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New Alleles of Rice *ebisu dwarf* (*d*2) Mutant Show Both Brassinosteroid-Deficient and -Insensitive Phenotypes

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ABSTRACT

ebisu dwarf (*d*2) is a mutant caused by mutation in a rice brassinosteroid biosynthetic enzyme gene, *CYP*90*D*2/*D*2, thereby conferring a brassinosteroid-deficient dwarf phenotype. Three newly isolated *d*2 alleles derived from a Nipponbare mutant library (*d*2-3, *d*2-4, and *d*2-6) produced more severe dwarf phenotypes than the previously characterized null allele from a Taichung 65 mutant library, *d*2-1. Linkage analysis and a complementation test clearly indicated that the mutant phenotypes in *d*2-6 were caused by defects in *CYP*90*D*2/*D*2, and exogenous treatment with brassinolide, a bioactive brassinosteroid, rescued the dwarf phenotype of three Nipponbare-derived *d*2 mutants. However, the content of endogenous bioactive brassinosteroid, castasterone, and the expression of brassinosteroid-response genes indicated that partial suppression of the brassinosteroid response in addition to a brassinosteroid deficiency has occurred in the Nipponbare-derived *d*2 mutants. Based on these results, we discuss the possibility that wild-type Nipponbare has some defects in an unknown factor or factors related to the brassinosteroid response in rice.

Keywords: Brassinosteroids; Brassinosteroid Biosynthetic Enzymes; Brassinosteroid Sensitivity; Cytochrome P450 Monooxygenase; Mutant Allele; Rice

1. Introduction

Brassinosteroids are endogenous phytohormones that are involved in the regulation of various growth and developmental processes in higher plants, including cell and stem elongation, dark-adapted morphogenesis (skotomorphogenesis), environmental stress responses, and the differentiation of tracheary elements [1-3]. Brassinosteroids have been shown to enhance various aspects of crop production in rice (*Oryza sativa* L.), including brassinosteroid-induced disease resistance [4] and abiotic stress tolerance [5]. Interestingly, overproduction and deficiency of brassinosteroids both increased grain and biomass yield, although they did this by different mechanisms [6-8]. These findings suggest that genetic improvement of crop production is feasible by modulating brassinosteroid metabolism and the content of bioactive brassinosteroids.

Several brassinosteroid-related mutants have been isolated in rice. The brassinosteroid-insensitive mutant *d*61 is caused by defects in the brassinosteroid receptor gene, *OsBRI*1 [9,10]. In addition, *brassinosteroid-deficient dwarf*1 (*brd*1) is caused by mutations in the brassinosteroid biosynthetic enzyme gene that encodes C-6 oxidase,

*CYP*85*A*1 [11,12]. *ebisu dwarf* (*d*2) is a loss-of-function mutant of the brassinosteroid biosynthetic enzyme gene that encodes C-23 hydroxylase, *CYP90D2/D*2 [13,14]. *osdwarf*4 and *d*11 are mutants of brassinosteroid biosynthetic enzyme genes that encode two C-22 hydroxylases, *CYP*90*B*2 and *CYP*724*B*1, respectively [6,15]. *brassinosteroid-deficient dwarf*2 (*brd*2) is caused by mutations in the sterol biosynthetic enzyme gene *DIMINUTO/ DWARF*1, which functions in the conversion of 24-methylenecholesterol to campesterol [16]. Characterization of these brassinosteroid-deficient mutants has helped us to understand the regulation of brassinosteroid biosynthesis in rice and the functions of brassinosteroids in rice growth and development. However, there are several enzymes involved in rice brassinosteroid biosynthesis for which mutants have not yet been isolated.

Although the major pathway for brassinosteroid biosynthesis has been established [17-19], recent studies demonstrated the complexity of the pathway by revealing new branches and shortcut routes [20,21]. To clarify the function of the other biosynthetic enzymes involved in brassinosteroid biosynthesis for which mutants have not been identified, we screened rice mutant libraries and isolated three new candidate lines. Here, we describe the characterization of these three mutants, which have uni-

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que phenotypes that exhibit both brassinosteroid deficiency and insensitivity. Based on the results of our analysis, we discuss the possibility that the well-known wild-type rice, Nipponbare, has some previously unreported defect in its brassinosteroid response.

2. Materials and Methods

2.1. Characterization of Mutants

To obtain brassinosteroid-related mutants, we performed a large-scale screening of rice mutant collections that were produced using a retrotransposon (*Tos*17), chemical mutagens, and γ-ray irradiation. Three candidate lines (*d*2-3, *d*2-4, and *d*2-6) were obtained from a Nipponbare library with tissue culture–induced mutations [22]. Seeds of wild-type rice (*Oryza sativa* L. "Nipponbare") and the mutants were sterilized in 1% NaClO for 30 min and sown on Murashige and Skoog agar medium. For the initial phytohormone treatment, seedlings were grown in a growth chamber at 28°C under continuous light for 1 week, then transplanted into media containing 10 nM brassinolide. For the subsequent field experiments, seedlings were grown for 1 month in a greenhouse, and then transplanted into a paddy field. For the gene expression analysis, we selected 2-week-old seedlings that exhibited uniform growth and adapted them to hydroponic culture for 2 days before treatment. Brassinolide treatment (100 nM) was carried out by adding the pure chemical to the culture medium. Whole seedlings were harvested 3 h after the treatment.

2.2. Mapping and Sequence Analyses

For the mapping of each mutant, we performed linkage analysis using an F_2 population of about 2000 plants derived from the cross between the Nipponbare-derived mutant (*japonica*) and Kasalath (*indica*) varieties. The mutation sites of all three candidate lines were mapped onto the short arm of chromosome 1 and were tightly linked to the *d*2 locus. The nucleotide sequence of the wild-type *CYP*90*D*2/*D*2 gene from these three lines was determined using a dideoxynucleotide chain-termination method using an automated sequencing system (ABI377; Applied Biosystems, Foster City, CA, USA), and was analyzed using the Lasergene software (DNAStar, Inc., Madison, WI, USA). We also determined the nucleotide sequence of a 9.3-kb genomic segment containing the *CYP*90*D*3 gene (from positions −4191 to +5156, taking the translation initiation site as +1) from Nipponbare and Taichung 65.

2.3. Complementation of the *d*2 Mutant

For complementation of the *d*2-6 mutation, a 15.2 kb restriction fragment (from −6063 to +9182 bp; taking the translation initiation site as +1) between *Sma*I and *Eco*RI sites was obtained from PCR products which amplified the genomic sequence of the *D*2 gene from wild-type rice Nipponbare. The fragment was cloned into pBluescript II SK (Stratagene, La Jolla, CA, USA) to confirm the sequence, and then inserted between *Sma*I and *Eco*RI sites of the hygromycin-resistance binary vector pCAMBIA1300 (Cambia, Canberra, Australia). The resulting construct was introduced into *Agrobacterium tumefaciens* strain EHA105, and *Agrobacterium*-mediated transformation of the *d*2-6 mutant was performed as described [23]. Transgenic plants were selected on media containing 50 mg·L$^{−1}$ hygromycin.

2.4. Analysis of Endogenous Brassinosteroid Levels

Shoots from wild-type and mutant plants were harvested 4 weeks after germination. To analyze the endogenous brassinosteroids, lyophilized shoots (equivalent to 20 g fresh weight) were extracted twice with 250 mL of MeOH, and deuterium-labeled internal standards (1 ng/g fresh weight) were added to the extract. After evaporation of the solvent in vacuo, the extract was partitioned between $CHCl_3$ and water three times. The $CHCl_3$-soluble fraction was purified with a silica gel cartridge column (Sep-Pak Vac 10 g; Waters, Milford, MA, USA). The column was subsequently eluted with 100 mL of $CHCl_3$, 2% (v/v) MeOH in $CHCl_3$, and 7% (v/v) MeOH in $CHCl_3$. Each of 2% MeOH and 7% MeOH fractions was purified by Sephadex LH-20 column chromatography (column volume of 200 mL; column eluted with MeOH-$CHCl_3$ [4:1]). The effluents of the elution volume to total column volume 0.6 to 0.8 were collected as BR fractions. The eluates were subjected to ODS-HPLC (Senshu Pak Pegasil ODS, 10 × 30 mm + Senshu Pak Pegasil ODS, 20 × 250 mm; Senshu Scientific, Tokyo) at a flow rate of 8 mL/min. Ninety percent acetonitrile was used as a solvent for the eluate derived from the 2% MeOH fraction, and 70% acetonitrile was used as a solvent for the eluate derived from the 7% MeOH fraction. HPLC purification from the 7% MeOH fraction yielded a brassinolide fraction (retention time (Rt) from 8 to 10 min), a castasterone fraction (Rt from 10 to 14 min), a teasterone fraction (Rt from 17 to 20 min), a typhasterol fraction (Rt from 26 to 32 min), and a 6-deoxocastasterone fraction (Rt from 38 to 44 min). HPLC purification from the 2% (v/v) MeOH fraction yielded 3-dehydroteasterone fraction (Rt from 12 to 16 min), a cathasterone fraction (Rt from 16 to 20 min), a 6-deoxoteasterone fraction (Rt from 28 to 32 min), a 3-dehydro-6-deoxoteasterone fraction (Rt from 32 to 36 min), and a 6-deoxotyphasterol fraction (Rt from 44 to 48 min). To analyze 6-deoxocathasterone, a portion (1 g fresh weight equivalent) of $CHCl_3$ fraction from a silica gel cartridge

column was purified with an octadecyl silane (ODS) cartridge column (Sep-Pak Plus C18, Waters), which was eluted with MeOH, and subjected to ODS-HPLC (Senshu Pak ODS 1151-D, 4.6 × 150 mm, Senshu Scientific, Tokyo) at a flow rate of 1 mL/min with 100% MeOH. The HPLC purification yielded a 6-deoxocathasterone fraction (Rt from 3 to 4 min). Each BR fraction was analyzed by GC-MS after derivatization. GC-MS analysis was carried out on a mass spectrometer (JMS-AM SUN200, JEOL, Tokyo) connected to a gas chromatograph (6890A, Agilent Technologies, Wilmington, DE) with a capillary column DB-5 (0.25 mm × 15 m, 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The analytical conditions were the same as previously described [13].

2.5. Gene Expression Analysis

Total RNA was extracted from whole seedlings using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Single-strand cDNAs were synthesized by using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA). Quantitative RT-PCR was performed with an iCycler iQ real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences were 5'-GTAGCCAGCTTGATCTCATCTC-3' and 5'-GGGA CGACTCTACTGCATCA-3' for *BU1*, 5'-TGATCCAT-TCCTGTACCCTG-3' and 5'-TACCTTCTTCCTC CCA-TCTG-3' for *CYP85A*1, 5'-CCACTAGCCACCAC TAC-TAC-3' and 5'-GGTGTGTGCAACTTGGCTTG-3' for *CYP*90*D*2/*D*2, 5'-CGAGGAATGCCCATCAAGG T-3' and 5'-ACCTTGATGGGCATTCCTCG-3' for *CYP*90*D*3, and 5'-CGCCAGTTTGGTCGCTCTCGATT TCG-3' and 5'-TCAGGAGCTCCGTGCTCTTCTGGTA C-3' for *Histone H*3. These primers specifically amplified the target gene sequences. Expression levels were normalized against the value obtained for *Histone H*3, which was used as the internal reference gene in each analysis.

2.6. Statistics

For gene expression experiments, we performed 3 biological repeats for wild-type and mutant plants. Statistical analysis of the data (the ratio of the target value to the internal reference) was performed using Version 5.1.2 of the JMP statistical package (SAS Institute Japan Ltd., Tokyo, Japan). Differences in the relative expression levels were analyzed by ANOVA followed by Holm's method for multiple comparisons.

3. Results and Discussion

3.1. Isolation of Three Mutants with Brassinosteroid-Related Phenotypes

As the result of a large-scale screening of rice mutant collections, we identified three lines that showed the

morphological characteristics of brassinosteroid-related mutants, namely dwarf and erect-leaf phenotypes. These mutants were obtained from a Nipponbare library with tissue culture-induced mutations [22]. Exogenously applying a bioactive brassinosteroid, brassinolide, rescued the dwarfism of these mutants and restored plant heights similar to that of the wild-type Nipponbare (**Figure 1**), suggesting that these mutants are brassinosteroid-deficient. Previously isolated brassinosteroid-related rice mutants have been categorized into three groups on the basis of leaf morphology and gross morphology. Plants exhibiting a group-1 phenotype formed only abnormal leaves with stiff, tortuous blades, and their leaf sheaths were scarcely developed. These plants did not flower, exhibit internode elongation, or bear seeds. These phenotypes were observed in the brassinosteroid-insensitive mutants, *d*61-3, *d*61-4, *d*61-5, and *d*61-6 [10]. Plants exhibiting a group-2 phenotype had severe dwarfing (about 10 cm in height) and malformed leaves with twisted leaf blades. The leaves of these plants were erect, and the ratio of leaf sheath length to leaf blade length was reduced. The floral organs did not form and internodes did not elongate in the group-2 phenotype plants, and they did not bear any seeds. These phenotypes were observed in the brassinosteroid-deficient mutants, *brd*1-1 and *brd*1-2 [11,12]. Plants exhibiting a group-3 phenotype showed a range of semi-dwarf phenotypes (about 70% ~ 95% height of wild-type). The leaves of these plants were erect, but the other abnormal phenotypes such as twisted leaf blades were not observed. Reproductive development of group-3 phenotype plants seems normal. These phenotypes were observed in the brassinosteroid-

Figure 1. Phenotypic rescue of the abnormal mutant phenotype by treatment with exogenous brassinolide. We transplanted 1-week-old seedlings of three Nipponbare-derived mutants, *d*2-3, *d*2-4, and *d*2-6, into MS medium containing 10 nM brassinolide (right three plants) or solvent (ethanol, left three plants), and grew them for 3 weeks. Although the root growth was inhibited by high concentration of brassinolide, above-ground morphology was rescued by the brassinolide treatment. Bar, 5 cm.

deficient mutants, *brd*2-1, *d*2-1, *d*2-2, *d*11-1, *d*11-2, and *osdwarf*4-1 [6,13,15,16], and in the brassinosteroid-insensitive mutants, *d*61-1 and *d*61-2 [9]. Because the gross morphology of newly isolated three mutants were ranged between group-2 and group-3 (discuss below), we hypothesized that these mutants had defects in one or more novel brassinosteroid biosynthetic enzymes or had novel defects in one or more previously identified brassinosteroid biosynthetic enzymes.

## 3.2. The Three Brassinosteroid-Deficient Mutants Were New *d*2 Alleles

For the mapping of each mutant, we performed linkage analysis using an F_2 population of about 2000 plants derived from the cross between the Nipponbare-derived mutant (*japonica*) and Kasalath (*indica*) varieties. The mutation sites of all three lines were mapped onto the short arm of chromosome 1 and were tightly linked to *CYP*90*D*2/*D*2. *CYP*90*D*2/*D*2 encodes a cytochrome P450 monooxygenase that functions in brassinosteroid biosynthesis, and its loss-of-function mutation causes the *ebisu dwarf* (*d*2) mutant [13]. Sequence analysis of *CYP*90*D*2/*D*2 from the three lines revealed that *d*2-3 had a single nucleotide substitution at the junction of exon 5 and intron 5 (G to C), *d*2-4 had a single nucleotide substitution (G to T) in exon 2 that induced an amino acid residue change (from Gly to Cys), whereas *d*2-6 had a 40-bp deletion in exon 4 (**Figure 2(a)**). Because this deletion caused a frame shift after Lys-313, the mutant protein lacks most of the enzyme, including the catalytic domain. The mutant phenotype of *d*2-6 was complemented (**Figure 2(b)**) by the introduction of a 15.2-kb genomic segment containing the *CYP*90*D*2/*D*2 gene (from positions −6063 to +9182 bp, taking the translation initiation site as +1). Based on these results, we concluded that *d*2-6 is a null allele of *d*2, and considered that all three lines are novel alleles of the *d*2 mutant.

3.3. Phenotypic Variation in the *d*2 Mutants

Previously, two *d*2 alleles had been isolated from the Taichung 65 mutant library, and one of them (*d*2-1) was considered to be a null allele because it had a single nucleotide substitution to produce a stop codon at Lys-83 (**Figure 2(a)**) [13]. *d*2-1 showed a semi-dwarf phenotype, in which plants reached 70% of the height of the wild-type, Taichung 65 (**Figure 3**) [13]. Interestingly, the plant heights of the *d*2-3, *d*2-4, and *d*2-6 mutants were about 30 cm, whereas that of Nipponbare, the wild-type that gave rise to *d*2-3, *d*2-4, and *d*2-6, was about 90 cm (**Figure 3**). These results indicate that the three newly obtained alleles, which included two knock-down alleles, showed a more severe dwarf phenotype than that shown by plants with the previously identified null allele, *d*2-1.
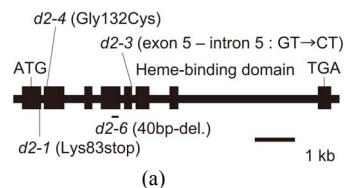
Figure 2. Characterization of the *d*2 mutants. (a) Genomic structure of the *CYP*90*D*2/*D*2 gene and positions of the mutations in the three *d*2 alleles discovered in the present study (*d*2-3, *d*2-4, and *d*2-6). Black boxes represent exons. (b) Complementation of the abnormal morphology of the *d*2-6 mutant by introduction of the *CYP*90*D*2/*D*2 gene. The *d*2-6 mutant containing the empty vector (left) and containing the DNA fragment encompassing the entire *CYP*90*D*2/*D*2 gene (right). Bar, 20 cm.
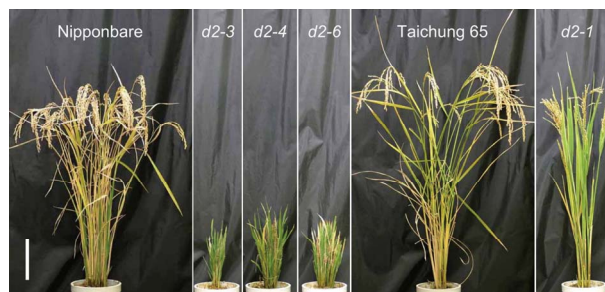
Figure 3. Phenotypic variation in the *d*2 mutants. Comparison of gross morphology between the wild-type (Nipponbare and Taichung 65) and *d*2 mutants. Nipponbare is the wild-type for *d*2-3, *d*2-4, and *d*2-6; Taichung 65 is the wild-type for *d*2-1. Bar, 20 cm.

Because the three newly obtained alleles and the previously identified *d*2-1 were derived from two different *japonica* varieties, Nipponbare and Taichung 65, respectively, our results suggest that the genetic background of the mutant plants affects the magnitude of the change in the phenotype.

3.4. Two *CYP*90*D* Genes in Rice

There are two *Arabidopsis CYP*90*D*1 orthologs, *CYP*90*D*2/*D*2 and *CYP*90*D*3, in the rice genome, and *D*2 encodes CYP90D2/D2 [13]. In wild-type Nipponbare, our quantitative reverse-transcription PCR analysis revealed
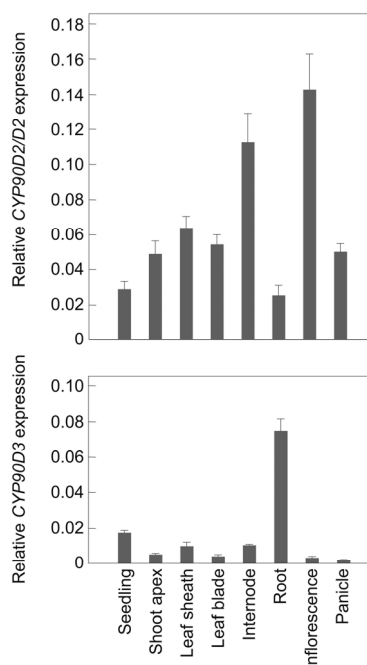
that both *CYP*90*D* genes were expressed at different levels in different organs (**Figure 4**). *CYP*90*D*2/*D*2 was expressed in all the organs of wild-type rice, but at a lower level in the roots and higher levels in the elongating internode and inflorescence, whereas *CYP*90*D*3 was expressed at the highest level in the roots and at lower levels in the other organs. These results suggest that both *CYP*90*D*2 and *CYP*90*D*3 are functional in wild-type rice.

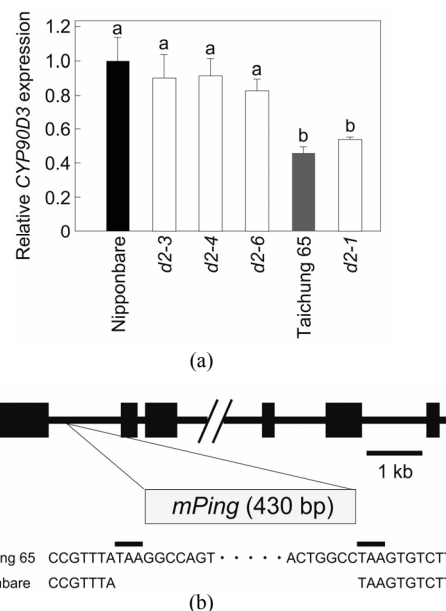## 3.5. *CYP*90*D*3 in the *d*2 Mutants

Because *CYP*90*D*2/*D*2 and *CYP*90*D*3 have similar sequences (75% nucleotide identity) and both *CYP*90*D*2 and *CYP*90*D*3 appear to be functional in wild-type rice (**Figure 4**), we hypothesized that the homeostatic system controlling the levels of bioactive brassinosteroids would up-regulate the expression of *CYP*90*D*3 in the *d*2-1 plants to compensate for the defects in brassinosteroid production caused by the loss-of-function mutation of *CYP*90*D*2/*D*2. *CYP*90*D*3 expression increased slightly (but not significantly) in *d*2-1 compared to its wild-type, Taichung 65 (about 1.2 times that in Taichung 65; **Figure 5(a)**), however, the steady-state level of *CYP*90*D*3 mRNA was significantly lower in Taichung 65 than in Nipponbare (about 46% that in Nipponbare; **Figure 5(a)**). In contrast, *CYP*90*D*3 mRNA did not show an increase in the *d*2-3, *d*2-4, and *d*2-6 mutants derived from wild-type Nipponbare (90%, 91%, and 83% of that in Nip-

ponbare, respectively; **Figure 5(a)**). Although the steady-state level of *CYP*90*D*3 mRNA was lower in Taichung 65 than in Nipponbare, increase or decrease in the levels of *CYP*90*D*3 expression in mutants were consistent with the magnitude of the mutant phenotype; that is, *d*2-1 showed a semi-dwarf phenotype, whereas *d*2-3, *d*2-4, and *d*2-6 plants showed more severe dwarfing than *d*2-1 plants, and this difference in *CYP*90*D*3 expression pattern may explain why *d*2-1 showed a less extreme phenotype. These results led us to hypothesize that Nipponbare has a mutant *CYP*90*D*3 gene, and that the expression of *CYP*90*D*3 is misregulated in Nipponbare and in the Nipponbare-derived *d*2 mutants.

To test this hypothesis, we compared the nucleotide sequence of a 9.3-kb genomic segment containing the *CYP*90*D*3 gene (from positions −4191 to +5156, taking the translation initiation site as +1) from Nipponbare and Taichung 65. We found the insertion of a *Tourist*-like miniature inverted-repeat transposable element (MITE) of 430 bp, called *miniature Ping* (*mPing*) [24-26], in the first intron of *CYP*90*D*3 from the Taichung 65 genome (**Figure 5(b)**). This insertion was flanked on both ends by a three-nucleotide sequence, TAA, which is the typi-

(a)

(b)

Figure 5. Expression of *CYP*90*D*3 gene in the *d*2 mutants. (a) Expression of *CYP*90*D*3 in wild-type rice and the *d*2 mutants. Expression levels were normalized against the values obtained for *Histone H*3. The values obtained from the wild-type Nipponbare were then normalized to a value of 1.0 to permit a comparison with the associated mutants. Nipponbare is the wild-type for *d*2-3, *d*2-4, and *d*2-6; Taichung 65 is the wild-type for *d*2-1. Each bar represents the mean ± s.d. of three biological repeats. Bars labeled with different letters differ significantly (The Holm-adjusted Student's *t* test, $P < 0.05$); (b) Genomic structure of *CYP*90*D*3 and the position of the *mPing* insertion in Taichung 65. Black boxes represent exons.

Figure 4. Expression of *CYP*90*D*2/*D*2 and *CYP*90*D*3 genes in various organs of wild-type Nipponbare. Expression levels were normalized against the values obtained for *Histone H*3. Each bar represents the mean ± s.d. of three biological repeats.

cal target sequence for duplication upon *mPing* transposition. These structural features suggest that *mPing* was transposed into the first intron of *CYP*90*D*3 in Taichung 65. Ohmori *et al*. [27] reported that the insertion of *Ping*, an autonomous transposon that can activate *mPing* transposition, in the fourth intron of the *DROOPING LEAF* (*DL*) gene decreased the transcript level of *DL*. In our case, however, *mPing* was inserted into the Taichung 65 *CYP*90*D*3 gene, and the steady-state level of *CYP*90*D*3 mRNA was lower in Taichung 65 than in Nipponbare. Although the expression of *CYP*90*D*3 in Taichung 65 seemed to be regulated normally by the homeostatic system that controls the levels of bioactive brassinosteroids, these results indicate that the magnitude of the mutant phenotype caused by the mutations in *CYP*90*D*2/*D*2 could not be explained simply by the insertion of *mPing* in the Taichung 65 *CYP*90*D*3 gene or by the genetic redundancy of the *CYP*90*D*2/*D*2 and *CYP*90*D*3 genes in rice.

3.6. Bioactive Brassinosteroids Accumulate in the Nipponbare-Derived *d*2 Mutants

To clarify what happens in the Nipponbare-derived *d*2 mutants, we compared the endogenous levels of brassinosteroids in *d*2-3 and *d*2-6 to those in wild-type Nipponbare. Because the most biologically active brassinosteroid, brassinolide, has been detected in *Arabidopsis* and some dicot plants but not in rice, Kim *et al*. [28] proposed that the precursor of brassinolide, castasterone, acts as the primary bioactive brassinosteroid in rice. *D2* gene encodes a cytochrome P450 monooxygenase, CYP-90D2, that catalyze C-23 hydroxylation of various 22-hydroxylated brassinosteroids [14]. The levels of 6-deoxoteasterone and teasterone, which are synthesized only by the C-23 hydroxylation of 6-deoxocathasterone and cathasterone, respectively, were decreased in *d*2 mutants (**Table 1**). These results are consistent with the fact that CYP90D2 act as brassinosteroid C-23 hydroxylases in rice. Surprisingly, castasterone did not decrease in *d*2-3 and *d*2-6; on the contrary, it accumulated slightly (**Table 1**). This result is opposite to the previous report that castasterone content decreased in *d*2-1 [13], and is inconsistent with the dwarf phenotype of the Nipponbare-derived *d*2 mutants if these mutants are simply caused by a brassinosteroid deficiency. Because accumulation of castasterone was observed in the brassinosteroid-insensitive dwarf mutants *d*61-2 and *d*61-4, which are caused by knock-down mutations of the rice brassinosteroid receptor gene *OsBRI*1 [9,10], the accumulation of castasterone in *d*2-3 and *d*2-6 plants suggests that these dwarf mutants are brassinosteroid-insensitive. However, as described above, exogenous brassinolide treatment rescued the dwarf phenotype of the Nipponbare-derived *d*2 mutants, indicating that these mutants retain

an ability to respond to bioactive brassinosteroids, at least when they are applied constitutively in excess amounts. Based on these results, we hypothesized that the biosynthesis of brassinosteroids and the response to these compounds are partially suppressed by the mutations in *CYP*90*D*2/*D*2 and by an unknown factor or unknown factors, respectively, in the Nipponbare-derived *d*2 mutants.

### 3.7. Brassinosteroid Response Is Partially Suppressed in the Nipponbare-Derived *d*2 Mutants

To confirm whether the response to brassinosteroids is partially suppressed in the Nipponbare-derived *d*2 mutants, we monitored the effect of brassinolide treatment on the expression of two brassinosteroid-responsive genes, *CYP*85*A*1 and *BU*1. *CYP*85*A*1 encodes brassinosteroid C-6 oxidase, and its expression is regulated by a homeostatic system that controls bioactive brassinosteroid levels; *i.e. CYP*85*A*1 expression was increased in the brassinosteroid-related mutants, and was decreased by the brassinolide treatment [11]. *BU*1 encodes a helix-loop-helix protein that participates in the regulation of rice lamina inclination [29]. *BU*1 is thought to be a primary brassinosteroid-response gene, because brassinosteroids increase *BU*1 expression even in the presence of the protein synthesis inhibitor cycloheximide.

The level of *CYP*85*A*1 mRNA in brassinolide-treated Taichung 65 seedlings significantly decreased to about 35% of the untreated control level (**Figure 6**). Similarly, *CYP*85*A*1 expression decreased in brassinolide-treated *d*2-1 plants (to 43% of that in untreated *d*2-1), although the steady-state level of *CYP*85*A*1 mRNA in the absence of brassinolide treatment was significantly higher in *d*2-1 than in wild-type Taichung 65 (about 1.2 times that in untreated Taichung 65; **Figure 6**). This expression pattern of *CYP*85*A*1 in *d*2-1 plants can be explained by the homeostatic system as described above. In *d*61-1, a Taichung 65-derived brassinosteroid-insensitive mutant [9], *CYP*85*A*1 expression was also significantly higher than wild-type in the absence of brassinolide treatment (1.1 times that in untreated Taichung 65) and unaffected by brassinolide treatment (**Figure 6**). Expression of *CYP*-85*A*1 in brassinolide-treated Nipponbare seedlings significantly decreased to about 44% of the untreated Nipponbare (**Figure 6**). Interestingly, the level of *CYP*85*A*1 mRNA in untreated *d*2-3, *d*2-4, and *d*2-6 was lower than that of Nipponbare (89%, 82%, and 84% of that in untreated Nipponbare, respectively), and was further decreased by brassinolide treatment (77%, 81%, and 81% of that in untreated mutants, respectively; **Figure 6**). This expression pattern of *CYP*85*A*1 in Nipponbare-derived *d*2 mutants cannot be explained simply by brassinosteroid-deficiency, as was the case of Taichung 65-derived

Table 1. Endogenous content of brassinosteroids in the wild-type nipponbare and *d*2 mutants.

Brassinosteroid	Content (ng·g⁻¹ fresh weight)				
	Nipponbare	*d*2-3 rep. 1[a]	*d*2-3 rep. 2	*d*2-6 rep. 1	*d*2-6 rep. 2
6-deoxocathasterone	0.38	0.44	0.49	0.40	0.50
6-deoxoteasterone	0.05	0.03	0.04	0.04	0.04
3-dehydro-6-deoxoteasterone	0.48	0.40	0.41	0.45	0.48
6-deoxotyphasterol	5.40	5.53	5.68	5.58	5.91
6-deoxocastasterone	1.67	2.17	2.45	1.93	2.26
cathasterone	n.d.	n.d.	n.d.	n.d.	n.d.
teasterone	0.06	0.03	0.04	0.04	0.03
3-dehydroteasterone	n.d.	n.d.	n.d.	n.d.	n.d.
Typhasterol	1.08	1.54	1.44	1.51	1.38
Castasterone	0.54	0.69	0.66	0.59	0.58
Brassinolide	n.d.	n.d.	n.d.	n.d.	n.d.

[a]Brassinosteroid levels in *d*2-3 and *d*2-6 mutants were measured in two independent experiments.
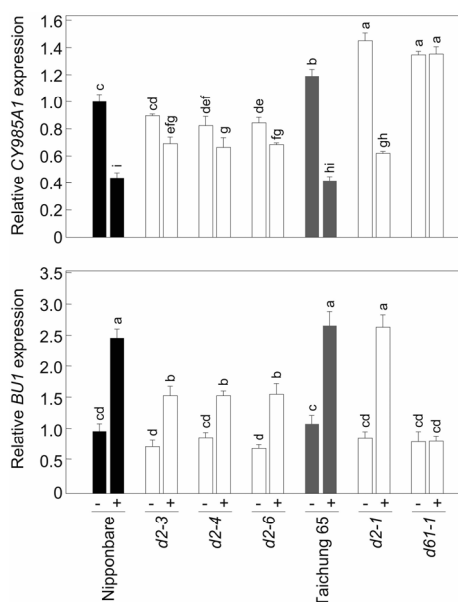
Figure 6. Expression of *CYP*85*A*1 and *BU*1 genes in the *d*2 mutants. We treated 2-week-old wild-type and mutant seedlings with 100 nM brassinolide (+) or a control solvent (ethanol, –) for 3 h. Expression levels were normalized against the values obtained for *Histone H*3. The values obtained from the wild-type Nipponbare seedlings treated with solvent were then normalized to a value of 1.0. Nipponbare is the wild-type for *d*2-3, *d*2-4, and *d*2-6; Taichung 65 is the wild-type for *d*2-1. Each column represents the mean ± s.d. of three biological repeats. Bars labeled with different letters differ significantly (The Holm-adjusted Student's *t* test, $P < 0.05$).

*d*2-1. Because the bioactive brassinosteroid castasterone accumulated in Nipponbare-derived *d*2 mutants (**Table 1**), the expression of *CYP*85*A*1 may also be regulated directly by its catalytic product, castasterone.

The level of *BU*1 mRNA in Taichung 65 seedlings was significantly increased by brassinolide treatment (about 2.4 times that in untreated Taichung 65; **Figure 6**). Similarly, *BU*1 expression was increased by brassinolide

treatment in the Taichung 65-derived *d*2-1 mutant (about 3.1 times that in untreated *d*2-1), reaching the same level as in the brassinolide-treated wild-type Taichung 65, although the steady-state level of *BU*1 mRNA in the absence of brassinolide treatment was slightly (but not significantly) lower in *d*2-1 than in Taichung 65 (about 78% that in Taichung 65; **Figure 6**). This expression pattern of *BU*1 in *d*2-1 plants can be explained by brassinosteroid deficiency. In *d*61-1, *BU*1 expression slightly (but not significantly) decreased both with and without brassinolide treatment (about 74% that in Taichung 65; **Figure 6**). Expression of *BU*1 in the Nipponbare seedlings was also significantly increased by brassinolide treatment both in the wild-type (about 2.5 times that in untreated plants) and in the *d*2-3, *d*2-4, and *d*2-6 mutants (about 2.1, 1.8, and 2.2 times that in untreated plants, respectively; **Figure 6**). Although, the steady-state levels of *BU*1 mRNA in all three Nipponbare-derived *d*2 mutants were lower than that of wild-type Nipponbare (74%, 88%, and 72% of that in untreated Nipponbare, respectively), and the increase in *BU*1 expression caused by the brassinolide treatment was to lower level in these mutants than in wild-type Nipponbare. These results support our hypothesis that the brassinosteroid response is partially suppressed in the Nipponbare-derived *d*2 mutants.

The difference in the severity of the mutant phenotype between the Taichung 65-derived *d*2-1 mutant and the Nipponbare-derived *d*2 mutants (*d*2-3, *d*2-4, and *d*2-6) can be explained by the finding that *d*2-1 showed a typical brassinosteroid-deficient phenotype, which was confirmed by the endogenous brassinosteroid content and expression of brassinosteroid-response genes, whereas *d*2-3, *d*2-4, and *d*2-6 represent partial suppression of the brassinosteroid response in addition to a brassinosteroid deficiency. Because our linkage analysis and complementation test clearly indicated that the mutant phenotypes in *d*2-3, *d*2-4, and *d*2-6 plants were caused by defects in the *CYP*90*D*2/*D*2 gene, we speculated that the

parent of the *d*2-3, *d*2-4, and *d*2-6 plants, Nipponbare, has one or more defects in its brassinosteroid response. Although double mutants of brassinosteroid-biosynthesis and -response genes have been generated in *Arabidopsis* [30,31], details of what happened in these double mutants (*i.e*., the levels of endogenous brassinosteroids, levels and patterns of expression of brassinosteroid-response genes) have not been described. At present, we cannot determine what factor or factors affect the brassinosteroid response in a Nipponbare background, but our results suggest that the brassinosteroid-stimulated growth responses in rice will differ among genetic backgrounds and that possible changes in cellular sensitivity to brassinosteroids must be considered when the effects of brassinosteroids on the regulation of various growth and developmental processes are examined in different rice cultivars. We predict that isolation and characterization of the unknown factor or factors will help us to understand how brassinosteroids regulate rice growth and development and will let us utilize the genes related to biosynthesis of and responses to these phytohormones to improve rice crop production.

4. Conclusion

In this study, we characterized three new alleles of the rice *d*2 mutation. In contrast to the previously isolated null allele derived from Taichung 65, *d*2-1, which shows a semi-dwarf phenotype combined with a typical brassinosteroid-deficient phenotype, Nipponbare-derived *d*2-3, *d*2-4, and *d*2-6 plants showed severe dwarfing and partial suppression of the brassinosteroid response, in addition to a brassinosteroid deficiency. Because the mutant phenotypes in *d*2-3, *d*2-4, and *d*2-6 are caused by defects in the *CYP*90*D*2/*D*2 gene, we hypothesize that their wild-type parent, Nipponbare, has one or more defects in unknown factors related to the response of rice to brassinosteroids.

5. Acknowledgements

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