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Non-target-site herbicide tolerant gene, CYP72A31

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Research area
Ecophysiology and sustainability
A novel rice cytochrome P450 gene, *CYP72A31*, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and Arabidopsis

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A summary of the most important findings and/or advance set out in the article

A novel cytochrome P450 monooxygenase involved in multi-herbicide detoxification that could be useful in the field of herbicide development and molecular breeding in crops.
Footnotes

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Abstract

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 non-lethal 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 multi-herbicides.

Bispyribac sodium (BS) is a herbicide that inhibits the activity of acetolactate synthase (ALS). Rice 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 mRNA levels in transgenic plants of rice and Arabidopsis. Moreover, Arabidopsis overexpressing CYP72A31 showed tolerance to bensulfuron-methyl (BSM), which belongs to a different class of ALS-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 to CYP81A6. Thus, the CYP72A31 gene is a potentially useful genetic resource in the field of weed control, herbicide development, and molecular breeding in broad range of crop species.
Introduction

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 the reduction to a non-lethal 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 oxidization 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 multi-herbicides 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 multi-herbicide tolerant crops could be produced easily by 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.

Acetolactate synthase (ALS, also known as acetohydroxy acid synthase or AHAS) plays a key role in the biosynthesis of branched-chain amino acids such as valine, leucine and isoleucine in many organisms. ALS is the primary target site for at least 4 classes of herbicides, i.e., sulfonylurea, imidazolinone, pyrimidinyl carboxylates and triazolopyrimidine herbicides (Shimizu et al., 2002; Shimizu et al., 2005). These herbicides can inhibit ALS activity, resulting in plant death caused by 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 pin-point 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 to target-site herbicide tolerance, little is known of the molecular mechanism of herbicide metabolism mediated by cytochrome P450. In rice, a herbicide-sensitive mutant has been produced by gamma-ray irradiation (Zhang et al., 2002). This mutant showed 60-fold higher sensitivity to bensulfonyl-methyl (BSM)—a sulfonylurea herbicide—compared to 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 non-target 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 to 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 acquirement of non-target 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.

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 to 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 BST gene

In further analyses, japonica elite rice variety Koshihikari, and an 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) gene, was located to a 12.7-Mb region at the marker interval C178-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) between Koshihikari harboring segments of Kasalath, 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 F$_2$ population of SL202 × Koshihikari. BS tolerance was observed in 138 of 190 F$_2$ 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 BC$_1$F$_3$ generation derived from a cross between SL202 and Koshihikari, and the BC$_2$F$_1$ generation derived from a cross between BC$_1$F$_3$ #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 a japonica model variety Nipponbare in the RAP-DB [http://rapdb.dna.affrc.go.jp/, (Kawahara et al., 2013; Sakai et al., 2013)]. In the cytochrome P450 homepage [http://drnelson.utmem.edu/cytochromeP450.html, (Nelson et al., 2004)], these genes correspond to CYP72A31, CYP72A32 and CYP72A33, respectively (see 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 1st and 2nd 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 RT-PCR (data not shown). However, sequence analysis of the CYP72A31 putative coding region revealed a −1 frame-shift mutation in the 4th 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 cysteine heme-iron ligand signature (Phe-x-x-Gly-x-Arg-x-Cys-x-Gly) is located downstream of this deletion site in the 4th exon (Supplemental Fig. S3). Therefore, CYP72A31 of Koshihikari lacks this motif 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 cDNA (see Supplemental Fig. S4). As expected, CYP72A31-overexpressing calli showed higher tolerance to 0.25 μM BS compared to 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 to 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 7 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 to non-transformant (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 non-transformant (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, growth of Kasalath callus was similar to that of Nipponbare under BS conditions, although seedlings of Kasalath showed higher BS tolerance compared to Nipponbare (Taniguchi et al., 2010). Since there is a positive relationship between CYP72A31 mRNA level and BS tolerance (Figs. 4 and 5, and Supplemental Fig. S6), it was assumed that the 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 callus. As expected, expression levels of CYP72A31 gene in shoots and roots of 7-day-old seedlings were much higher than those in primary and secondary calli (Table 1), 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 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 CYP72A31 gene confers BSM tolerance, the BSM sensitivity in CYP72A31 overexpressing Arabidopsis was checked. As expected, Arabidopsis overexpressing CYP72A31 (#31-4-2) showed >28-fold higher tolerance to BSM, although Arabidopsis overexpressing CYP72A32 or CYP72A33 (#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) (cv. Nipponbare, #5-5) and checked the sensitivity to BS. The mRNA levels of the
CYP81A6 gene in #5-5 were 10- to 20-fold lower compared to non-transformant (Supplemental Fig. S9). Plants of rice line #5-5 showed high sensitivity to BSM compared to non-transformant (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 #5-5 showed sensitivity to BS similar to that of non-transformant (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, 9 CYP72A genes (CYP72A17-25) are localized to an ~80 kb region of chromosome 1. On the other hand, CYP72A31-33 genes are localized to ~23 and ~28 kb regions of chromosome 1 distinct from a cluster of 9 CYP72A genes in the Nipponbare and Kasalath genome, respectively. In Kasalath, the amino acid identities between CYP72A31 and 32, CYP72A31 and 33, and CYP72A32 and 33 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 catalyzes (ω-1)-hydroxylation of the herbicide pelargonic acid (Imaishi and Matumoto, 2007). These previous reports together with our present results suggest that CYP72A genes are indeed widely involved in xenobiotics responses.

The biological functions of CYP72A family 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 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* genes 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 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 has been reported (McCourt et al., 2006). It has also been shown that it is difficult to predict substrate preferences from primary sequence 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 \textit{CYP72A31} gene conferred BS tolerance on rice and Arabidopsis, although overexpression of the \textit{CYP72A32} or \textit{CYP72A33} genes did not confer BS tolerance (Figs. 4 and 5, Supplemental Figs. S5 and S8). Moreover, \textit{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 \textit{CYP72A31} gene is a useful genetic resource to develop herbicide tolerance in rice. We are currently producing a near isogenic line of Koshihikari harboring a functional \textit{CYP72A31} gene derived from Kasalath. Moreover, we succeeded in using somaclonal mutagenesis to introduce the point mutation W548L into the \textit{ALS} gene to confer tolerance to multi \textit{ALS}-inhibiting herbicides in Koshihikari (data not shown). We expect to produce herbicide-tolerant Koshihikari easily by genetic stacking of the mutated \textit{ALS} gene and the functional \textit{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 to 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 \textit{CYP81A6} gene is involved in tolerance to two different classes of herbicides, bentazon and sulfonylurea herbicides such as BSM, in rice, Arabidopsis and tobacco (Zhang et al., 2002; Pan et al., 2006; Liu et al., 2012). The heterologous expression of \textit{CYP72A31} and/or \textit{CYP81A6} genes by transgenic approaches provides the potential for much greater flexibility of weed management in crops.

\textbf{Application of the \textit{CYP72A31} gene to a broad array of technologies}

In this study, we demonstrated that the \textit{CYP72A31} gene can confer tolerance to \textit{ALS}-inhibiting herbicides in rice and Arabidopsis, suggesting that, like \textit{CYP81A6}, the \textit{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 (Sato et al., 2013), Arabidopsis (Kawai et al., 2010) and soybean (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 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, and 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 not to be functional in *japonica* but it is in *indica* varieties, since *indica* and *indica*-derived varieties showed higher BS tolerance compared to *japonica* varieties (Ohno et al., 2008; Taniguchi et al., 2010).

Third, knock-down 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 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 knock-down 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 L., cv. Akebono, RD-23, Nipponbare, Koshihikari and Kasalath) and Arabidopsis (Arabidopsis thaliana var. Col-0) were used in this study. BILs and CSSLs originated from a cross between Koshihikari and Kasalath were provided by the Rice Genome Resource Center [http://www.rgrc.dna.affrc.go.jp/ineKKBIL182.html, http://www.rgrc.dna.affrc.go.jp/ineKKCSSL39.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 μM BS (Kumiai Chemical Industry), 1.6 g/L Hoagland’s mix (Sigma-Aldrich) and 3 g/L gelrite (Wako Pure Chemical Industries) at 27 °C for 7-9 days. 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 31-33 °C for 14 days. To test BS sensitivity in transgenic rice plants overexpressing the CYP72A31 gene, plantlets were transferred to MS medium (Toki, 1997; Toki et al., 2006) containing 1 μM BS and grown at 27 °C for 10 days. 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 \textit{CYP72A31} gene, \(T_3\) seeds were sown on MS medium containing various concentrations of BS or 3 nM BSM and grown at 22 °C for 10 or 18 days, respectively. We evaluated the BS sensitivity based on the emergence of true leaves.

\textit{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 \(F_1\) plant was crossed with Koshihikari to obtain \(BC_1F_1\). This plant was selfed twice to obtain \(BC_1F_3\) seeds. To narrow down the location of the \textit{BST} gene, we evaluated the BS tolerance of 82 \(BC_1F_3\) plants. One \(BC_1F_3\) plant, \#78, was crossed to Koshihikari to obtain \(BC_2F_1\) seeds, and 45 \(BC_2F_1\) plants were evaluated for their BS tolerance. The molecular markers used for genotyping are listed in Supplemental Table S2.

\textit{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 N\(_2\) and stored at -80°C. Total RNA was extracted from samples using an RNeasy Plant mini kit (QIAGEN) according to the manufacturer’s protocol. For RT-PCR for \textit{CYP72A31}, \textit{CYP72A32} and \textit{CYP72A33} genes, total RNA was used for reverse transcription 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 manufacturers’ protocols with the primer sets \textit{CYP72A31} RT-F/\textit{CYP72A31} RT-R, \textit{CYP72A32} RT-F/\textit{CYP72A32} RT-R, \textit{CYP72A33} RT-F/\textit{CYP72A33} RT-R, \textit{OsAct1} RT-F/\textit{OsAct1} RT-R and \textit{AtEF1\textsubscript{α}A4} RT-F/\textit{AtEF1\textsubscript{α}A4} RT-R. A PCR fragment of each gene was used to make standard curves for quantification. For RT-PCR of the \textit{CYP81A6} gene, total RNA treated with DNaseI [Deoxyribonuclease RT Grade for Heat Stop (NIPPON GENE)] was used for reverse transcription using the Oligo dT primer with PrimeScript RT reagent Kit (TaKaRa BIO). Transcript levels of each gene were measured by real-time PCR using a
Thermal Cycler Dice TP800 (TaKaRa BIO) and SYBR Green I (TaKaRa BIO) according to the manufacturers’ protocols with the primer sets CYP81A6 RT-F/CYP81A6 RT-R and CA000683_F/CA000683_R (for rice actin-1). 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 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 in an LR clonase-catalyzed reaction (Life technologies) 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.
were performed according to our previous studies (Toki, 1997; Toki et al., 2006). *Agrobacterium*-mediated transformation in Arabidopsis was performed by the inflorescence infiltration method (Bechtold et al., 1993).

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Figure Legends

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 10-15 cm 3rd leaf were grown on medium containing BS and/or 1-aminobenzotriazole (1-ABT) for 5 days. Diamonds, squares, triangles and crosses indicate treatments with 0, 0.8, 1.6 and 4.0 μM BS, respectively. Values are average±SE (n = 4 or 5).

Figure 2. BS sensitivity of *japonica* rice Koshihikari and *indica* rice Kasalath

BS sensitivity among rice cultivars Kasalath and Koshihikari, and 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.

Figure 3. **BST** encodes a novel cytochrome P450 gene, *CYP72A31* of rice

A. Graphical representation of the genotype of chromosome 1 in BC$_1$F$_3$ plants derived from a cross between SL202 and Koshihikari. Black, white and grey bars indicate the regions homozygous for the Kasalath and Koshihikari alleles, and heterozygous region, respectively. Out of 45 F$_1$ (#78 x 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 - 41834-50 was heterozygous, 7 were BS tolerant, while 13 were sensitive. This result was similar to that of #78 x Koshihikari. B. Schematic representation of the **BST** gene (Os01g0602200, *CYP72A31*) in Kasalath. Black and white boxes show the putative untranslated region and coding region of *CYP72A31*, respectively. Dotted square and white triangles show the 3.4-kb and 1-bp deletions in *CYP72A31* gene of Nipponbare and Koshihikari, respectively. The disruptions of *CYP72A31* gene in Nipponbare and Koshihikari are thought to be due to these deletions among many mutations. Bar = 1 kb.
Figure 4. Overexpression of the CYP72A31 gene confers BS tolerance in Nipponbare.
A. BS sensitivity in transformed calli overexpressing CYP72A31 gene. Rice calli in the T₀ generation were transferred to fresh medium containing 0.25 μM BS and cultivated for 14 days. Transgenic calli overexpressing CYP72A31 gene grew vigorously, although control calli transformed with green fluorescent protein (gfp) expressing vector ceased growth. B. BS sensitivity in transgenic rice seedlings overexpressing CYP72A31 gene. Transgenic seeds were germinated and grown on medium containing 0.01, 0.1, 1, or 10 μM BS.

Figure 5. Overexpression of the CYP72A31 gene confers BS tolerance in Arabidopsis.
A, B. BS sensitivity in transgenic Arabidopsis seedlings overexpressing CYP72A31 gene (#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-day cultivation, plants were checked for emergence of true leaves. Data are mean±SE of three experiments. NT; non-transformant. C. mRNA levels of CYP72A31 gene in the leaves of #4-2 and #6-5. mRNA levels were normalized to the AtEF1aA4 mRNA level as a control. The CYP72A31 mRNA level in #4-2 was higher than that in #6-5.

Figure 6. BSM sensitivity upon overexpression of CYP72A31 in Arabidopsis.
The BSM sensitivity in non-transformant (NT) and transgenic Arabidopsis seedlings. Transgenic seeds were germinated on medium containing 3 nM BSM.

Figure 7. CYP81A6 gene is barely involved in BS tolerance.
BSM (A) and BS (B) sensitivity in CYP81A6 knockdown rice seedlings. Rice seeds were germinated and grown on medium containing 0.001, 0.01, 0.1, 1, or 10 μM BSM or BS for 7 days. Black diamonds and white circles indicate non-transformant (NT) and CYP81A6 knockdown plants (#5-5), respectively. Values are the average±SD (n = 4).
Table 1. Expression analysis of *CYP72A31* gene in Nipponbare and Kasalath

<table>
<thead>
<tr>
<th></th>
<th>Nipponbare</th>
<th>Kasalath</th>
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<tbody>
<tr>
<td>Shoots</td>
<td>N.D.</td>
<td>0.139±0.017</td>
</tr>
<tr>
<td>Roots</td>
<td>N.D.</td>
<td>0.636±0.141</td>
</tr>
<tr>
<td>Primary callus</td>
<td>N.D.</td>
<td>0.011±0.001</td>
</tr>
<tr>
<td>Secondary callus</td>
<td>N.D.</td>
<td>0.033±0.005</td>
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Relative mRNA levels of the *CYP72A31* gene in the shoots and roots of 7-day-old seedlings, 7-day-old primary callus and 21-day-old secondary callus. All mRNA levels were normalized to the *OsActin1* mRNA level as a control. Data show mean±SD of three separate PCR analyses. RT-PCR experiments were performed twice using different RNA samples as template with similar results.
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