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Rice *bending lamina 2* (*bla2*) Mutants Are Defective in a Cytochrome P450 (CYP734A6) Gene Predicted to Mediate Brassinosteroid Catabolism

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Desirable morphological traits in rice plants, such as shorter stalks and erect leaves, are being pursued in breeding programs. Rice brassinosteroid (BR)-deficient mutants display the phenotype of reduced heights and erect lamina. Therefore, biotechnology can be used for controlled deactivation of bioactive BRs in specific tissues. Here, we isolated a gene encoding for a BR-deactivating enzyme. Based on its sequence identity with a known enzyme, *Arabidopsis* Cytochrome P450 734A1, we identified a rice homolog named OsCYP734A6 (LOC_Os01g29150). A search of the rice T-DNA mutant population for insertional mutants of this gene revealed two alleles. These T-DNA loss-of-function mutants displayed a strongly bending lamina phenotype similar to the morphology of rice plants treated with BRs. These two mutants were named *bending lamina* (*bla*)*2-1* and *bla2-2*. In *Arabidopsis*, the transcript level of a putative BR biosynthetic gene, *OsDWARF4*, is feed-back down-regulated in response to exogenous application of BRs. Similarly, a steady-state level of *OsDWARF4* transcripts was significantly down-regulated here in the *bla2* mutant background. Based on their sequence similarity, the bending-lamina phenotype in the *bla2* mutant, and down-regulation of the BR biosynthetic gene *OsDWARF4*, we therefore propose that OsCYP734A6 (BLA2) is likely to be involved in BR deactivation.

Keywords: brassinolide, Cytochrome P450, erect leaves, rice T-DNA mutant, short stature

Brassinosteroids (BRs) collectively refer to a group of plant-originated steroidal compounds. BRs are engaged in many developmental aspects, such as stimulation of cell division and elongation, conference of stress tolerance, vascular system differentiation, leaf development, and photo-/skoto-morphogenesis. When *Arabidopsis* plants are defective in BR biosynthesis or their signal transduction pathways, they exhibit characteristic growth-deficient phenotypes, such as short stature, round and curled leaves, short petioles and pedicles, and reduced fertility. In rice, disrupted BR biosynthesis or signaling pathways leads to mutants with shorter stems, reduced second internodes, and erect leaves (Hong et al., 2003; Sakamoto et al., 2006). Analyses of these rice BR mutants are filling the gaps that have arisen from research on *Arabidopsis* alone. Although numerous BR-biosynthetic or -signaling mutants can be found in rice, none of the genes engaged in the BR deactivation process have yet been reported.

Cytochrome P450 proteins refer to enzymes that form superfamilies across the biological kingdoms of bacteria, animals, and plants. They mediate various types of metabolic reactions, e.g., substrate hydroxylation, which uses one of the two oxygen atoms in molecular dioxygen (Werck-Reichhart and Feyereisen, 2000). Nelson et al. (2004) have performed comparative genomic analyses with the P450 sequences of *Arabidopsis* and rice, and have reported that the genome of the former possesses 246 genes and 26 pseudogenes, whereas the latter has 356 genes and 99 pseudogenes. Based on their phylogenetic analyses, they have found that all of these P450s are clustered into 10 clans: 71, 72, 85, 86, 51, 74, 97, 710, 711, and 727. Phylogenetic clustering of the functional P450s also has shown that these clans can be divided into two clades: the A-type and the non-A type (Paquette et al., 2000). The A-type P450s are important in the biosynthesis of plant-specific metabolites, while the non-A-type carries out the reactions conserved across kingdoms, including sterol and brassinosteroid biosynthesis, plus catabolism.

Of the 10 clans, all the BR-biosynthetic enzymes belong to Clan 85 (Kwon et al., 2005), whereas those involved in BR deactivation are grouped into Clan 72. That latter clan is subdivided into seven CYP families: CYP72, 709, 714, 715, 721, 734, and 735. Of these, only CYP734A1 from *Arabidopsis* has been extensively studied (reviewed in Choe, 2006). A functional clue to the CYP73A1 protein has been manifested in the analysis of *Arabidopsis* T-DNA activation-tagged mutant *phyb-4 activation-tagged suppressor1* (BAS1, At2g26710). Examination of the endogenous levels of BRs in the *bas1-D* mutant have revealed that the level of both castasterone (CS) and brassinolide (BL) is significantly reduced relative to the wild-type plants. Heterologous expression of this gene in yeast, followed by feeding experiments with CS and BL, has shown that BAS1 (CYP734A1) converts CS and BL to C-26 hydroxylated compounds. Further experiments have confirmed that the C-26 hydroxylated BRs are indeed not bioactive (Turk et al., 2003). Ohnishi et al. (2006) also have reported that the tomato version of BAS1, which is assigned as CYP734A7, is involved in BR deactivation based on results obtained from studies of

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transgenic tobacco plants that over-express the CYP734A7 gene.

Furthermore, activation tagging mutagenesis of *Arabidopsis* has revealed another dwarf mutant that arises from elevated activity in BR deactivation. Three research groups -- Nakamura et al. (2005), Takahashi et al. (2005), and Turk et al. (2005) - have independently isolated dwarf mutants, respectively naming them *chibi2* (*chi2*), *shrinked1-D* (*shk1-D*), and *suppressor of phyb-4 7-D* (*sob7*). These dwarf mutants have been discovered via activation tagging of a Cytochrome P450 gene (CYP72C1, At1g17060). Based on the sequence similarity of CYP72C1 with CYP734A1, and the dwarf phenotypes caused by overexpression of the *CYP72C1* gene, the gene product has been predicted to mediate BR-deactivating steps. Further biochemical analysis, such as heterologous expression of the gene in bacteria or yeast, followed by metabolism tests, would reveal a precise function for this CYP72C1 enzyme. Here, we isolated a rice gene involved in BR deactivation. After searching the rice amino acid sequence database, we identified a Cytochrome P450 gene that shares significantly high identity, at the sequence level, with *Arabidopsis* CYP734A1. Furthermore, we isolated rice mutants corresponding to this gene, and found that these lines display phenotypes similar to plants treated with BRs.

MATERIALS AND METHODS

In Silico Cloning of the OsCYP734A6 Gene and Sequence Analysis

To obtain a homolog of the *Arabidopsis* CYP734A1 protein, we searched The Institute of Genome Research (TIGR) pseudomolecules database, using the *Arabidopsis* CYP734A1 amino acid sequence as a probe. Of the sequences that showed significant identity, the deduced amino acid sequence from LOC_Os01g29150 had the top score. Cytochrome P450 amino acid sequences are systematically assigned an official number via standard naming convention. Thus, we first checked if this CYP number was already assigned for the LOC_Os01g29150 sequence, using the BLASTX utility at the Cytochrome P450 homepage (http://drnelson.utmem.edu/CytochromeP450.html), and found that LOC_Os01g29150 is the same as CYP734A6. This identical procedure was used to name the other five P450 sequences analyzed in the study presented here. To better understand the conserved residues among this group of proteins, we performed multiple sequence alignment with CLUSTAL W (Ver 1.83) software. Based on those aligned multiple sequences, a phylogenetic tree was constructed using the Neighbor-joining algorithm and a Gonnett protein weight matrix. Default parameters for alignments were utilized if not otherwise denoted. The tree was then illustrated via NJ Plot software (Perriere and Gouy, 1996). Bootstrap values of 1000 iterations were marked when new branches were formed. *Arabidopsis* CYP734A1 (At2g26710) and CYP72C1 (At1g17060) were archived from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), and were included to ascertain their relationship with functionally described proteins. Similarly, CYP90B1 (DWF4, At3g50660), which is involved in BR biosynthesis, was included as an outgroup.

Plant Materials and Growth Conditions

Our *bla2* mutant was obtained from a rice (*Oryza sativa*) T-DNA (pGA2715) mutant population that had been regenerated via callus culture derived from a japonica variety, 'Dongjin' (Jeong et al., 2002). The rice T-DNA flanking sequence database was searched for any insertional mutant in the locus LOC_Os01g29150 (http://www.postech.ac.kr/life/pfg/). Two lines, 1B-24336 and 1B-02440, were shown to have insertions in the coding sequence of this locus. Mixed seeds of the T3 generation were sown in soil and grown in a greenhouse from May to October 2006.

Genomic DNA Isolation for PCR

Tissue samples from the flag leaves were pulverized with a micropestle, under liquid nitrogen, before being thawed and homogenized in 1.5 mL microcentrifuge tubes. DNA was prepared via the modified hexadecyltrimethylammonium bromide (CTAB) method (Hong et al., 2005). Briefly, the ground tissues were suspended in 750 μL of warm (65°C) CTAB buffer (2% [w/v] CTAB, 1.42 mM NaCl, 20 mM EDTA, 100 mM tris-HCl [pH 8.0], 2% [w/v] polyvinylpyrrolidone-40, and 5.0 mM ascorbic acid). After the addition of 7 μL of RNase A (20 mg/mL), the samples were incubated at 65°C for 5 min to remove the RNA. Nucleic acids were extracted with 0.7 volumes of chloroform, and the two phases were separated by centrifuging for 10 min at 15,000 rpm in a tabletop microcentrifuge. The upper aqueous phase was transferred to new tubes, and the DNA was precipitated by adding 0.7 volume of isopropanol, mixing, and centrifuging at 12,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, dried, and re-suspended in 50 μL of TE buffer (10 mM Tris [pH 8.0] and 1mM EDTA). Oligonucleotide sequences for genotyping included, from the 5′ to 3′ end: tagctgtgaaggtattggag (GT1-F), cgcaaattcgcaagcaaaca (GT1-R), gatcaccttgcattgacatc (GT2-F), cgaggaccacatctatacat (GT2-R), and aacgctgatcaattccacag (RB).

Total RNA Isolation and RT-PCR

Fresh tissues of rice were ground to a fine powder in liquid nitrogen and transferred to new microcentrifuge tubes containing TRIzol reagent (Invitrogen, USA). Afterward, 200 μL of a 24:1 chloroform:isoamylalcohol mixture was added and mixed well by vortexing. The extract was centrifuged for 20 min at 13,000 rpm and the supernatant was transferred to new tubes. An equal volume of isopropanol was added and incubated for 10 min at -80°C. The RNA mixture was centrifuged for 20 min at 13,000 rpm, washed with 70% ice-cold ethanol, and centrifuged for an additional 5 min at 10,000 rpm. The pellet was then re-suspended with DEPC-treated water. In all, 2 μg of total RNA was subjected to synthesis of complementary DNA using Superscript Reverse Transcriptase and the oligo-dT_{12-18} primer in a 20 μL reaction mixture (Promega, USA). The oligonucleotide sequences for RT-PCR analysis included: 5′-ctcgccatcttcttcctga-3′ (OsDWF4-RTF) and 5′-atgaaccctaatgggaaggc-3′ (OsDWF4-RTR), 5′-ctacatacaactccatcatgaagtg-3′ (ACT-F), and 5′-cctcatcaggcatctgattaaa-3′ (ACT-R). The thermocycling pro-

gram for RT-PCR was initial denaturation at 95°C for 5 min; 25 cycles of amplification at 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and a final gap-filling for 10 min at 72°C.

Lamina Joint Inclination Assay

Our lamina joint inclination assay was done as described previously (Han et al., 1997). Rice seedlings were etiolated by growing them in a dark chamber at 26°C for 8 d, after which those at a uniform developmental stage were selected. Sections encompassing the upper and lower 1 cm from the second leaf lamina joint were excised and floated on distilled water in a dark chamber at 28°C for 24 h to exhaust any endogenous hormones. Afterward, uniformly inclined segments (at approximately 45°) were selected. Ten samples were incubated in 6-well plates containing 5 mL of *epi*-brassinolide (Sigma, USA, catalog #E1641) at various concentrations (10^{-9}, 5×10^{-9}, 10^{-8}, or 10^{-7} M). These were incubated in a dark chamber at 28°C for 48 h before their inclined angles of lamina joint were measured.

MPSS Data Search

Genes with multiple copies in an organism are tending to have differential expression patterns according to space, time, and treatment. To examine this, we searched the Massively Parallel Signature Sequencing (MPSS) database (http://mpss.udel.edu/rice/). MPSS databases publicly deposit results of the specific gene expression analysis. When queries as the code for a specific gene are made in several different ways, the databases return the abundance data for the specific conditions. To measure the general expressivity, we obtained the results of OsCYP734s.

RESULTS AND DISCUSSION

Putative BR Deactivating P450s Are Identified through Database Search

Arabidopsis CYP734A1 hydroxylates the C-26 position of BRs to deactivate them (Turk et al., 2003). The roles played by BR-deactivating enzymes are important to maintaining the homeostasis of plant BRs. To isolate a homolog, we searched the rice pseudomolecules database, using the CYP734A1 amino acid sequence as a probe. The top six protein sequences with the highest degrees of similarity were selected for further analysis. Not surprisingly, they belonged to the Cytochrome P450 proteins. To assign names for them, we searched the "Cytochrome P450 Homepage" (http://drnelson.utmem.edu/CytochromeP450.html), and found, in order of their identity to CYP734A1, the following: OsCYP734A6 (LOC_Os01g29150), OsCYP734A5 (LOC_Os07g45290), OsCYP734A4 (LOC_Os06g39880), OsCYP734A2 (LOC_Os02g11020), OsCYP72A18 (LOC_Os01g43710), and OsCYP72A35 (LOC_Os01g52790). To investigate the phylogenetic relationships among these proteins, we constructed a tree after including the following *Arabidopsis* P450 protein sequences: AtDWARF4 (AtDWF4, CYP90B1), AtBAS1 (CYP734A1), and AtSOB7 (CYP72C1) (Fig. 1). This tree clearly showed that the biosynthetic enzyme AtDWF4 was separate from the rest of the proteins,

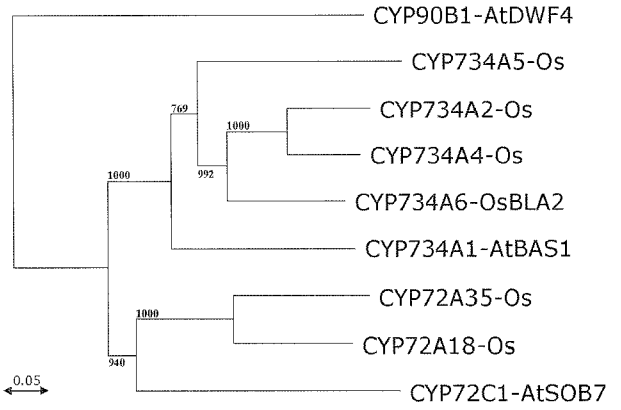


Figure 1. Phylogenetic tree of selected Cytochrome P450 protein sequences, which was visualized using NJ Plot software. Bootstrap values of 1000 iterations are marked when new branches are formed. *Arabidopsis* CYP734A1 and CYP72C1 are included to better understand relationship with functionally described proteins; CYP90B1 (DWF4), known to be involved in BR biosynthesis, is included as outgroup. OsCYP734A6 is clustered with *Arabidopsis* CYP734A1 with high confidence value (bootstrap value, 1000/1000 iterations).

suggesting that such enzymes possess divergent sequences from the biosynthetic enzyme (Fig. 1). The rice CYP734 proteins were all clustered with *Arabidopsis* CYP734A1, while the two proteins belonging to CYP72 were grouped with *Arabidopsis* CYP72C1, which has been shown to mediate BR deactivation. Boot strap numbers of >700 out of 1000 iterations might suggest that the representation of this clustering was fairly robust. Scores for the degree of amino acid sequence identity and similarity of CYP73A6 with CYP734A1 were 62% and 79%, respectively. Based on these phylogenetic tree analyses and sequence similarity tests, it is likely that the functioning of rice CYP734A6 is similar to that of *Arabidopsis* CYP734A1.

To evaluate whether a specific group of CYP proteins shared sequence similarity in the substrate recognition sequences (SRS), we performed multiple sequence alignment (Fig. 2). It is significant that the three sequences belonging to the same CYP72 group shared similarity in the SRS region, just as the CYP734 groups did. In contrast, functionally different P450s, such as CYP90B1, had barely any similarity to CYP72C and CYP734 in the SRS region. The conserved amino acid sequences in that SRS region among the CYP734s suggest that their substrates might have been the same as CS and BL, as was reported previously (Turk et al., 2003). We also noted that these P450 proteins had an oxygen binding domain, implying that hypoxic conditions, such as when rice plants are submerged by flooding, may regulate enzyme activity or the status of transcript levels.

Two Independent T-DNA Mutants Are Identified for the OsCYP734A6 Gene

To determine the functioning of OsCYP734A6, we searched the rice T-DNA mutant database for any T-DNA insertional mutants and found that two T-DNA lines, 1B-24336 and 1B-02440, possess T-DNA insertions in their first intron and fourth exon, respectively (Fig. 3). To better represent the mutant allele of these genes, we named these two

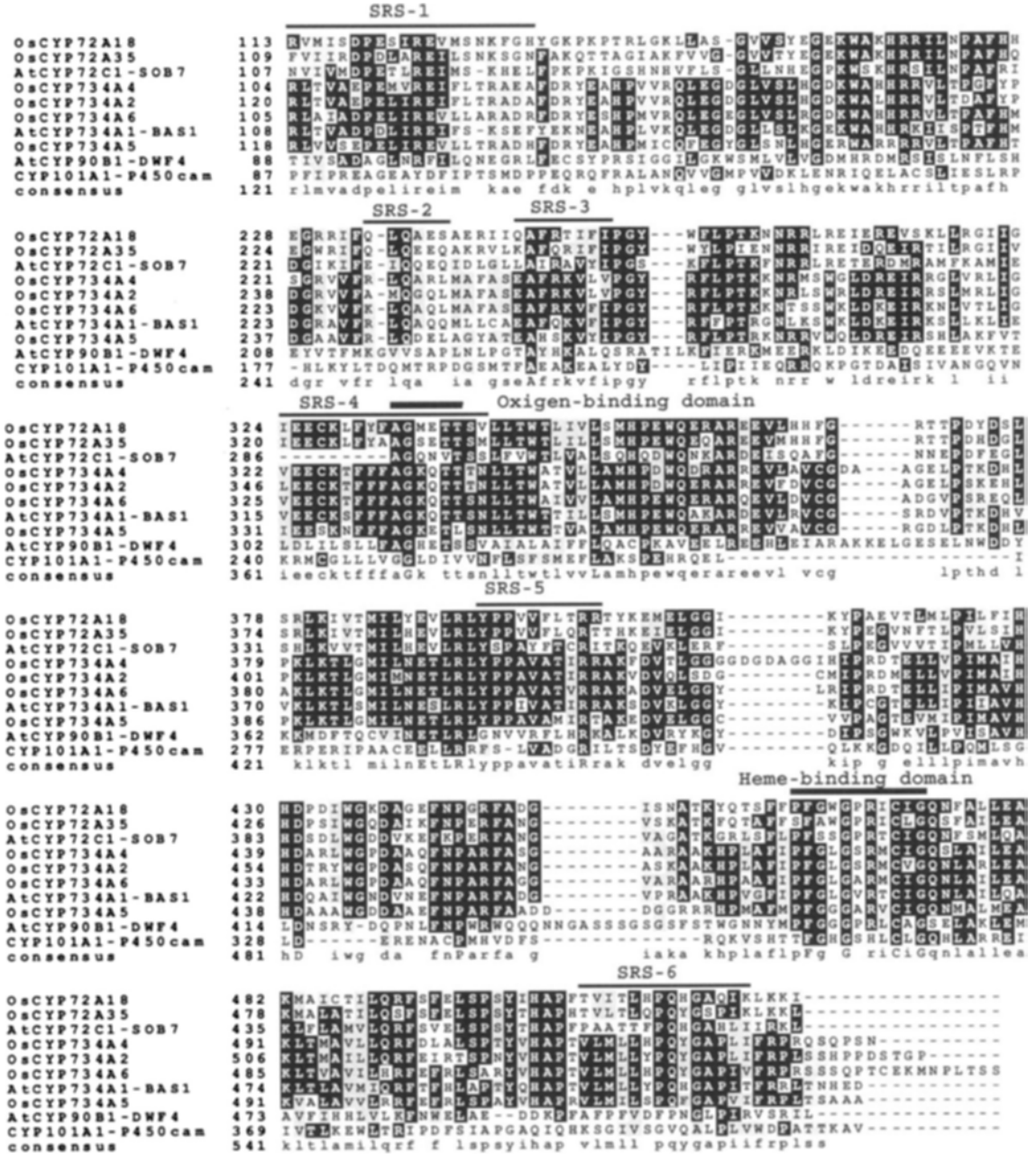


Figure 2. Box-shaded multiple sequence alignment of selected sequences. Six substrate binding sites (SRS) identified by Gotoh (1992) are marked as SRS-1~ -6. Typically conserved sequences in Cytochrome P450 proteins, e.g., O_2-binding and Heme-binding domain, are also shown. Note that three sequences belonging to CYP72 share same degree of similarity in SRS region as CYP734 groups do within group. Functionally different P450s, such as CYP90B1, share only minimal similarity with CYP72C and CYP734 in SRS region. CYP101A1 (P450cam, GenBank accession number P00183) sequence, whose molecular structure has been resolved, is included to map SRS regions. Shown are parts of sequences corresponding to known SRS and molecule-binding domains.

mutant lines *bending lamina2-1* (*bla2-1*) and *bending lamina2-2* (*bla2-2*). The T-DNA vector (pGA2715) used for these lines was originally designed to produce activation tagging mutant lines (Jeong et al., 2002). This T-DNA insertion is located in the coding region, so we could predict that these insertions resulted in loss-of-function mutations.

To isolate homozygous lines for the *bla2-2* allele, we planted 24 seeds from the T3 lines. The genomic DNAs isolated from those 24 individual lines were subjected to genotyping, using a right-border primer (RB) and a gene-specific primer (GT2-R). Successful amplification of the DNA indicated that they possessed this insertion. In addition, to test if any wild-type allele was present without a T-DNA insertion, we used a gene-specific primer set (GT2-F and GT2-R). Successful amplification demonstrated the occurrence of such an allele, and the existence of the T-DNA insertion was manifested by the inability to amplify any DNA from those primers. Based on these genotyping experiments, we identified 7 segregating wild-type, 5 heterozygous, and 9 homozygous plants among the 24 (Fig. 4). The remaining 3

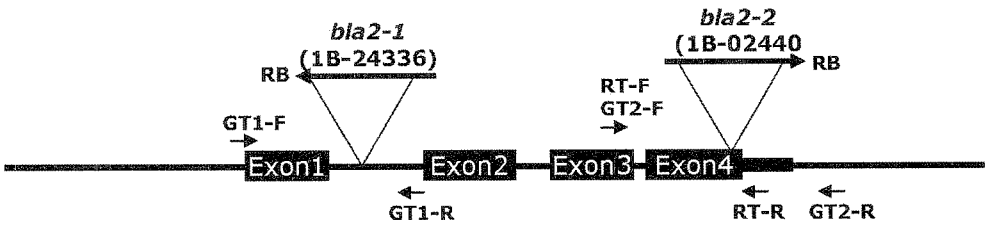
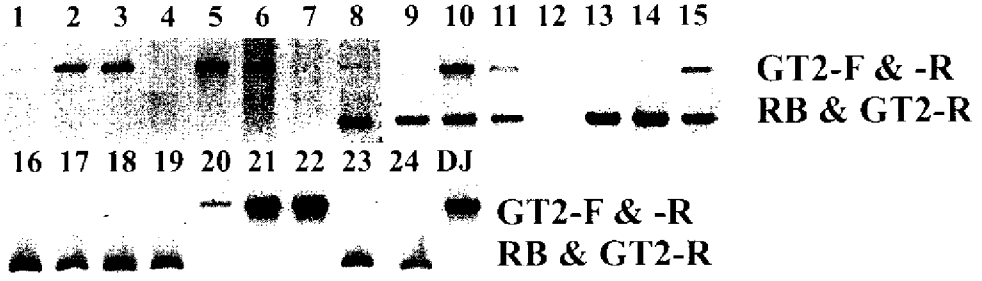


Figure 3. Schematic representation of rice LOC_Os01g29150 (CYP734A6) locus. Exons and introns are indicated by thick- and light-filled rectangles, respectively. Primer name and positions used for inverse PCR (iPCR) are shown as arrows and RB. Primer positions and directions for genotyping are written as GT1-F, etc. *bla2-1* and *bla2-2* represent T-DNA insertional mutant lines that were originally named 1B-24336 and 1B-02440, respectively.



Line	1	2	3	4	5	6	7	8	9	10	11	12
Genotype	SWT	Het	SWT	-	SWT	SWT	-	Het	Homo	Het	Het	-

Line	13	14	15	16	17	18	19	20	21	22	23	24
Genotype	Homo	Homo	Het	Homo	Homo	Homo	Homo	SWT	SWT	SWT	Homo	Homo

SWT, segregating wild type; Het, heterozygote; Homo, homozygote; -, ambiguous.

Figure 4. Genotyping of *bla2-2* mutant plants. Genomic DNA was isolated from individual plants of *bla2-2* segregating population. To amplify wild-type DNA without any insertion, GT2-F and GT2-R primers were used, and RB and GT2-R oligonucleotides were used to amplify the T-DNA flanking DNA. Heterozygous lines produced DNA fragments corresponding to both wild-type DNA and flanking DNA in Lanes 10, 11, and 15, whereas homozygous mutant plants produced only T-DNA flanking DNA in lanes 9, 13, and 14. Interpretation for each line is determined in accompanying table.

lines were not considered because their DNA band-patterning was ambiguous. In addition to *bla2-2*, we genotyped *bla2-1* and isolated 3 homozygous lines out of 15 segregating plants (data not shown).

Rice *bla2* Mutants Display Phenotypes of Exaggerated Bending Lamina

We carefully examined the mutants for any phenotype caused by the T-DNA insertion (Table 1). Overall, plants of the *bla2* mutant were significantly shorter than the wild-type

Table 1. Morphometric analysis for the key characters of the wild-type 'Dongjin', *Osbri1*, and *bla2-2* mutants. The *bla2-2* plants were shorter than from 'Dongjin', but the angle of the leaf lamina was characteristically increased relative to both the wild type and the *Osbri1* mutant.

	Height	Leaf blade		
		Length	Joint angle*	Width
'Dongjin'	120.3 ± 3.2	40.1 ± 5.5	32.8 ± 5.6	1.5 ± 0.1
Osbri1 (d61-2)	108 ± 7.5	51.3 ± 4.3	9.6 ± 0.7	1.7 ± 0.2
bla2-2	86.6 ± 8.8	23.3 ± 5.8	96 ± 27.7	1.1 ± 0.2

Unit in cm.
*Degree (°)

('Dongjin') or even the BR receptor-deficient mutant *Osbri1* (d61-2; Yamamuro et al., 2000). Similarly, leaf blades were shorter in the *bla2* mutant. However, the joint angle was greatest in the *bla2* mutant (96°), and smallest in the *Osbri1* mutant (9.6°). Representative lines of the wild type, *Osbri1*, and *bla2-2* displayed differences in this lamina-bending phenotype more clearly. For example, the lamina of the *bla2* mutants were prominently bent away from the main axis of the leaf sheath (Fig. 5) and, compared with the 'Dongjin' wild type, almost all the older leaves were angled away. Even more obvious were the phenotypic differences between the *bla2* and *Osbri1* mutants, the latter being defective in the BR receptor of rice (Yamamuro et al., 2000). Their lamina were all erect and robust, without any of the leaves being collapsed (Yamamuro et al., 2000). Exogenous treatment of BRs to etiolated rice seedlings results in a degree of lamina-bending dependent upon the concentration applied. In performing those same experiments here, we found that, as expected, the detached portions of etiolated seedlings responded to BRs by bending away in proportion to the concentration tested (Fig. 5A). For example, when 100 nM *epi*-BL was applied, the lamina were extremely bent, nearly touching the leaf sheath. The bending-lamina phenotypes observed in the *bla2* plants implied that this mutant contains an elevated level of endoge-

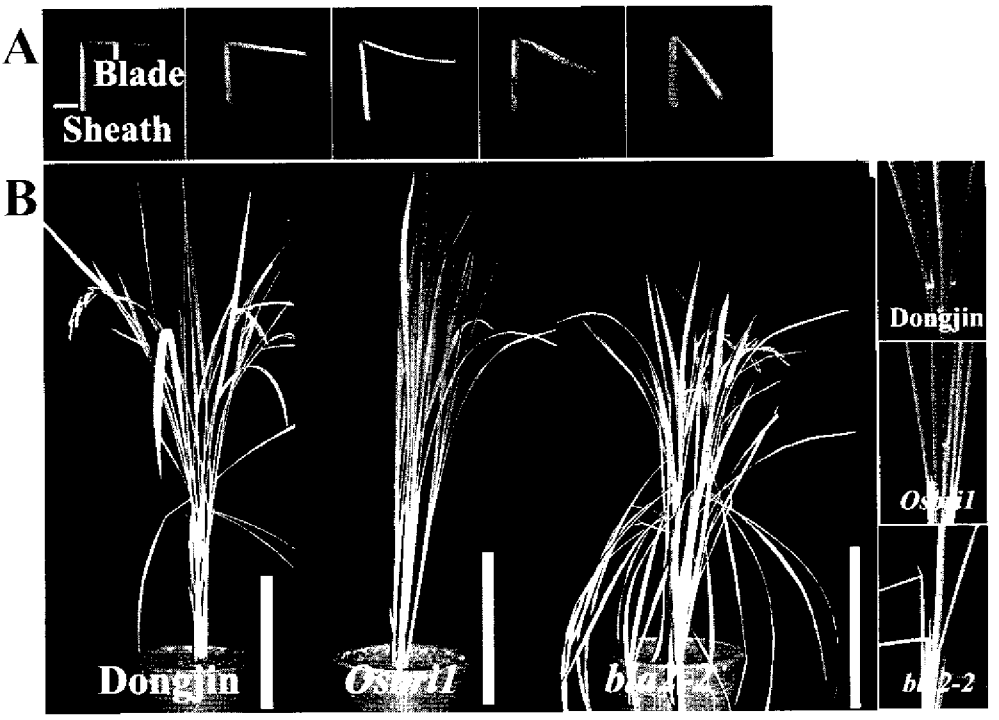


Figure 5. Lamina bending assay (**A**) and phenotypes of *bla2-2* mutant (**B**). (**A**) Etiolated rice seedlings were treated with indicated concentrations of *epi*-BL before representative samples were photographed. (**B**) 4-month-old rice plants were compared. In the inset, lamina-joint tissues are enlarged for better resolution. Overall height of both *Osbri1* and *bla2-2* is less than that of wild-type 'Dongjin'. BR receptor-deficient mutant *Osbri1* displays robustly erect leaves whereas all *bla2-2* leaves are bent away from top-to-bottom axis of plants. This opposite phenotype indicates that *bla2-2* may have an increased level of endogenous BRs due to knock-out of OsCYP734A6, which is predicted to mediate BR catabolism. Unit bar = 30 cm.

nous BRs. Again, the completely opposite phenotypes observed between *Osbri* and the *bla2* mutant clearly suggested that BR-signaling is constitutively turned on in the latter, possibly due to a loss-of-function mutation in the gene that encodes for a BR-deactivating enzyme.

Putative *OsDWARF4* Is Down-Regulated in the *bla2* Mutant

Previously we reported that the *Arabidopsis* BR-biosynthetic gene *DWF4* is feedback down-regulated either upon treatment with exogenous BRs or when the endogenous level of BRs is increased (Choe et al., 2001; Kim et al., 2006). We hypothesized that the *bla2* mutants accumulated BRs due to a knock-out of the BR deactivation process mediated by BLA2, so that a putative BR-biosynthetic gene *OsDWARF4* (Loc_Os03g12660) showed a decline in its transcript level. Consistent with this, we found that the steady-state level of *OsDWARF4* transcript was down-regulated by exogenous BR treatment of the 'Dongjin' wild-type plants (Fig. 6). Similarly, the level was decreased in the *bla2* mutant, supporting the idea that BLA2 is involved in BR deactivation.

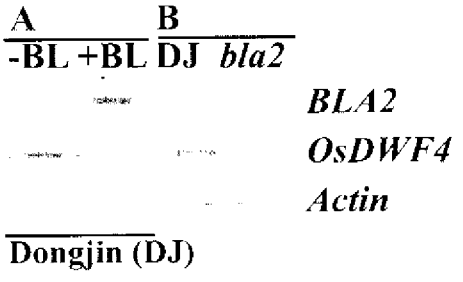


Figure 6. Feedback regulation of *BLA2* and putative BR-biosynthetic gene *OsDWARF4*. *BLA2* transcript was induced in response to BL, implying that gene product is involved in disposal of excess BL (**A**). Rice homolog of *Arabidopsis DWARF4* gene was identified based on nucleotide sequence homology, and primers were designed to amplify 3′ untranslated region. When rice seedlings were treated with 0.1 M *epi*-BL, transcripts were almost undetectable (**B**). Similarly, expression was significantly decreased in *bla2-2* mutant, suggesting that BR-signaling is constitutively turned on in this mutant, possibly due to block in BR catabolism that is mediated by BLA2 protein.

Differential Expression Patterns for the OsCYP734 Genes

In general, genes with multiple copies in an organism are predicted to have differential expression patterns according to space, time, and treatment. To confirm this, we searched the Massively Parallel Signature Sequencing (MPSS) database with these genes (http://mpss.udel.edu/rice/). Of the six putative genes belonging to the OsCYP734 group, OsCYP734A3 is believed to represent a pseudogene (Nelson et al., 2004). In our study, all OsCYP734A4 signature values were "0", possibly due to extremely low expressivity. Therefore, we omitted their expression levels from Table 2. To measure the general expressivity of OsCYP734A2, OsCYP734A5, and OsCYP734A6, we summed the abundance values for each gene induced under different experimental conditions. Their respective values were 275, 208, and 38 (Table 2), suggesting that the expression of OsCYP734A6 was relatively lower than that of the other two. Furthermore,

Table 2. Tissue-specific expression of the OsCYP734 genes. Massively Parallel Signature Sequencing (MPSS) database was searched for these genes (http://mpss.udel.edu/rice/). Tissues lacking any "abundance" data (i.e., from OsCYP734A4) were omitted from this table. Numbers represent the expression levels. Acronyms are defined as, NIP, 90-d-old immature panicle; NOS, ovary and mature stigma; NCA, 30-d-old callus; NDL, young, 14-d-old leaves drought-stressed for 5 d; PSL, 6-d-old developing seeds; PLW, water weevil-damaged leaves; PLC, mechanically damaged leaf; NCR, young roots cold-stressed at 4°C for 24 h; NSR, young roots stressed in 250 mM NaCl for 24 h; NDR, young roots drought-stressed for 5 d; NRB, 60-d-old mature root replica B; NME, 60-d-old crown vegetative meristematic tissues; NYR, young roots; NR2, root combined; NRA, 60-d-old mature root replica A.

Tissues	OsCYP734A6	OsCYP734A2	OsCYP734A5
NIP	**21**	0	**4**
NOS	**9**	**3**	0
NCA	**6**	**63**	**3**
NDL	**2**	0	0
PSL	0	0	**5**
PLW	0	0	**4**
PLC	0	0	**6**
NCR	0	0	**49**
NSR	0	**13**	**13**
NDR	0	**13**	**9**
NRB	0	**16**	**31**
NME	0	**16**	0
NYR	0	**42**	**7**
NR2	0	**42**	**36**
NRA	0	**67**	**41**
Expressivity	**38**	**275**	**208**

among our 32 testing scenarios, OsCYP734A6 signatures were only detected from the immature panicles, ovaries and mature stigmas, calli, and young leaves that were drought-stressed for 5 d. These results imply that the steady-state level of OsCYP734A6 transcript is tightly controlled. By contrast, the transcript level of OsCYP734A2 was highest in the root systems (NRA, NR2, NYR) and in the calli. Similar to OsCYP734A2, transcript of OsCYP734A5 was most highly detected in the root systems. Interestingly, and unlike the other two genes, expression of OsCYP734A5 increased when plants were subjected to low temperature. Therefore, the MPSS data for these three OsCYP734 genes suggest that they play important roles that depend on the tissue type or the environmental challenge to which they are subjected.

Our database search of rice pseudomolecules revealed that the rice genome contains six homologs of *Arabidopsis* CYP734A1, which mediates C-26 hydroxylation of BRs to deactivate the bioactive compounds. Among these six homologs, one (OsCYP734A3) proved to be a pseudogene, while OsCYP734A6 shared the highest degree of identity with *Arabidopsis* CYP734A1 at the amino acid sequence level. This indicates that OsCYP734A6 may encode for a similar protein involved in BR deactivation.

Two lines of evidence support the possibility of OsCYP734A6 being a BR-deactivator. First, the bending-lamina phenotypes of the knock-out mutant are very similar to the morphology of plants treated with BRs, suggesting that the mutants accumulate bioactive BRs due to a knock-out of the gene that encodes for a BR-deactivating enzyme. Second, the BR-biosynthetic genes are feedback down-regulated in response to either exogenous application of BRs or endogenous overexpression of BR biosynthesis (Kim et al., 2006). Similarly, a steady-state level of the putative BR-biosynthetic gene, *OsDWARF4* (Loc_Os03g12660), is strongly down-regulated in the *bla2* mutant, possibly because of the increased level of bioactive BRs in that mutant.

Based on their sequence similarities, the phenotype of strongly bending lamina in the *bla2* mutant, and the down-regulation of the BR-biosynthetic gene OsDWF4, we propose that OsCYP734A6 (BLA2) is involved in BR deactivation, as has already been demonstrated in *Arabidopsis* (Turk et al., 2003) and tomato (Ohnishi et al., 2006).

Phenotypes such as shorter stems and erect leaves are considered target traits for any rice breeder. Careful expression of the putative BR-deactivating gene in the lamina joint may result in clearance of bioactive BRs in these tissues, eventually producing the desired erect-leaf phenotypes. Therefore, future exploitation of this gene would expedite the success of such a breeding program.

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