bel* gene, ▶ PCR-RFLP detection of two mutation sites, and amino acid sequence characterization of CYP81A6. (**A**) High-resolution linkage and physical map of the *bel* gene. The vertical lines represent the molecular makers, and the numbers of recombinant plants are indicated above the linkage map. The physical distances between adjacent markers are shown in parentheses. The bel^a mutant co-segregated with PCR-RFLP maker DP1 and bel^b with DP2. (**B**) Structure of the wild-type *Bel* gene. It consists of two exons (black boxes) and one intron (white box). The positions of 5′- and 3′-UTRs (gray boxes), translation initiation codon (ATG), translation termination codon (TGA), single-base deletions and their exact positions identified in bel^a and bel^b, and highly conserved heme-binding domain (HBD) are indicated. (**C**) PCR-RFLP detection of bel^a and bel^b mutation sites. (i) PCR-RFLP detection of bel^a. M: 100-bp DNA ladder (TOYOBO). The resulting PCR-RFLP marker in this experiment was designated as DP1. (ii) Co-segregation of PCR-RFLP marker DP1 with 231 recessive bel^a plants from an F2 mapping population. Lanes 1–5: each lane represents a DNA sample pooled from 46 to 47 recessive bel^a plants; M: 100-bp DNA ladder. (iii) PCR-RFLP analysis of the mutant Norin8m and its wild-type progenitor. M: 100-bp DNA ladder. The resulted PCR-RFLP maker in this experiment was designated as DP2. (**D**) Sequence comparison of CYP81A6 and the other CYP81 proteins. According to the nomenclature for the P450 superfamily (http://drnelson.utmem.edu/CytochromeP450. html), rice Bel was named CYP81A6. Dashes indicate gaps introduced for maximal alignment. White-on-black letters represents identical amino acids. Triangles indicated the mutation sites. The locations of conserved domains are indicated above or under the sequences. Multiple sequences alignment was performed using the CLUSTAL X analysis tool in DDBJ. (**E**) The phylogenetic relationship between CYP86A6 and related hydroxylase P450 proteins. The analysis was performed using CLUSTAL X and illustrated using Treeview


(A)
RM416
8a
3a
Bel
DP1(DP2)
7a
14a
RM3867
1
1
2
1
(40kb)
(70kb)
AC079887(168758bp)
AC084282(128016bp)
AC090713(145921bp)
(B)
A TG
TGA
GCC507G→GC G (belb)
GGG2058G→GG G (bela)
(C)
8077S
W6154S
M
1
2
3
4
5
9311
PA64Sm
Nonin8m
Nonin8
i
ii
iii
(E)
CYP720B1
CYP71B1
CYP71A1
CYP71A11
CYP98A13v1
CYP73A1
CYP75B1
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CYP81B1 ---MEIPYLLTTTLLLLFTTLYLLLRRRSSTLPPTIPRSLPIIGHLYLLKPPLYRTLAKLSAKHGQILRLQLGERRVLIVSSPSAAEE 85
CYP81B2 -MVNMPTPIIYAPLLIAFYIITKHFLRKLRNMPPAPFLTFPFIGHLYLFKKPLQRTLAKISERYGSVLLLEFGSRKVLLVSSPSAAEE 87
CYP81E1 -MEILSLLSYSVFYLALFFIFNIVIRARKFKNLPPGPPSLPIIGNLHHLKRPLHRTFKGLSEKYGHVFSLWFGSRLVVVVSSASEFQQ 87
CYP81E9 ---ALFYYSLLSLSFIITIKILLKITSRRLKNLPPGPPTIPIIGNLHHLKHPLHRTFTTLSQTYGDIFSLWFGSRLVVVVSSPSLAHE 85
CYP81A6 CFTEHDVTFANRPRPESQLLVSFNGAALATASYGAHWRNLRRIVAVQLLSAHRVGLMSGLIAGEVRAMVRFMYRAAAASPAGAARIQL 176
CYP81B1 CFTKNDIVFANRPKMLFGKIIGVNYTSLAWSPYGDNWRNLRRIASIEILSIHRLNEFHDIRVEETRLLIQKLLSACNSGSS---QVTM 170
CYP81B2 CLTKNDIIFANRPLLMAGKHLGYNFTSLAWSSYGDHWRNLRRITSVEMFSTHRLQMLHGIRIDEVKSMVKRLN---SSAIA---EKSV 169
CYP81E1 CFTKNDVVLANRPRFLSGKYIFYNYTTLGSTSYGEHWRNLRRITALDVLSNHRINSFSGIRRDETQRLITRLADDSSTN---FAEMEL 172
CYP81E9 CFTKNDILLANRPRFLTGKYIFYNYTTLGSASYGDHWRNLRRITTIDVLSNNRLNSFLGVRRDETNRLIQKLLKDVVSEGFGFTKVEL 173
CYP81A6 KRRLFEVSLSVLMETIAHTKATRPETDPDTDMSVEAQEFKQVWDEIIPHIGAANLWDYLPALRWFDVFGVRRKILAAVSRRDAFLRRL 264
CYP81B1 KFSFYELTLNVMMRMISGKRYFGG-DNPELEE--EGKRFRDMLDETFVLAGASNVGDYLPVLSWLGVKGLEKKLIKLQEKRDVFFQGL 255
CYP81B2 DMKSMFFELNVMMFTIAGKRYYG--ENVEDIE--EAIRFKGLVQETFRIGGATNIGDFLPALKLLVRK-LEKSLIVLQENRDEFMQEL 252
CYP81E1 SSRLYDMTFNNIMRMISGKRYYGEDCDTSDLQ--EASQFRDMVSELLQLSGANMKTDFMPLLRFLDFENLEKRLKDISGKTDAFLRGL 258
CYP81E9 RPRLTEMTFNAMMRMISGKRYYGDDGDVSDVE--EAKQFREIISEMMSLLGANNKGDFLPLLRVVDLDNLEKRCKRIAKRSNAFLEGL 259
CYP81A6 IDAERRRLD-------DGDEGEKKSMIAVLLTLQKTEPEVYTDNMITALTANLFGAGTETTSTTSEWAMSLLLNHPDTLKKAQAEIDA 345
CYP81B1 IDQLRKSK-------GTEDVNKKKTMIELLLSLQETEPEYYTDAMIRSFVLVLLAAGSDTSAGTMEWVMSLLLNHPQVLKKAQNEIDS 336
CYP81B2 IKDCRKRMEKEGTVTDSEIEGNKKCLIEVLLTLQENEPEYYKDIIIRSLMLVLLSAGTDTSVGTMEWALSLNLNHPETLKKAQAEIDE 340
CYP81E1 IEEHRTKK----------ERAN--TMIDHLLNLQDSQPEYYTDQIIKGLALAMLLAGTDSSAVTLEWSMSNLLNHPEVLKRVKDELDT 334
CYP81E9 IEEHRRGN----------IHSDGGTMIDHLLKLSESQPEYYSDHLIKGLIQGMLLAGTDTSAVTIEWVMSELLNHPEVLKKAKEELDT 337
CYP81A6 SVGNSRLITADDVTRLGYLQCIVRETLRLYPAAPMLLPHESSADCKVGGYNIPRGSMLLINAYAIHRDPAVWEEPEKFMPERFED--- 430
CYP81B1 VIGKNCLVDESDIPNLPYLRCIINETLRLYPAGPLLVPHEASSDCVWGGYNVPRGTILIVNQWAIHHDPKVWDEPETFKPERFEGLEG 424
CYP81B2 HIGHERLVDESDINNLPYLRCIINETFRMYPAGPLLVPHESSEETTVGGYRVPGGTNLLVNLWAIHNDPKLWDEPRKFKPERFEGLEG 428
CYP81E1 HVGQDRLVDESDLPKLTYLKNVINETLRLYTPAPLLLPHSTSDECNIGGYKVPQDTIVLINAWAIHRDPELWTEATTFKPERFE---- 418
CYP81E9 QIGKNKLVDEQDLSKLPYLQNIISETLRLHPPAPLLLPHYSSEDCTIGEFNVPKDTIILINVWGIHRDPKHWNDALSFKPERFE---- 421
CYP81A6 -GGCDGNLLMPFGMGRRRCPGETLALRTVGLVLGTLIQCFDWERVDGVEVDMTEGGGLTIPKVVPLEAMCRPRDAMGGVLRELV 513
CYP81B1 T--RDGRKLLPFGSGRRSCPGEGLAVRMLGMTLGSIIQCFDWERTSEELVDMTEGPGLTMPKAIPLVAKCKPRVEMTNLLSEL- 505
CYP81B2 VRVRDGYKMMPFGSGRRSCPGEGLAIRMVALSLGCIIQCFDWQRLGEGLVDKTEGTGLTLPKAQPLVAKCSPRFIMANLLSQI- 511
CYP81E1 -KKGELEKLIAFGMGRRACPGEGLAIRAISMTLALLIQCFDWKLINGDKIDLAERDGFTLTKLVPLKAMCKSRPVINKVFKQ-- 499
CYP81E9 -KEEEVNKVMAFGLGRRACPGLSLAQRTVGFTVGLLIQCFEWERESEEKLDMMEGKGITMPMKIPLRAMCKALPIANDVTK--- 501

(Pauli and Kutchan 1998; Whitbred and Schuler 2000), CYP92A1/A5 (Persans et al. 2001; Ralston et al. 2001), CYP98A13v1 (Gang et al. 2002), and CYP720B1 (Ro et al. 2005) and CYP82C1p (Siminszky et al. 1999), suggesting that CYP81A6 is involved in endogenous secondary compound and xenobiotic hydroxylation. The sequencing-verified plasmid was named as pC450-2 (Fig. 3A).

Herbicide test of *Bel*

To confirm that *CYP81A6* corresponds to the *bel* locus, we performed experiments to rescue the mutant phenotype of 8077S with the wild-type *CYP81A6* and also to phenocopy the *bel* phenotype with an antisense *CYP81A6* construct. pC450-2 containing a 4,187-bp fragment (GenBank Accession No. DQ341412) of *CYP81A6* promoter and its coding sequence and pAANT1 containing an antisense *CYP81A6* fragment (Fig. 3B) were introduced into 8077S and W6154S, respectively, via *Agrobacterium tumefaciens*-mediated transformation. PCR analysis of all 42 plants obtained from 7 independent transformants confirmed presence of the *hph* gene in the recipient genome (data not shown). Since an endogenous mutant allele was present in the mutant 8077S, the transformants were further confirmed by PCR using two primers with one being specific to the transgene and the other being specific to the endogenous genomic sequence (Fig. 3C, D), and by DNA sequencing (Fig. 3E, F). For 8077S plants transformed with pC450-2, the transformants were resistant to 1250 mg/l bentazon and 2.6 mg/l BSM, similar to the positive control plant (Fig. 3G, middle; I, third from left), while the non-transgenic control 8077S died (Fig. 3G, left; I, first from left). On the other hand, W6154S transformants with the antisense construct pAANT1 displayed the bentazon- and BSM-sensitive phenotype similar to 8077S (Fig. 3H, middle; I, second from left), for all plants that were *hph* positive as detected by PCR analysis (Fig. 3J). These results clearly show that the novel P450 monooxygenase *CYP81A6* gene represents the rice *Bel* locus and confers resistance to both bentazon and sulfonylurea-type herbicides.

Expression analysis of *CYP81A6*

To confirm the *CYP81A6* is expressed in all organs of rice or not, RT-PCR analysis was performed using the specific primers. The PCR products were amplified from RNAs isolated from various organs (Fig. 4),

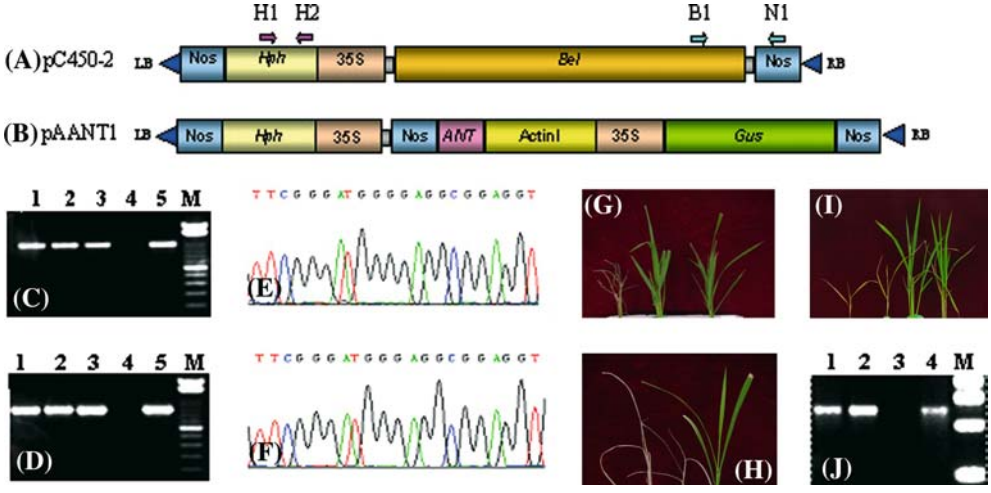

Fig. 3 The schematic diagram of expression vectors, and the molecular analysis and herbicide test of the transgenic plants. (**A**) pC450-2 with sense *Bel* including its coding region, promoter and terminator for complementation test of *bel*[a]. (**B**) pAANT1 containing the antisense *Bel* fragment for down-regulating endogenous *Bel*. (**C**) PCR detection of the exogenous transgene (lanes 1–3, transgenic plants; lane 4, 8077S; lane 5, plasmid pC450-2; M, 100-bp DNA ladder) and (**D**) PCR detection of the endogenous mutant allele (lanes 1–3, transgenic plants; lane 4, plasmid pC450-2; lane 5, 8077S; M, 100-bp DNA ladder) in the plants transformed with pC450-2 using *Bel*-specific primers B1 plus N1 and B1 plus G2, respectively. (**E**, **F**) Sequence confirmation of the PCR products from exogenous transgene (**E**) and from the endogenous mutant allele (**F**). (**G**, **H**) Bentazon test of the transgenic plants derived from sense (**G**) and antisense (**H**) constructs at seedling stage (left: negative control; middle: transgenic plant; right: positive control). (**I**) BSM test of the transgenic plants derived from both antisense (second from left) and sense (third from left) constructs as well as negative (left) and positive (last) control plants at seedling stage. (**J**) PCR analysis of the plants transformed with pAANT1 using *hph*-specific primers (lanes 1–2, transgenic plants; lane 3, W6154; lane 4, plasmid pAANT1; M, molecular marker DL2000). The concentration of bentazon and BSM for herbicide tests was 1250 mg/l and 2.6 mg/l, respectively

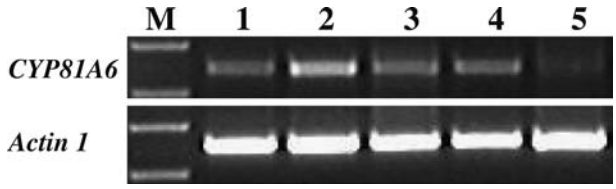

Fig. 4 Expression of *CYP81A6* in various organs of the rice. Total RNAs were isolated from immature panicles (1), leaf sheaths (2), leaf (3), shoots (4) and roots (5). The isolated RNAs were subjected to RT-PCR. *Actin 1* was used as a control. M is the DNA marker

suggesting that *CYP81A6* gene is transcribed in all organs of rice. And the *CYP81A6* gene was highly expressed in shoots, leaf, leaf sheaths and immature panicles, while it is transcribed at low level in the roots (Fig. 4).

Discussion

The present study describes the molecular mapping, cloning, and characterization of two rice bentazon-lethal mutant genes bel^a and bel^b, from 8077S and Norin8m, respectively. Both 8077S and Norin8m started to show symptoms at a concentration of 100 mg/l. We observed that the threshold concentrations of their bentazon-sensitivity were about 60-fold lower than those of their wild-type parents, and also 8077S plants were slightly more tolerant than Norin8m plants (Table 1 and Fig. 1A). Using a line with bel^a to cross with a line with bel^b, followed by herbicide test of their progeny, we determined that these two mutants created independently in the different subspecies were genetically allelic to each other (Fig. 1C). Furthermore, on the base of the following evidences, we conclude that the bentazon- and sulfonylurea-lethal phenotypes of the bel^a and bel^b mutants were due to mutations in the cytochrome gene *CYP81A6*, which likely resulted in defects in the metabolism of these xenobiotics. First, two independent single base deletions were identified in the coding region of *Bel* corresponding to either bel^a or bel^b locus (Fig. 2B). Second, the *Bel* locus encodes a P450 protein that is classified in the CYP81A group, and is highly similar to other subfamily P450 proteins, such as CYP81B1 (Cabello-Hurtado et al. 1998), CYP81B2 (Yamada et al. 2000), CYP81E1 (Akashi et al. 1998), and CYP81E9 (Liu et al. 2003) involved in the xenobiotics and endogenous substrate hydroxylation (Fig. 2D). Third, introduction of the wild-type *CYP81A6* into the bel^a mutant 8077S restored its resistance to both bentazon and sulfonylurea-type herbicides (Fig. 3G, I), whereas introduction of the antisense fragment of *CYP81A6* into the wild-type W6154S made the transformed plants sensitive to these herbicides (Fig. 3H, I).

The first plant P450s analyzed for herbicide metabolism were *CYP73A1* and *CYP81B1*, which were involved in phenolic or lipid pathway (Cabello-Hurtado et al. 1998; Pierrel et al. 1994). However, the enzyme kinetic assays indicate that these P450s do not play a significant role in herbicide detoxification in vivo. It was reported in recently that two plant P450s could metabolize herbicides effectively (Robineau et al. 1998; Siminszky et al. 1999). However, both of them metabolize phenylurea, but not the other classes of the herbicides tested. One gene, *CYP76B1*, was isolated from *Helianthus tuberosus* on the base of its inducibility by Mn^{2+} ions or drugs such as abinopyrine and phenobartital. When expressed in yeast, the *Helianthus CYP76B1* together with an *Arabidopsis* P450 reductase catalyzed detoxification of phenylurea, with a high turnover rate comparable to the rate of physiological substrates (Robineau et al. 1998). The second gene, *CYP71A10*, was isolated from soybean based on sequence conservation of P450 genes (Siminszky et al. 1999). The soybean CYP71A10 protein expressed in yeast converted chlortoluron to ring-methyl hydroxylated, and to a lesser extent, mono-*N*-demethylated compounds. The catalytic turnover rate of phenylurea by CYP71A10 was many times lower than that by CYP76B1 because CYP71A10 had a looser regiospecificity. More recently, three more chlortoluron-metabolism P450s were reported, including *CYP81B2* and *CYP71A11* from *Nicotiana tobacca* (Yamada et al. 2000), and *CYP71B1* from *Thlaspi arvensae* (Lamb et al. 1998). The CYP81B2 and CYP71A11 proteins expressed in yeast could metabolize chlortoluron into a non-toxic compound, but the catalytic efficiency for CYP71A11 was relatively low (Yamada et al. 2000). To date, all these P450 genes reported in different studies have only been shown to metabolize the phenylurea herbicides such as chlortoluron. *CYP81A6* encoded by *Bel* described in the present study however can metabolize two different classes of herbicides including bentazon and sulfonylurea. For the latter class, *CYP81A6* could metabolize four members such as MSM, TBE, BSM and PSM, as demonstrated in an herbicide test (data not shown). Furthermore, the wild-type *Bel* gene could fully rescue the bel^a mutant and confer the resistance to bentazon and sulfonylurea at a level comparable to that of the wild-type W6154S plants. To our knowledge, this is the first plant P450 gene capable to metabolize two different classes of herbicides.

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