

A Novel Rice Cytochrome P450 Gene, *CYP72A31*, Confers Tolerance to Acetolactate Synthase-Inhibiting Herbicides in Rice and Arabidopsis¹[C][W][OPEN]

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Target-site and non-target-site herbicide tolerance are caused by the prevention of herbicide binding to the target enzyme and the reduction to a nonlethal dose of herbicide reaching the target enzyme, respectively. There is little information on the molecular mechanisms involved in non-target-site herbicide tolerance, although it poses the greater threat in the evolution of herbicide-resistant weeds and could potentially be useful for the production of herbicide-tolerant crops because it is often involved in tolerance to multiherbicides. Bispyribac sodium (BS) is an herbicide that inhibits the activity of acetolactate synthase. Rice (*Oryza sativa*) of the *indica* variety show BS tolerance, while *japonica* rice varieties are BS sensitive. Map-based cloning and complementation tests revealed that a novel cytochrome P450 monooxygenase, *CYP72A31*, is involved in BS tolerance. Interestingly, BS tolerance was correlated with *CYP72A31* messenger RNA levels in transgenic plants of rice and Arabidopsis (*Arabidopsis thaliana*). Moreover, Arabidopsis overexpressing *CYP72A31* showed tolerance to bensulfuron-methyl (BSM), which belongs to a different class of acetolactate synthase-inhibiting herbicides, suggesting that *CYP72A31* can metabolize BS and BSM to a compound with reduced phytotoxicity. On the other hand, we showed that the cytochrome P450 monooxygenase *CYP81A6*, which has been reported to confer BSM tolerance, is barely involved, if at all, in BS tolerance, suggesting that the *CYP72A31* enzyme has different herbicide specificities compared with *CYP81A6*. Thus, the *CYP72A31* gene is a potentially useful genetic resource in the fields of weed control, herbicide development, and molecular breeding in a broad range of crop species.

The mechanism of herbicide tolerance can be classified roughly into two groups: target-site and non-target-site herbicide tolerance (Powles and Yu, 2010). Target-site herbicide tolerance is caused by the prevention of herbicide binding to the target enzyme, caused by point mutations occurring in the latter. It is relatively easy to elucidate the molecular mechanisms of target-site herbicide tolerance, because it is regulated mostly by a single gene encoding a target enzyme harboring point

mutations. On the other hand, non-target-site herbicide tolerance is caused by reduction to a nonlethal dose of herbicide reaching the target enzyme, caused by mechanisms such as activation of herbicide detoxification, decrease of herbicide penetration, and herbicide compartmentation in plant cells (Yuan et al., 2007). Among these mechanisms, the oxidation of herbicides by endogenous cytochrome P450 monooxygenase is thought to be a major pathway in plants (Werck-Reichhart et al., 2000; Siminszky, 2006; Powles and Yu, 2010). From the point of view of weed control, non-target-site herbicide tolerance is a greater threat to crop production and in the evolution of herbicide-resistant weeds, because it is often involved in resistance to multiherbicides that inhibit different target proteins, including never-used and potential plant growth regulators (Yuan et al., 2007; Powles and Yu, 2010). Conversely, it is expected that multiherbicide-tolerant crops could be produced easily by the application of non-target-site herbicide tolerance. Moreover, information gained from study of the molecular mechanisms of non-target-site herbicide tolerance can be applied to the research and development of novel herbicides and plant growth regulators.

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Acetolactate synthase (ALS; also known as aceto-hydroxy acid synthase) plays a key role in the biosynthesis of branched-chain amino acids such as Val, Leu, and Ile in many organisms. ALS is the primary target site for at least four classes of herbicides: sulfonyleurea, imidazolinone, pyrimidinyl carboxylates, and triazolopyrimidine herbicides (Shimizu et al., 2002, 2005). These herbicides can inhibit ALS activity, resulting in plant death caused by a deficiency of branched-chain amino acids. ALS-inhibiting herbicides control many weed species in addition to exhibiting high selectivity in major crops and low toxicity to mammals, which lack the branched-chain amino acid biosynthetic pathway. However, various mutations in ALS that confer ALS-inhibiting herbicide tolerance have been found in many weeds (Shimizu et al., 2005; Powles and Yu, 2010). Similar mutations in ALS have also been reported in crops (Shimizu et al., 2005). To date, crops that show tolerance to ALS-inhibiting herbicides have been produced by various approaches, such as conventional mutation breeding, conventional transformation, and pinpoint mutagenesis via gene targeting based on information obtained from analyses of ALS mutants (Shimizu et al., 2005; Endo and Toki, 2013). On the other hand, weeds that show tolerance to ALS-inhibiting herbicides by cytochrome P450-mediated detoxification have also been reported (Powles and Yu, 2010). However, compared with target-site herbicide tolerance, little is known of the molecular mechanism of herbicide metabolism mediated by cytochrome P450. In rice (*Oryza sativa*), an herbicide-sensitive mutant has been produced by γ -ray irradiation (Zhang et al., 2002). This mutant showed 60-fold higher sensitivity to bensulfuron-methyl (BSM), a sulfonyleurea herbicide, compared with wild-type rice (Pan et al., 2006). Genetic mapping and complementation tests revealed that a cytochrome P450, *CYP81A6*, is involved in BSM tolerance (Pan et al., 2006). As far as we know, this is the only example of the isolation and characterization of a cytochrome P450 gene involved in nontarget herbicide tolerance in rice.

Bispyribac sodium (BS), a pyrimidinyl carboxylate herbicide, is effective in controlling many annual and perennial weeds, with excellent selectivity on direct-seeded rice (Shimizu et al., 2002). Recently, it was reported that *japonica* rice varieties show higher sensitivity to BS compared with *indica* rice varieties at the early stages of plant growth (Ohno et al., 2008; Taniguchi et al., 2010). A mutated *ALS* gene confers BS tolerance in plants including rice (Shimizu et al., 2005; Endo and Toki, 2013). However, the deduced amino acid sequences were shown to be highly conserved among *japonica* and *indica* rice varieties, and ALS levels of sensitivity to BS were similar in *japonica* and *indica* rice varieties (Taniguchi et al., 2010). These results suggest the possibility that *indica* rice varieties might show higher tolerance to BS due to the acquisition of nontarget herbicide tolerance.

In this study, we isolated and characterized a novel cytochrome P450 gene, *CYP72A31*, involved in BS tolerance in rice. We also demonstrated that overexpression of *CYP72A31* confers tolerance to ALS-inhibiting herbicides, including BS and BSM, in *Arabidopsis thaliana*.

RESULTS

Cytochrome P450 Is Involved in BS Tolerance in *indica* Rice Varieties

To check whether cytochrome P450 monooxygenase is involved in BS tolerance, we compared BS sensitivity in a *japonica* rice variety, Akebono, and an *indica* rice variety, RD-23, in the presence of 1-aminobenzotriazole (1-ABT), an inhibitor of cytochrome P450 monooxygenase. Growth of RD-23 seedlings treated with BS in combination with 1-ABT was suppressed compared with growth without 1-ABT (Fig. 1A). On the other hand, growth of Akebono seedlings treated with BS was similar both with and without 1-ABT (Fig. 1B), suggesting that cytochrome P450 monooxygenase is involved in BS tolerance in RD-23.

Positional Cloning of the *BS-TOLERANT* Gene

In further analyses, *japonica* elite rice variety Koshihikari and the *indica* model variety Kasalath were used to locate the quantitative trait locus (QTL) for BS tolerance. Composite interval mapping was performed using a population of 183 backcross inbred lines (BILs) derived from a cross of Koshihikari and Kasalath. A QTL with major effect for BS tolerance, named the *BS-TOLERANT* (*BST*)

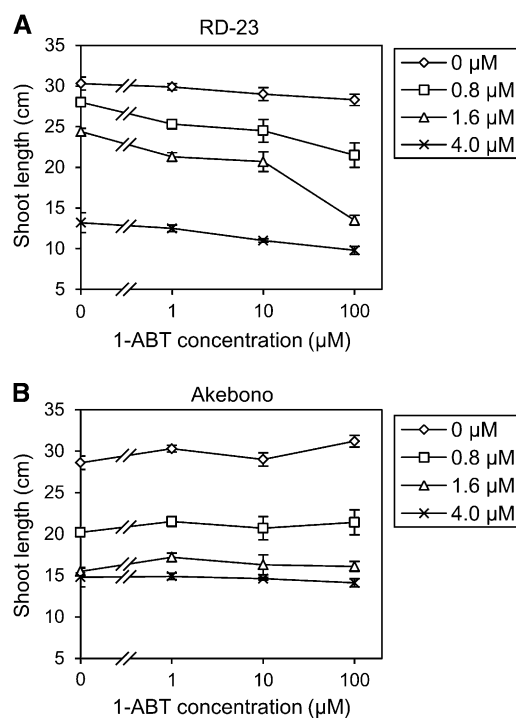


Figure 1. Inhibition of cytochrome P450 monooxygenase enhanced BS sensitivity in RD-23. RD-23 (A) and Akebono (B) seedlings at the stage having a 10- to 15-cm third leaf were grown on medium containing BS and/or 1-ABT for 5 d. Diamonds, squares, triangles, and crosses indicate treatments with 0, 0.8, 1.6, and 4.0 μM BS, respectively. Values are averages \pm SE ($n = 4$ or 5).

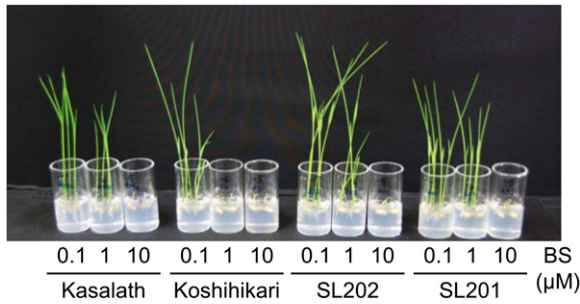


Figure 2. BS sensitivity of *japonica* rice Koshihikari and *indica* rice Kasalath. BS sensitivity was tested among rice varieties Kasalath and Koshihikari as well as SL201 and SL202, which are CSSLs carrying Kasalath chromosome segments in the Koshihikari genetic background. BS was added to the medium at 0.1, 1, and 10 μM , respectively. [See online article for color version of this figure.]

gene, was located to a 12.7-Mb region at the marker interval C178 to C122 on chromosome 1, and the Kasalath allele increased tolerance (Supplemental Table S1). In addition, we checked BS sensitivity in chromosome segment substitution lines (CSSLs) carrying Kasalath segments in the Koshihikari background. SL201 and SL202, which harbor segments of chromosome 1 in Kasalath, showed BS tolerance similar to that of Kasalath (Fig. 2). This result was consistent with the result of composite interval mapping of BS sensitivity using BILs (Supplemental Table S1).

To isolate the *BST* gene, we attempted to delimit a candidate chromosomal region for the QTL detected by using an F₂ population of SL202 \times Koshihikari. BS tolerance was observed in 138 of 190 F₂ plants derived from a cross between SL202 and Koshihikari, which fits a 3:1 ratio ($\chi^2 = 0.34$; $P > 0.05$). This result implies that BS tolerance is a dominant trait. Through genetic

mapping using the progeny of the BC₁F₃ generation derived from a cross between SL202 and Koshihikari and the BC₂F₁ generation derived from a cross between BC₁F₃ line 78 and Koshihikari, the *BST* gene was narrowed down to a 372-kb region between RM9 and 41834-50 (Fig. 3A). Three putative genes encoding cytochrome P450 monooxygenase (Os01g0602200, Os01g0602400, and Os01g0602500) are annotated at this locus of the *japonica* model variety Nipponbare in the Rice Annotation Project Database (<http://rapdb.dna.affrc.go.jp/>; Kawahara et al., 2013; Sakai et al., 2013). On the cytochrome P450 home page (<http://dmnelson.uthsc.edu/CytochromeP450.html>; Nelson et al., 2004), these genes correspond to *CYP72A31*, *CYP72A32*, and *CYP72A33*, respectively (Supplemental Figs. S1 and S2). We compared the putative coding sequences of *CYP72A31*, *CYP72A32*, and *CYP72A33* in Nipponbare and Kasalath. In Kasalath, all genes are thought to be functional based on information from the genomic sequence of chromosome 1 (Kanamori et al., 2013). On the other hand, it was predicted that the *CYP72A31* gene is not functional in Nipponbare due to a 3.4-kb deletion including the region that corresponds to the first and second exons (Fig. 3B), although the *CYP72A32* and *CYP72A33* genes are functional. In Koshihikari, the large deletion seen in Nipponbare is not found in the *CYP72A31* locus, and unlike in Nipponbare, *CYP72A31* mRNA was detected by reverse transcription (RT)-PCR (data not shown). However, sequence analysis of the *CYP72A31* putative coding region revealed a -1 frame-shift mutation in the fourth exon of *CYP72A31*, creating a stop codon 15 nucleotides downstream of this deletion site (Fig. 3B; Supplemental Fig. S3). It is noteworthy that the cytochrome P450 Cys heme-iron ligand signature (Phe-x-x-Gly-x-Arg-x-Cys-x-Gly) is located downstream of this deletion site in the fourth exon (Supplemental Fig. S3). Therefore, *CYP72A31* of Koshihikari lacks this motif

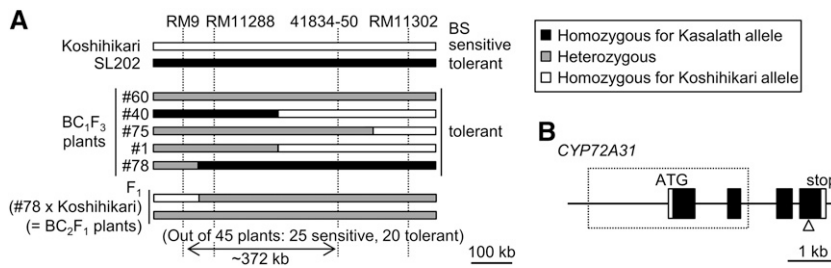


Figure 3. *BST* encodes a novel cytochrome P450 gene, *CYP72A31*, of rice. A, Graphical representation of the genotype of chromosome 1 in BC₁F₃ plants derived from a cross between SL202 and Koshihikari. Black, white, and gray bars indicate the regions homozygous for the Kasalath and Koshihikari alleles and the heterozygous region, respectively. Out of 45 F₁ (line 78 \times Koshihikari) plants, 20 were BS tolerant while 25 were sensitive. As a control experiment, out of 20 plants in which the region at the marker interval RM9 to 41834-50 was heterozygous, seven were BS tolerant while 13 were sensitive. This result was similar to that of line 78 \times Koshihikari. B, Schematic representation of the *BST* gene (Os01g0602200, *CYP72A31*) in Kasalath. White and black boxes show the putative untranslated region and coding region of *CYP72A31*, respectively. The dotted rectangle and white triangle show the 3.4-kb and 1-bp deletions in the *CYP72A31* gene of Nipponbare and Koshihikari, respectively. The disruptions of *CYP72A31* in Nipponbare and Koshihikari are thought to be due to these deletions among many mutations. Bar = 1 kb.

and thus would not play a role as a monooxygenase even if the transcripts were translated. These results indicated that *BST* is likely to correspond to the *CYP72A31* gene.

BS Sensitivity Test in *CYP72A31*-Overexpressing Plants

To confirm whether the *BST* gene is identical to *CYP72A31*, we checked BS sensitivity in transgenic rice of Nipponbare overexpressing *CYP72A31*, *CYP72A32*, or *CYP72A33* complementary DNA (cDNA; Supplemental Fig. S4). As expected, *CYP72A31*-overexpressing calli showed higher tolerance to 0.25 μM BS compared with control calli, while *CYP72A32*- or *CYP72A33*-overexpressing calli showed no obvious tolerance to 0.25 μM BS (Fig. 4A; Supplemental Fig. S5). Interestingly, transgenic callus lines in which *CYP72A31* mRNA accumulated at a relatively lower level showed no significant tolerance to 0.75 μM BS (Supplemental Fig. S6A), suggesting that there is a positive correlation between BS tolerance and *CYP72A31* expression level. To determine the *CYP72A31* expression level sufficient to confer 0.75 μM BS tolerance, we checked BS sensitivity in *CYP72A31*-overexpressing calli of Kasalath. A BS sensitivity test showed that 40- to 100-fold more *CYP72A31* mRNA was required to confer tolerance to 0.75 μM BS compared with control callus of Kasalath (Supplemental Fig. S6B).

Twenty-five lines of regenerated plantlets overexpressing *CYP72A31* of Nipponbare were transferred to medium containing 1 μM BS. The roots of *CYP72A31*-overexpressing seedlings grew vigorously under BS treatment in seven of these lines, although roots of control plants ceased growth in all lines (Supplemental Fig. S7). Surprisingly, we found that *CYP72A31*-overexpressing rice plants of Nipponbare showed over 100-fold higher tolerance to BS compared with nontransformant (Fig. 4B).

To test whether the *CYP72A31* gene confers BS tolerance in dicots, we produced transgenic *Arabidopsis* plants overexpressing *CYP72A31*, *CYP72A32*, and *CYP72A33*. As in rice, *Arabidopsis* seedlings overexpressing *CYP72A31* showed higher BS tolerance, although BS sensitivity in *Arabidopsis* seedlings overexpressing *CYP72A32* or *CYP72A33* was comparable to that of nontransformant (Fig. 5; Supplemental Fig. S8). Line 4-2, in which the *CYP72A31* mRNA level was higher, showed higher tolerance to BS than line 6-5 (Fig. 5). Taking these results together, we concluded that the *CYP72A31* gene is the *BST* gene and that the expression level of the *CYP72A31* gene parallels the level of BS tolerance.

Analysis of *CYP72A31* Gene Expression

In a previous study, the growth of Kasalath callus was similar to that of Nipponbare under BS conditions, although seedlings of Kasalath showed higher BS tolerance compared with Nipponbare (Taniguchi et al., 2010). Since there is a positive relationship between *CYP72A31* mRNA level and BS tolerance (Figs. 4 and 5; Supplemental Fig. S6), it was assumed that the

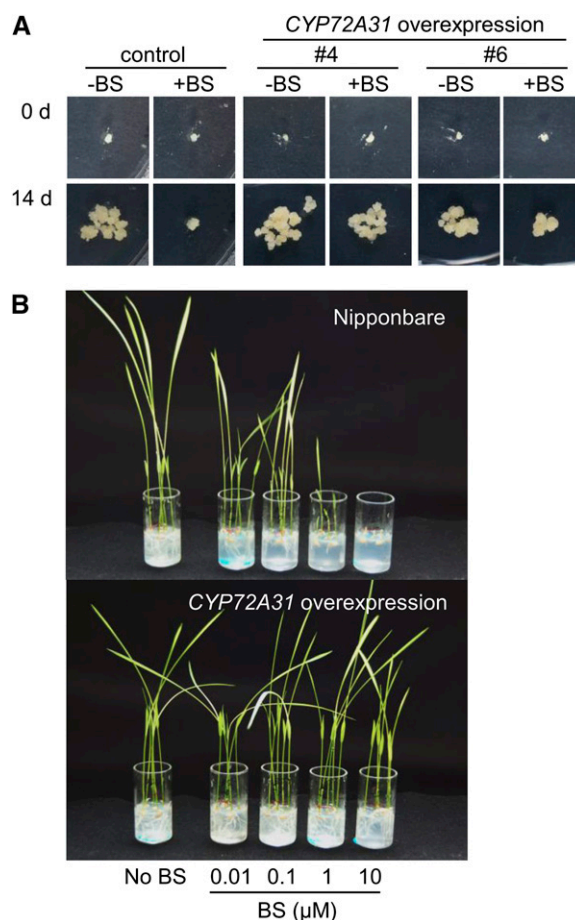


Figure 4. Overexpression of the *CYP72A31* gene confers BS tolerance in Nipponbare. A, BS sensitivity in transformed calli overexpressing the *CYP72A31* gene. Rice calli in the T0 generation were transferred to fresh medium containing 0.25 μM BS and cultivated for 14 d. Transgenic calli overexpressing the *CYP72A31* gene grew vigorously, although control calli transformed with *GFP*-expressing vector ceased growth. B, BS sensitivity in transgenic rice seedlings overexpressing the *CYP72A31* gene. Transgenic seeds were germinated and grown on medium containing 0.01, 0.1, 1, or 10 μM BS. [See online article for color version of this figure.]

difference in BS sensitivity between seedlings and callus of Kasalath is due to expression levels of the *CYP72A31* gene. To confirm this, we determined the mRNA levels of the *CYP72A31* gene in shoots, roots, and calli. As expected, expression levels of the *CYP72A31* gene in shoots and roots of 7-d-old seedlings were much higher than those in primary and secondary calli (Table I), suggesting that BS tolerance levels among organs in Kasalath are caused by differences in *CYP72A31* mRNA amounts.

Comparison of ALS-Inhibiting Herbicide Tolerance by *CYP72A31* and *CYP81A6*

It was reported that *cyp81a6* mutant rice showed high sensitivity to bentazon and sulfonylurea herbicides (e.g. BSM), which have different chemical structures

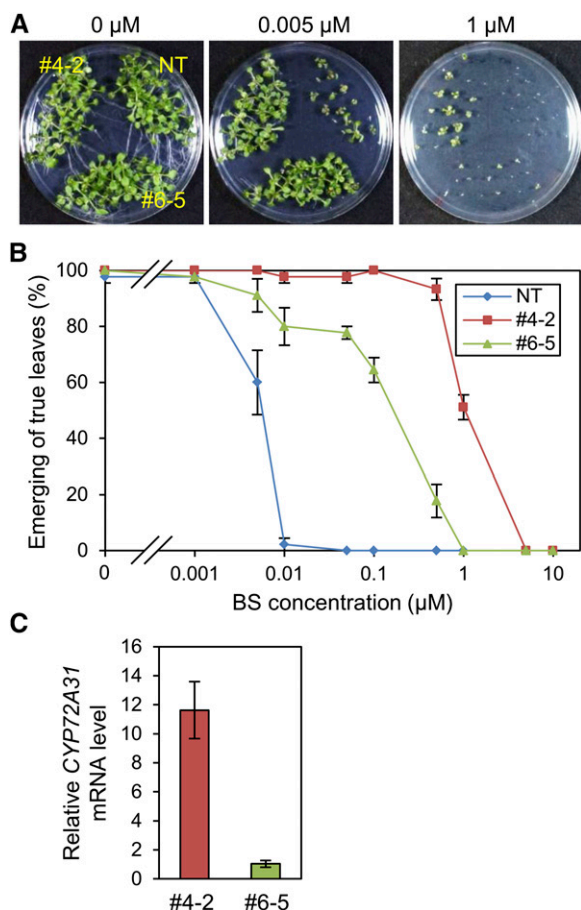


Figure 5. Overexpression of the *CYP72A31* gene confers BS tolerance in Arabidopsis. A and B, BS sensitivity in transgenic Arabidopsis seedlings overexpressing *CYP72A31* (lines 4-2 and 6-5). Transgenic seeds were germinated on medium containing 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 μM BS. After 10 d of cultivation, plants were checked for the emergence of true leaves. Data are means \pm SE of three experiments. NT, Nontransformant. C, mRNA levels of the *CYP72A31* gene in the leaves of lines 4-2 and 6-5. mRNA levels were normalized to the Arabidopsis *elongation factor1 α 4* (*AtEF1 α 4*) mRNA level as a control. The *CYP72A31* mRNA level in line 4-2 was higher than that in line 6-5. [See online article for color version of this figure.]

from pyrimidinyl carboxylate herbicides (e.g. BS; Zhang et al., 2002; Pan et al., 2006; Wang et al., 2012). It was also reported that *japonica* rice varieties Kamenoo-4 and Joshu tended to be more sensitive to BSM than *indica* rice varieties IR-8 and IR-26 (Kobayashi et al., 1995), suggesting the possibility that, in addition to the *CYP81A6* gene, the *CYP72A31* gene is involved in BSM tolerance. To check whether the *CYP72A31* gene confers BSM tolerance, the BSM sensitivity in *CYP72A31*-overexpressing Arabidopsis was checked. As expected, Arabidopsis overexpressing *CYP72A31* (line 31-4-2) showed more than 28-fold higher tolerance to BSM, although Arabidopsis overexpressing *CYP72A32* or *CYP72A33* (line 32-1-1 or 33-1-3, respectively) showed no BSM tolerance, as with BS (Fig. 6). On the other hand, we produced transgenic rice plants in

which *CYP81A6* expression levels are repressed by RNA interference (RNAi; Nipponbare line 5-5) and checked their sensitivity to BS. The mRNA levels of the *CYP81A6* gene in line 5-5 were 10- to 20-fold lower compared with nontransformant (Supplemental Fig. S9). Plants of rice line 5-5 showed high sensitivity to BSM compared with nontransformant (Fig. 7A), similar to a previous report (Lin et al., 2008). These results suggested that the combination of *CYP72A31* and *CYP81A6* genes could confer additive tolerance to BSM.

We further checked whether the *CYP81A6* gene is involved in BS tolerance. The sensitivity test revealed that line 5-5 showed sensitivity to BS similar to that of nontransformant (Fig. 7B). This result shows that, unlike the *CYP72A31* gene, the *CYP81A6* gene is barely involved, if at all, in BS tolerance.

DISCUSSION

The *CYP72A31* Gene Encodes a Novel Cytochrome P450 Monooxygenase and Is Involved in ALS-Inhibiting Herbicide Tolerance

Of the total of 14 *CYP72A* genes in the rice genome, 13 are located on chromosome 1 (Supplemental Fig. S1). Among them, nine *CYP72A* genes (*CYP72A17*–*CYP72A25*) are localized to an approximately 80-kb region of chromosome 1. On the other hand, genes *CYP72A31* to *CYP72A33* are localized to approximately 23- and 28-kb regions of chromosome 1, distinct from a cluster of nine *CYP72A* genes in the Nipponbare and Kasalath genomes, respectively. In Kasalath, the amino acid identities between *CYP72A31* and *CYP72A32*, *CYP72A31* and *CYP72A33*, and *CYP72A32* and *CYP72A33* are 85%, 84%, and 90%, respectively (Supplemental Fig. S2). *CYP72A21* expression is reported to be induced by treatment with various herbicides, including chlorsulfuron, a sulfonylurea herbicide (Hirose et al., 2007). *CYP72A18*

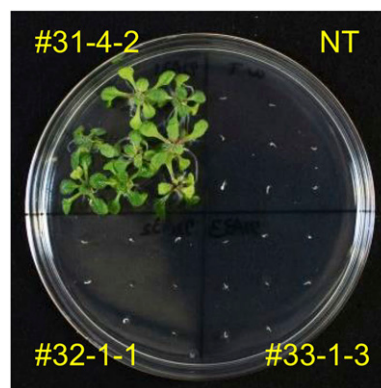


Figure 6. BSM sensitivity upon overexpression of *CYP72A31* in Arabidopsis. The BSM sensitivity in nontransformant (NT) and transgenic Arabidopsis seedlings is shown. Lines 31-4-2, 32-1-1, and 33-1-3 are transgenic Arabidopsis plants overexpressing *CYP72A31*, *CYP72A32*, and *CYP72A33*, respectively. Transgenic seeds were germinated on medium containing 3 nM BSM. [See online article for color version of this figure.]

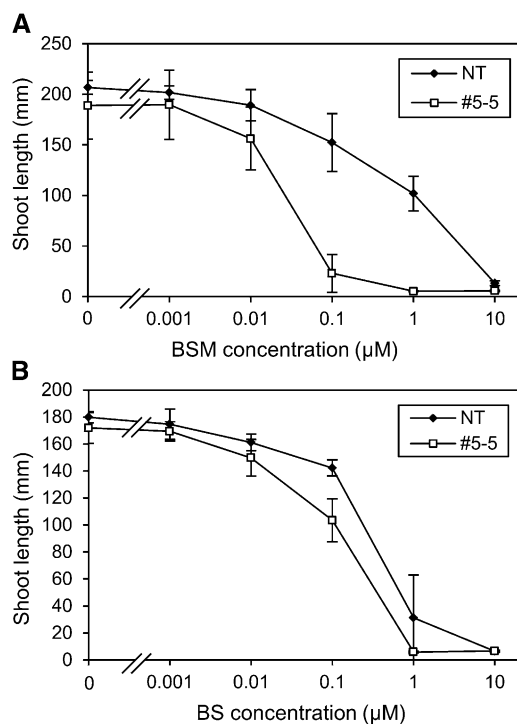


Figure 7. The *CYP81A6* gene is barely involved in BS tolerance. BSM (A) and BS (B) sensitivity in *CYP81A6* knockdown rice seedlings is shown. Rice seeds were germinated and grown on medium containing 0.001, 0.01, 0.1, 1, or 10 μM BSM or BS for 7 d. Black diamonds and white squares indicate nontransformant (NT) and *CYP81A6* knockdown plants (line 5-5), respectively. Values are averages ± SD ($n = 4$).

catalyzes (ω -1)-hydroxylation of the herbicide pelargonic acid (Imaishi and Matumoto, 2007). These previous reports together with our results here suggest that *CYP72A* genes are, indeed, widely involved in xenobiotic responses.

The biological functions of *CYP72A* proteins are only poorly elucidated to date, although they are hugely diverse (Nelson and Werck-Reichhart, 2011). In *Catharanthus roseus*, the *CYP72A1* gene encodes a secologanin synthase that converts loganin into secologanin (Irmler et al., 2000). *CYP72A154* and *CYP72A63* are involved in the glycyrrhizin biosynthesis pathway in licorice (*Glycyrrhiza glabra*) and *Medicago truncatula*, respectively (Seki et al., 2011). Such findings reveal that *CYP72A* proteins work as ring-opening and triterpene-oxidizing enzymes. In rice, expression levels of *CYP72A18*, *CYP72A19*, *CYP72A22*, and *CYP72A23* were reported to be altered by infection with rice blast (Wang et al., 2004), suggesting the possibility that the *CYP72A31* gene might be involved not only in herbicide detoxification but also in secondary metabolism. It will be of great interest to discover which compounds are targets of the *CYP72A31* enzyme and to elucidate its evolutionary significance.

Application of *CYP72A31* and *CYP81A6* Genes in Weed Management and Herbicide Screening

It is important and useful for the development of novel herbicides to analyze the biochemical and enzymatic

properties of *CYP72A31*. Moreover, it is of interest to identify the products of herbicides that are metabolized by *CYP72A31*. In a previous report, *O*-demethylation of BS was detected as one of the major pathways of BS metabolism in rice and wheat (*Triticum aestivum*) seedlings (Matsushita et al., 1994). This metabolite and/or further processing of it might be catalyzed by *CYP72A31*. We showed that the *CYP72A31* gene confers tolerance not only to BS but also to BSM in Arabidopsis (Figs. 5 and 6). This result suggested that the *CYP72A31* and *CYP81A6* genes have redundant functions in BSM detoxification, to a varying degree, although they belong to different families, clades in cytochrome P450 being deeply divergent. It is thought that herbicide detoxification activities are determined by factors such as substrate specificity and amount of enzyme, which are regulated by expression levels and patterns. The elucidation of the molecular mechanism of ALS-inhibiting herbicides involving *CYP72A31* and/or *CYP81A6* will be the subject of future research.

Structural data describing the ALS protein in complex with ALS-inhibiting herbicides have been reported (McCourt et al., 2006). It has also been shown that it is difficult to predict substrate preferences from primary sequences in *CYP72A* proteins (Nelson and Werck-Reichhart, 2011). Thus, resolution of the crystal structure of *CYP72A31* will lead to new insights in the development of novel herbicides with novel crop-weed selectivity. Our results show that overexpression of the *CYP72A31* gene conferred BS tolerance on rice and Arabidopsis, although overexpression of the *CYP72A32* or *CYP72A33* gene did not confer BS tolerance (Figs. 4 and 5; Supplemental Figs. S5 and S8). Moreover, *CYP81A6* knockdown rice plants did not exhibit enhanced BS sensitivity (Fig. 7). On the other hand, we showed that both *CYP81A6* and *CYP72A31* are involved in BSM tolerance (Figs. 6 and 7). Combined with these results, knowledge of the crystal structure and the cytochrome P450 substrate-binding motif among these proteins will offer new information about the specificity to bind BS and BSM, information that can be applied to the molecular evolution of cytochrome P450 for the production of herbicide-tolerant crops.

The *CYP72A31* gene is a useful genetic resource to develop herbicide tolerance in rice. We are currently

Table 1. Expression analysis of *CYP72A31* in Nipponbare and Kasalath

Relative mRNA levels of the *CYP72A31* gene in the shoots and roots of 7-d-old seedlings, 7-d-old primary callus, and 21-d-old secondary callus are shown. All mRNA levels were normalized to the *OsActin1* mRNA level as a control. Data shown are means ± SD of three separate PCR analyses. RT-PCR experiments were performed twice using different RNA samples as templates, with similar results. N.D., Not detected.

Sample	Nipponbare	Kasalath
Shoots	N.D.	0.139 ± 0.017
Roots	N.D.	0.636 ± 0.141
Primary callus	N.D.	0.011 ± 0.001
Secondary callus	N.D.	0.033 ± 0.005

producing a near-isogenic line of Koshihikari harboring a functional *CYP72A31* gene derived from Kasalath. Moreover, we succeeded in using somaclonal mutagenesis to introduce the point mutation W548L into the *ALS* gene to confer tolerance to multiple ALS-inhibiting herbicides in Koshihikari (data not shown). We expect to produce herbicide-tolerant Koshihikari easily by genetic stacking of the mutated *ALS* gene and the functional *CYP72A31* gene, which will confer target-site and non-target-site herbicide tolerance, respectively. Plants with both target-site and non-target-site herbicide tolerance are safer compared with plants exhibiting only one or the other, because excess herbicide that cannot bind the *ALS* protein can be detoxified rapidly in such plants. Moreover, the *CYP81A6* gene is involved in tolerance to two different classes of herbicides, bentazon and sulfonylurea herbicides such as BSM, in rice, Arabidopsis, and tobacco (*Nicotiana tabacum*; Zhang et al., 2002; Pan et al., 2006; Liu et al., 2012). The heterologous expression of *CYP72A31* and/or *CYP81A6* genes by transgenic approaches provides the potential for much greater flexibility of weed management in crops.

Application of the *CYP72A31* Gene to a Broad Array of Technologies

In this study, we demonstrated that the *CYP72A31* gene can confer tolerance to ALS-inhibiting herbicides in rice and Arabidopsis, suggesting that, like *CYP81A6*, the *CYP72A31* gene can be applied to a broad array of technologies as follows.

First, the *CYP72A31* gene can be used as a selectable marker in genetic transformation. A BS-insensitive mutant *ALS* gene has been used widely as a positive marker to obtain transformed cells and plants under BS selection. For example, a combination of a mutated rice *ALS* gene and BS selection has been applied in rice (Osakabe et al., 2005; Okuzaki et al., 2007; Wakasa et al., 2007; Taniguchi et al., 2010), wheat (Ogawa et al., 2008), tall fescue (*Festuca arundinacea*; Sato et al., 2013), Arabidopsis (Kawai et al., 2010), and soybean (*Glycine max*; Tougou et al., 2009). Like the mutated *ALS* gene, the *CYP81A6* gene has also been reported as a selection marker with BSM in Arabidopsis, cotton (*Gossypium hirsutum*), and tobacco plants (Ke et al., 2012; Liu et al., 2012). However, optimization of *CYP72A31* gene expression levels and the herbicide concentration in the selection medium might be necessary to establish a transformation system using the *CYP72A31* gene as a selection marker, since *CYP72A31* mRNA levels are crucial to confer BS tolerance in rice and Arabidopsis (Fig. 5; Supplemental Fig. S6).

Second, the *CYP72A31* gene can be used to ensure high varietal purity in hybrid seed production systems. Male-sterile rice plants in which the *CYP81A6* gene was disrupted with γ -ray irradiation and male-sterile tall fescue plants transformed with a mutated *ALS* gene have been developed (Wang et al., 2012; Sato et al., 2013). No apparent negative phenotypes were observed in these male-sterile rice mutants and their F1 hybrids

with other varieties, and contamination with false hybrid plants caused by self-pollination can be addressed, as these are completely killed off by treatment with bentazon (Wang et al., 2012). It is expected that the difference in BS sensitivity depending on the *CYP72A31* gene between *japonica* and *indica* rice varieties can be more easily applied to the hybrid seed production system in rice than the *CYP81A6* gene. This is because, unlike *CYP81A6*, the *CYP72A31* gene is thought to be non-functional in *japonica* but it is functional in *indica* varieties, since *indica* and *indica*-derived varieties showed higher BS tolerance compared with *japonica* varieties (Ohno et al., 2008; Taniguchi et al., 2010).

Third, knockdown of the *CYP72A31* gene could work as a useful negative selection marker in rice plants. Varieties that show dominant herbicide sensitivity can be applied to so-called "terminator technology" in crops (i.e. gene containment systems to suppress gene flow, including the spread of transgenes into the environment) using techniques such as maternal inheritance, male sterility, and seed sterility (Daniell, 2002). Indeed, transgene containment was achieved successfully by knockdown of the *CYP81A6* gene in rice (Lin et al., 2008).

MATERIALS AND METHODS

Primers

Primers used in this study are listed in Supplemental Table S2.

Plant Materials and Treatments

Rice (*Oryza sativa* varieties Akebono, RD-23, Nipponbare, Koshihikari, and Kasalath) and Arabidopsis (*Arabidopsis thaliana* Columbia-0) were used in this study. BILs and CSSLs originated from a cross between Koshihikari and Kasalath and were provided by the Rice Genome Resource Center (<http://www.rgrc.dna.affrc.go.jp/ineKKBIL182.html> and <http://www.rgrc.dna.affrc.go.jp/ineKCCSSL39.html>; Ma et al., 2002; Ebitani et al., 2005).

For genetic mapping of the *BST* gene, germinated rice seeds were grown on medium composed of 1 μ M BS (Kumiai Chemical Industry), 1.6 g L⁻¹ Hoagland mix (Sigma-Aldrich), and 3 g L⁻¹ gelrite (Wako Pure Chemical Industries) at 27°C for 7 to 9 d. We evaluated BS sensitivity based on the shoot length of these plants. To test BS sensitivity in transgenic rice calli overexpressing the *CYP72A31* gene, clonally propagated calli were transferred to N6D medium (Toki, 1997; Toki et al., 2006) containing BS and grown at 31°C to 33°C for 14 d. To test BS sensitivity in transgenic rice plants overexpressing the *CYP72A31* gene, plantlets were transferred to Murashige and Skoog medium (Toki, 1997; Toki et al., 2006) containing 1 μ M BS and grown at 27°C for 10 d. For the test of BS or BSM sensitivity in *CYP81A6* knockdown rice plants, germinated rice seeds were grown on medium containing various concentrations of BS or BSM at 27°C for 1 week. We evaluated the sensitivity based on the shoot length of these plants. To test BS and BSM sensitivity in transgenic Arabidopsis plants overexpressing the *CYP72A31* gene, T3 seeds were sown on Murashige and Skoog medium containing various concentrations of BS or 3 nM BSM and grown at 22°C for 10 or 18 d, respectively. We evaluated the BS sensitivity based on the emergence of true leaves.

Genetic Mapping of the *BST* Gene

Line SL202, which has a Kasalath segment on chromosome 1 in a Koshihikari background, was crossed with Koshihikari, and the F1 plant was crossed with Koshihikari to obtain BC1F1. This plant was selfed twice to obtain BC1F3 seeds. To narrow down the location of the *BST* gene, we evaluated the BS tolerance of 82 BC1F3 plants. One BC1F3 plant, line 78, was crossed to Koshihikari to obtain BC2F1 seeds, and 45 BC2F1 plants were evaluated for their BS tolerance. The molecular markers used for genotyping are listed in Supplemental Table S2.

Extraction of Total RNA and RT-PCR

For extraction of RNA, primary and secondary calli, shoots, and roots in rice and true leaves in *Arabidopsis* were harvested, immediately frozen in liquid N₂, and stored at -80°C. Total RNA was extracted from samples using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. For RT-PCR for *CYP72A31*, *CYP72A32*, and *CYP72A33* genes, total RNA was used for RT using the oligo(dT)₂₀ primer with ReverTra Ace (Toyobo). Transcript levels of each gene were measured by real-time PCR using an ABI7300 (Life Technologies) and the Power SYBR Green PCR Master Mix (Life Technologies) according to the manufacturer's protocols with the primer sets *CYP72A31* RT-F/*CYP72A31* RT-R, *CYP72A32* RT-F/*CYP72A32* RT-R, *CYP72A33* RT-F/*CYP72A33* RT-R, *OsAct1* RT-F/*OsAct1* RT-R, and *AtEF1αA4* RT-F/*AtEF1αA4* RT-R. A PCR fragment of each gene was used to make standard curves for quantification. For RT-PCR of the *CYP81A6* gene, total RNA treated with DNaseI (DNase RT Grade for Heat Stop; Nippon Gene) was used for RT using the oligo(dT) primer with the PrimeScript RT Reagent Kit (TaKaRa Bio). Transcript levels of each gene were measured by real-time PCR using the Thermal Cycler TP800 (TaKaRa Bio) and SYBR Green I (TaKaRa Bio) according to the manufacturer's protocols with the primer sets *CYP81A6* RT-F/*CYP81A6* RT-R and CA000683_F/CA000683_R (for rice *Actin1*). A PCR fragment of each gene was used to prepare standard curves for quantification.

Vector Construction and Transformation

To construct overexpression vectors, reverse-transcribed cDNAs of *CYP72A31*, *CYP72A32*, and *CYP72A33* genes of Nipponbare and Kasalath were prepared with high-fidelity DNA polymerase KOD -Plus- (Toyobo) using primer sets *CYP72A31* F/*CYP72A31* R, *CYP72A32* F/*CYP72A32* R, and *CYP72A33* F/*CYP72A33* R. The resultant PCR fragments were cloned into the vector pBluescript SK⁻ using *EcoRV*. The sequences of cDNA clones were analyzed with an ABI3130 sequencer (Life Technologies). To construct overexpression vectors for *CYP72A31* and *CYP72A33* genes, the 1.8-kb fragment containing *CYP72A31* and *CYP72A33* cDNAs digested from these vectors using *EcoRI/SalI* was replaced with a GFP::Tnos fragment in the vector pCAMBIA1390-sGFP digested with *EcoRI/SalI*. To construct an overexpression vector for *CYP72A32*, a 1.8-kb fragment containing *CYP72A32* cDNA was amplified by PCR from a cDNA clone using KOD -plus- (Toyobo) with the primer set *CYP72A32* F2/M13 Rv. The resultant PCR fragment digested by *EcoRI/SalI* was replaced with a GFP::Tnos fragment in the vector pCAMBIA1390-sGFP digested with *EcoRI/SalI*. To construct the RNAi vector, a 328-bp fragment containing *CYP81A6* was amplified by PCR from cDNA using KOD FX (Toyobo) with the primer set *CYP81A6* F/*CYP81A6* R. The resultant PCR fragment was cloned into the vector pENTR/D-TOPO using directional TOPO cloning methods (Life Technologies) to yield an entry vector. The RNAi vector was produced with the use of an LR clonase (Life Technologies) that catalyzes the recombination between an entry vector and the pANDA vector (Miki and Shimamoto, 2004).

The binary vectors described above were transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) by electroporation. *A. tumefaciens*-mediated transformation using rice callus derived from mature seeds was performed according to our previous studies (Toki, 1997; Toki et al., 2006). *A. tumefaciens*-mediated transformation in *Arabidopsis* was performed by the inflorescence infiltration method (Bechtold et al., 1993).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers Kasalath *CYP72A31* (AB907219), Kasalath *CYP72A32* (AB907220), Kasalath *CYP72A33* (AB907221), Nipponbare *CYP72A32* (AB907222) and Nipponbare *CYP72A33* (AB907223).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The phylogenetic tree of *CYP72A* protein in rice, maize, and *Arabidopsis*.

Supplemental Figure S2. Alignment of *CYP72A31*, *CYP72A32*, and *CYP72A33* protein in Nipponbare and Kasalath.

Supplemental Figure S3. The alignment of *CYP72A31* in Koshihikari and Kasalath.

Supplemental Figure S4. Structure of *CYP72A31*, *CYP72A32*, and *CYP72A33* overexpression vectors.

Supplemental Figure S5. BS sensitivity of overexpression of *CYP72A32* and *CYP72A33* genes in Nipponbare.

Supplemental Figure S6. Correlation of *CYP72A31* expression levels and BS tolerance in rice callus.

Supplemental Figure S7. Overexpression of *CYP72A31* gene confers BS tolerance in regenerated seedlings of Nipponbare.

Supplemental Figure S8. BS sensitivity of overexpression of *CYP72A32* and *CYP72A33* genes in *Arabidopsis*.

Supplemental Figure S9. *CYP81A6* knockdown rice plants.

Supplemental Table S1. Putative QTL for BS tolerance detected through composite interval mapping using 183 BILs (Koshihikari/Kasalath/Koshihikari).

Supplemental Table S2. Primers used in this study.

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