The Role of OsBRI1 and Its Homologous Genes, OsBRL1 and OsBRL3, in Rice

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Since first identifying two alleles of a rice (Oryza sativa) brassinosteroid (BR)-insensitive mutant, d61, that were also defective in an orthologous gene in Arabidopsis (Arabidopsis thaliana) BRASSINOSTEROID INSENSITIVE1 (BRI1), we have isolated eight additional alleles, including null mutations, of the rice BRI1 gene OsBRI1. The most severe mutant, d61-4, exhibited severe dwarfism and twisted leaves, although pattern formation and differentiation were normal. This severe shoot phenotype was caused mainly by a defect in cell elongation and the disturbance of cell division after the determination of cell fate. In contrast to its severe shoot phenotype, the d61-4 mutant had a mild root phenotype. Concomitantly, the accumulation of castasterone, the active BR in rice, was up to 30-fold greater in the shoots, while only 1.5-fold greater in the roots. The homologous genes for OsBRI1, OsBRL1 and OsBRL3, were highly expressed in roots but weakly expressed in shoots, and their expression was higher in d61-4 than in the wild type. Based on these observations, we conclude that OsBRI1 is not essential for pattern formation or organ initiation, but is involved in organ development through controlling cell division and elongation. In addition, OsBRL1 and OsBRL3 are at least partly involved in BR perception in the roots.

Brassinosteroids (BRs) are plant hormones that have various effects on plant growth and development, including cell elongation, cell division, vascular development, abscission, and stress resistance (Clouse and Sasse, 1998; Sasse, 1999). The study of BRs began much later than that of other classical plant hormones, such as auxin, cytokinin, GA, abscisic acid, and ethylene. The effect of BR was first demonstrated in the 1960s, and the isolation of brassinolide (BL), the most active BR, was accomplished in 1979 (Mandava, 1988; Sasse, 1999). Since then, the study of BRs has rapidly progressed, coupled with successful molecular genetics approaches in Arabidopsis (Arabidopsis thaliana). The cloning of the BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1), the second plant hormone receptor ever to be cloned (Wang et al., 2001), was typical of the rapid progress in the study of BRs.

Recently, three homologous genes for BRI1, BRL1 to 3, were found in Arabidopsis (Caño-Delgado et al., 2004; Zhou et al., 2004). When these homologous genes, under the control of the BRI1 promoter, were expressed in the Arabidopsis bri1 mutant, the bri1 phenotype was rescued by introduction of BRL1 and BRL3, but not by BRL2 (Caño-Delgado et al., 2004). Chemical experiments support the above observation that BRL1 and BRL3 can interact with BL with high affinity, but BRL2 cannot (Kinoshiba et al., 2005). The triple mutant bri1/bri1/bri1 also enhanced the abnormal phenotype of bri1. Based on these observations, Chory and her colleagues concluded that Arabidopsis contains three BR receptors with specific functions in cell growth and vascular differentiation (Wang et al., 2001; Caño-Delgado et al., 2004; Kinoshiba et al., 2005).

BR receptors, which are homologous to the Arabidopsis BRI1 gene (AtBRI1), have been isolated from many plant species, including dicots such as tomato (Lycopersicon esculentum) and pea (Pisum sativum), and monocots such as rice (Oryza sativa) and barley (Hordeum vulgare); Yamamuro et al., 2000; Montoya et al., 2002; Chono et al., 2003; Nomura et al., 2003). In rice, the orthologous gene for Arabidopsis BRI1, OsBRI1, is expressed in almost all organs, as in Arabidopsis (Yamamuro et al., 2000). Loss-of-function mutants of OsBRI1, d61-1 and d61-2, are insensitive to BR and have erect leaves, dwarf culms, abnormal skotomorpho-

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genesis, and no organized microtubule arrangement in the cells from nonelongated internodes. Interestingly, several transgenic plants carrying the antisense strand of the OsBRII transcript exhibited phenotypes even more severe than those of d61-1 and d61-2 (Yamamuro et al., 2000).

In this study, we screened eight additional d61 alleles that showed various degrees of phenotypic severity. We analyzed a null mutant, d61-4, whose phenotype was the most severe of the 10, to characterize BRII function in rice. The phenotypic severity of d61-4 was much stronger and BR accumulation was much greater in shoots than in roots. In rice, the homologous genes for OsBRII, OsBRL1 and OsBRL3, were preferentially expressed in roots. Based on these observations, we propose that rice has three BR receptors and OsBRII dominantly functions in almost all organs, but the other two proteins, OsBRL1 and OsBRL3, also function in the roots.

RESULTS
Phenotypes of Various d61 Alleles in Rice

In addition to two previously reported alleles of the rice OsBRII gene (d61-1 and d61-2), which is homologous to Arabidopsis BRII (Yamamuro et al., 2000), we isolated eight new Osbrii alleles. The phenotypic severity of these Osbrii (d61) alleles varied (Fig. 1). We classified these mutants into three groups according to plant height and fertility. The height of plants with mild and intermediate alleles was approximately 80% to 90% and 60% to 70% of the wild type at the heading stage, respectively (Fig. 2A). Plants with mild and intermediate d61 alleles were fertile and formed dark green, erect leaves. In contrast, plants with severe alleles were severely dwarfed, and plant height 6 months after sowing was only approximately 5 cm (Fig. 2, B and C). These severe alleles resulted in sterile plants, because of the absence of flowers, with malformed dark green leaves (Fig. 2C). These severe mutants could not grow in soil. The overall phenotype of the severe d61 alleles was similar to the knockout mutant of BR C-6 oxidase, brd1-1 (Hong et al., 2002), although the severity of the d61 mutations was stronger.

Rice leaves can be separated into two parts, the leaf blade (upper region) and the sheath (lower region), by the lamina joint (Fig. 2D, arrowhead). The rice root system consists of three types of roots: seminal root formed in the embryo, crown (adventitious) root formed from the stem after germination, and lateral root. We measured the length of the leaf blades, leaf sheaths, and seminal root, and counted the number of crown roots and lateral roots in 10-d-old wild-type and d61 plants (Table I). The most dramatic defect was observed in leaf sheath length; the leaf sheaths of d61-4 were shortened to less than one-twenty-fifth the length of wild-type leaf sheaths. Leaf blade length was also shortened in d61-4, but the extent was less than in the sheaths, and no defect was observed in d61-1 or d61-2. Consequently, the ratio of leaf blade length to sheath length increased along with severity. In contrast to the severe defect in leaf sheath elongation, the d61 mutation did not appear to affect seminal root elongation. Seminal root length in d61-4 was almost three-quarters that of the wild type, whereas a slight promotion was observed in d61-1 and d61-2 relative to wild type. The formation of lateral roots in the seminal roots was also not severely affected by the mutations. Although the most plain root phenotype in d61 was the crown root formation in the stem, this may have been caused by defects in stem development, from which crown roots develop, resulting from the d61 mutation. These phenotypic analyses indicate that the d61 mutations preferentially cause defects in leaf formation and

![Figure 1. The mutation positions of the 10 d61 alleles.](image-url)
Figure 2. The phenotypes of the d61 mutants. A, The gross morphology of the d61 mild (d61-1, middle) and intermediate (d61-2, right) alleles. For comparison, the wild-type (WT) plant (left) is also shown (bar = 20 cm). B, The gross morphology of the wild type (left) and the severe allele (d61-4, right) at 2 months after sowing. A close-up view of the mutant was superimposed at the top right (bar = 5 cm). C, A close-up view of the d61-4 shoot at 6 months after sowing. The plant height was less than 5 cm. The d61-4 leaf was severely stunted and twisted (bar = 1 cm). D, The rice leaf can be divided into the blade (upper) and the sheath (lower) by the lamina joint (arrowhead). The sheath of the d61-4 leaf almost disappeared (bar = 5 cm). E, The first leaves of the wild type (left) and d61-4 (right). The first leaf of rice develops only a sheath and not a blade, while the mutant leaf develops a dominant blade (bar = 1 mm). Arrowheads indicate the lamina joint.

The 10 mutations were located at various domains: Leu-rich repeats (LRRs; four alleles), a 70-amino acid island (ID; two alleles), a transmembrane domain (one allele), and a kinase domain (three alleles; Fig. 1). The mutants d61-4 and d61-6 had a single nucleotide substitution to produce a stop codon at Glu-847 and an amino acid exchange of d61-10 and d61-1 at the kinase domain. These amino acid exchanges did not disrupt kinase activity because these amino acid exchanges did not disrupt kinase activity.

The amino acid changes of two alleles, d61-3 and d61-5, that were classified in the severe group were predicted to alter the secondary or tertiary structure or both of LRR and severely reduce its function. The intermediate phenotype d61-2 was associated with a Val residue in the LRR just in front of ID substituted with Met. The mutations in d61-8 and d61-9 occurred at Gly-522 and Gly-539 in ID, respectively. Although ID has been considered important for BR binding, both mutants exhibited the mild phenotype. An Arabidopsis bri1 mutation, bri1-113, whose mutation site corresponds to that of d61-9, causes a severe phenotype in Arabidopsis (Li and Chory, 1997). In Arabidopsis, the phenotypic severity of the bri1 mutant somehow depends on the ecotype (Caño-Delgado et al., 2004). Thus, the strong phenotype observed in Arabidopsis bri1-113 may depend on its ecotype. It is also possible that if endogenous active BRs in Arabidopsis and rice differ, the different phenotypic severity reflects the importance of the Gly residue for BR binding. Kinase activity should be essential for BRI1 function, but the amino acid changes of d61-10 and d61-1 at the kinase domain did not cause severe defects in BRI1 function, probably because these amino acid exchanges did not disrupt the BRI1 kinase activity. Thus, these amino acid residues are not considered important for kinase activity (Oh et al., 2000; Wang et al., 2005).

The Phenotype of d61-4

We further analyzed d61-4, which had lost almost all the kinase domain and was therefore considered to be a null allele. Actually, this allele showed the most severe phenotype of all the alleles. Germination of d61-4 was delayed 1 or 2 d relative to the wild type, and it developed severely stunted, rolled, and twisted dark green leaves, while the plastochron of d61-4 was nearly identical to that of the wild type (Fig. 2, B and C; data not shown). In addition to the abnormal leaf structure, the ratio of leaf blade to sheath length was altered in d61-4. The length of the blade and sheath of the fourth leaf were nearly the same as in the wild type, while in d61-4 the length of the leaf blade was slightly less relative to that of the wild type and the sheath length

| Table 1. Morphology of 10-d-old wild-type and d61 mutants |

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<tr>
<th></th>
<th>Wild Type</th>
<th>d61-1 (Mild)</th>
<th>d61-2 (Intermediate)</th>
<th>d61-4 (Severe)</th>
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<tbody>
<tr>
<td>Leaf blade (cm)</td>
<td>1.15 ± 0.15</td>
<td>1.19 ± 0.20</td>
<td>1.22 ± 0.38</td>
<td>0.69 ± 0.46</td>
</tr>
<tr>
<td>Leaf sheath (cm)</td>
<td>2.70 ± 0.37</td>
<td>1.75 ± 0.22</td>
<td>1.67 ± 0.23</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Leaf blade/sheath</td>
<td>0.42</td>
<td>0.68</td>
<td>0.73</td>
<td>7.11</td>
</tr>
<tr>
<td>Seminal root length (cm)</td>
<td>5.65 ± 0.96</td>
<td>6.78 ± 1.48</td>
<td>8.66 ± 1.46</td>
<td>4.36 ± 0.83</td>
</tr>
<tr>
<td>No. of lateral roots</td>
<td>16.9 ± 2.3</td>
<td>14.3 ± 2.0</td>
<td>11.3 ± 0.9</td>
<td>12.6 ± 2.6</td>
</tr>
<tr>
<td>No. of crown roots</td>
<td>7.7 ± 1.7</td>
<td>6.4 ± 0.9</td>
<td>5.0 ± 0.9</td>
<td>3.9 ± 0.9</td>
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*Data represent the means ± SD of 10 plants in each line.*
was drastically reduced (Fig. 2D). In 2-month-old d61-4, shoot development was strictly defective and leaves were still rolled and twisted; however, a number of roots were developed, as in the wild type, and unlike the leaves the roots were not rolled (Fig. 2B; see below).

Whereas the first leaf of the wild type had a unique structure and did not form a leaf blade, in d61-4 the same leaf blade was formed in the first leaf as in the other leaves (Fig. 2E). The formation of a leaf blade in the first leaf was also observed in the intermediate allele, d61-2, although the blade was much smaller than in d61-4 (data not shown). In d61-4, ligule formation was never observed (data not shown). These results indicate that BR is involved in the determination of leaf blade and sheath length ratios and ligule formation.

The Anatomical Characterization of d61-4

To examine the d61-4 phenotype in more detail, we observed its internal structure. One month after sowing, both the wild type and d61-4 developed six leaves, indicating that the leaf extraction rate was approximately the same in d61-4 and the wild type at the young seedling stage, as well as at more developed stages. At this stage, the node-internode structure became clear in the wild-type stem (nodes are indicated by arrowheads in Fig. 3A), but not in d61-4 (Fig. 3B). Instead of developing the node-internode structure, the mutant stem developed randomly oriented vascular bundles in the area below the shoot apical meristem (SAM).

The phyllotaxis was not disturbed in d61-4, and leaf initiation occurred in a distichous alternate manner (Fig. 3, C and D). After initiation, however, the vertical elongation of leaf primordia was apparently inhibited (as seen in the heights of P2, indicated by the bidirectional arrows in Fig. 3, A and B). In contrast to the inhibition of vertical elongation, lateral enlargement of leaf primordia was not inhibited in d61-4 (Fig. 3, C and D). The leaf primordia in d61-4 were slightly thicker than those of the wild type at the same stage; the shape of the primordia in d61-4 was more circular (Fig. 3D), while the wild-type primordia were elliptical (Fig. 3C). In the wild type, leaf primordia were densely encircled with younger primordia, whereas some space remained between adjacent primordia in the mutant (Fig. 3, C and D). The large and small vascular bundles were arranged by turns in leaf sheath as a similar manner both in wild-type and mutant plants (Fig. 3C), although the number of large and small vascular bundles was slightly decreased in the mutant (Fig. 3D; data not shown).

We also compared the anatomical organization of crown roots in 1-month-old wild-type and mutant plants. The overall structure around the root tip region was nearly identical in both plants (Fig. 3, E and F). However, the diameter of the d61-4 root was greater than that of the wild-type root, and sudden cell expansion was observed just above the divisional zone (indicated as “Dv”) in the d61-4 root. The longitudinal cell elongation of d61-4 was defective, while the width of cortex cells and, consequently, the diameter of the root were enlarged.

We also examined the vascular vein structure of the leaf sheaths (Fig. 3, G and H) and blades (Fig. 3, I and J) in the wild-type (Fig. 3, G and I) and mutant (Fig. 3, H and J) plants. The phloem tissues of the mutant vascular bundles both in the blades and sheaths appeared to be enlarged, and the number of sieve tubes and
companion cells was higher relative to the wild type. Similar abnormal proliferation of phloem regions has been reported in other plants with defective BR signaling (Nagata et al., 2001; Caño-Delgado et al., 2004), indicating that abnormal phloem proliferation is a common phenomenon in both dicots and monocots. The development of xylem tissue was defective in the mutant leaf sheath, and the development of metaxylem cells almost failed (Fig. 3H). However, xylem tissue in the leaf blade of d61-4 developed almost normally, and two well developed metaxylem cells were observed to be the same as those in wild-type leaves (Fig. 3J). These observations indicate that BR signaling is necessary for the normal development of xylem tissue as observed in dicots, whereas in rice the BRI1 contribution is weaker in the leaf blade than in the leaf sheath.

Development of the d61-4 Embryo

The abnormal phenotype of young seedlings just after germination leads us to speculate the abnormal morphology of embryonic organs. Therefore, we also examined embryo development in d61-4. Because d61-4 plants are sterile, we obtained d61-4 embryos produced by plants carrying the heterozygous alleles of d61-4. The frequency of the mutant embryo fit the normal segregation rate (approximately 25%; data not shown). The wild-type and d61-4 embryos could not be distinguished among more than 30 embryos until 4 d after pollination (DAP; Fig. 4A). At 5 DAP, wild-type and mutant embryos were distinguishable by the overall shape of the embryo; the SAM became clear, and the first leaf (L1) began to develop in both embryos (Fig. 4, B and C). At this stage, the wild-type embryo enlarged in the apical direction to develop the scutellum, and the dorsal side of the embryo facing the endosperm became flattened by the organized division of scutellum cells (Fig. 4B, right side). In mutant embryos, however, enlargement in the apical direction was less active and the leveling of the dorsal side did not occur. Consequently, the mutant embryo became more circular than the wild-type embryo (Fig. 4C). At 7 DAP, the wild-type embryo developed almost all tissues and organs, including three leaf primordia and a well organized root (Fig. 4D). The vascular bundle was also clearly present. At 7 DAP, it was difficult to prepare a section of d61-4 containing both the shoot and root, possibly because the embryo was arranged in a distorted fashion against the endosperm. When we prepared the longitudinal serial section of the embryo ball, one section of the median of the SAM did not contain radicle tissue (Fig. 4E), and the section with the center of the radicle did not contain the SAM (Fig. 4F). Although the coleoptile height was shortened, causing it to be dome-like in shape (Fig. 4, E and F), the d61-4 embryo contained all organs, the SAM, coleoptile, the first and second leaf primordia, scutellum, epiblast, vascular bundles, and radicle, as seen in the wild-type embryo (Fig. 4, E and F).
Expression of the Marker Genes in \textit{d61-4}

We performed in situ hybridization analysis using several molecular markers as probes to further examine the molecular mechanism behind the abnormal morphology in \textit{d61-4}. The histone H4 gene was used as a marker for the S phase of the cell cycle. \textit{OSH1}, a rice gene orthologous to maize (\textit{Zea mays}) \textit{KN1} and Arabidopsis \textit{STM}, is a marker of cells with an undetermined cell fate, including SAMs and young developing vascular bundles (Matsuoka et al., 1995; Sentoku et al., 1999). The rice \textit{SCARECROW1, OsSCR1}, is a marker of ground tissue (L2 layer of root; Kamiya et al., 2003a). \textit{RAmy1A} is a marker for the epithelium, the outermost cell of the scutellum (Kaneko et al., 2002; Kamiya et al., 2003b).

In the wild-type shoot apical region 1 month after sowing, the expression of histone H4 was observed in P1 to P4 with preferential localization in the epidermal and subepidermal cells (Fig. 5A). It was also preferentially observed in lateral cells of the stem, just below the SAM. In \textit{d61-4}, the expression of histone H4 was

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Table II. The numbers of cells in embryo coleoptile

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\includegraphics[width=\textwidth]{figure5.png}
\caption{The expression of the marker genes around the SAM in the vegetative stage and in the developing embryo. A and B. The expression of histone H4 around the SAM of wild-type (A) and \textit{d61-4} (B) plants at 1 month after sowing. C and D. The expression of \textit{OSH1} around the SAM of wild type (C) and \textit{d61-4} (D) at the same stage. The expression patterns of histone H4 and \textit{OSH1} in the mutant shoot apex region are similar to those in the wild type (A–D). E. The expression of \textit{OSH1} in the 4-DAP embryo. The expression of \textit{OSH1} was observed in the SAM and the epiblast (ep). \textit{OSH1} expression in wild-type and \textit{d61-4} embryos was indistinguishable at 4 DAP. F and G. The expression of \textit{OsSCR1} in the 7-DAP embryo in wild type (F) and \textit{d61-4} (G). \textit{OsSCR1} was expressed in the endodermis layer of the radicle. H and I. The expression of \textit{OSH1} in the 7-DAP embryo of wild type (H) and \textit{d61-4} (I). \textit{OSH1} expression was localized in the SAM, the ventral side of the radicle, and the epiblast of both embryos. J and K. The expression of \textit{RAmy1A} in the 9-DAP wild-type (J) and \textit{d61-4} (K) embryos. \textit{RAmy1A} expression was localized to the epithelium. L and M. The expression of histone H4 in the wild type (L) and \textit{d61-4} (M) embryos at 7 DAP. The signal was sporadically observed at a similar frequency in the both embryos. sc, Sclerenchyma; vb, vascular bundle; r, root. Bar = 200 μm.}
\end{figure}
also observed in P1 to P4 and in the stem, and the frequency of cells expressing H4 was slightly higher than in the wild type because its expression also occurred in internal stem cells, including the developing vascular bundles and leaf founder regions (Fig. 5B). OSH1 was expressed in the SAM, the junction regions between leaf primordia and the stem, and the developing vascular bundles in the wild type (Fig. 5C). The expression pattern was essentially the same in d61-4 as in the wild type (Fig. 5D).

We also examined the expression of marker genes in developing embryos to analyze whether basic pattern formation and cell-fate determination occurs normally in the mutant embryo. First, we examined the expression of OSH1 to analyze the development of the SAM. At 4 DAP, OSH1 expression was seen in the SAM and the epiblast (Fig. 5E), and the OSH1 expression patterns were the same in more than 20 embryos harvested from plants with the heterozygous allele (D61/ d61). This indicates that OSH1 expression in wild-type and d61 embryos was indistinguishable, and therefore the establishment of the SAM occurred normally in d61 embryos. The expression of OsSCR in the wild-type and d61 embryos was also indistinguishable during embryogenesis and in mature embryos (Fig. 5, F and G), indicating that establishment of the L2 layer, including the radicle endodermis layer, occurred normally. The OSH1 expression pattern in both d61 and the wild type was maintained in the mature embryo at 7 DAP, but the expression level was slightly lower in d61 than in the wild type (Fig. 5, H and I), as in the SAM at the vegetative stage. RAMy1A expression was specifically observed in the epithelium in 9-DAP embryos both in the wild type and d61-4 (Fig. 5, J and K). These results demonstrate that fate determination of various cells occurred normally in d61-4 during embryogenesis, but the subsequent morphogenesis or development or both are abnormal. We also observed the expression of histone H4 in 7-DAP wild-type and d61-4 embryos. The frequency of cell division was approximately the same in both wild-type and d61-4 embryos (Fig. 5, L and M).

**BR Accumulation in d61-4**

We measured the endogenous BR content to determine why the phenotypic severity varied between the shoot and the root. The shoots and roots of 6-week-old d61-4 and wild-type plants were separated and the amounts of BRs were analyzed. So far, we have never detected BL (the most active BR), only castasterone (CS), in rice. As expected, the shoots of d61-4 had large accumulations of bioactive CS, about 50-fold the accumulations in wild-type shoots (Table III), but no BL could be detected even in these materials. This suggests that rice may use CS as a bioactive BR, but not BL, or BL may be rapidly catabolized in rice. Interestingly, such high levels of CS accumulation were not observed in the d61-4 roots; instead, only a 1.5-fold accumulation occurred (Table III), suggesting that there may be different mechanisms in shoots and roots for accumulation of bioactive BR (see below).

### Table III. Endogenous BR contents in wild-type and d61-4 plants

<table>
<thead>
<tr>
<th>BRs4</th>
<th>Wild Type</th>
<th>d61-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>24-MC</td>
<td>15,100</td>
<td>1,110</td>
</tr>
<tr>
<td>CR</td>
<td>96,300</td>
<td>44,100</td>
</tr>
<tr>
<td>CN</td>
<td>1,790</td>
<td>1,360</td>
</tr>
<tr>
<td>6-OxoCN</td>
<td>74.9</td>
<td>27.9</td>
</tr>
<tr>
<td>6-DexoCT</td>
<td>0.89</td>
<td>5.79</td>
</tr>
<tr>
<td>6-DexoTE</td>
<td>0.12</td>
<td>1.89</td>
</tr>
<tr>
<td>6-Dexo3DT</td>
<td>0.39</td>
<td>7.97</td>
</tr>
<tr>
<td>6-DexoTY</td>
<td>2.95</td>
<td>25.1</td>
</tr>
<tr>
<td>6-DexoCS</td>
<td>0.82</td>
<td>1.22</td>
</tr>
<tr>
<td>CT</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TE</td>
<td>0.02</td>
<td>0.29</td>
</tr>
<tr>
<td>TY</td>
<td>0.83</td>
<td>1.58</td>
</tr>
<tr>
<td>CS</td>
<td>0.47</td>
<td>1.03</td>
</tr>
<tr>
<td>BL</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*BR contents are expressed as nanogram per gram fresh weight of tissues. MC, Methylenecholesterol; CR, campesterol; CN, campestanol; CT, cathasterone; TE, teasterone; DT, dehydroteasterone; TY, typasterol.*
Comparison of Phenotypes and Gene Expression in d61-4 and brd1-1

The phenotypic severity of d61-4 in the shoots was stronger than that of the strongest BR-deficient mutant, brd1-1 (Fig. 7A). In contrast, the severity in the roots was stronger in brd1-1 than in d61-4 (Fig. 7B). This type of severe phenotype in shoots and a mild phenotype in roots of d61-4 corresponded well with the accumulation pattern of CS (Table III). We observed in detail the internal structure of the fourth leaf primordium (P4) and the root elongation zone. The cell arrangement in the d61-4 leaf primordia was severely disturbed, but was organized in the wild-type leaf primordia (Fig. 7, C and E). In contrast to the leaf primordia, the cell layer in the d61-4 roots was maintained and the number of layers was approximately the same as in the wild type (Fig. 7, D and F). These internal cell structures corresponded well with the gross morphologies of the shoots and roots, that is, the longitudinal growth of the roots may be maintained by the cell layer (Fig. 7, B and F), while the rolled abnormal growth of leaves may be caused by the disturbed cell arrangement in leaf primordia (Fig. 7, A and E). When we observed the internal structure of brd1-1, the cell arrangement in the leaf primordia was almost maintained, while that in the roots was disturbed, also corresponding to the gross morphology of brd1-1 (Fig. 7, G and H).

We compared the expression level of BR-related genes between the wild type and d61-4 (Fig. 7I). In 6-week-old seedlings, the expression of both OsBRL1 and OsBRL3 was higher in d61-4 roots than in wild-type roots, whereas slightly increased, but still low, levels of expression occurred in the shoots (Fig. 7I). In d61-4 roots, the expression of OsBRI1 was also observed and was slightly higher relative to wild-type roots, although this transcript contained a stop codon in the kinase domain (Fig. 1). We also examined the expression of rice BR biosynthetic genes D2 (Hong et al., 2003), D11 (Tanabe et al., 2005), and OsDWARF (Hong et al., 2002). The expression of the D2 gene was only detected in the shoots and was more expressed in d61-4 than in the wild type. D11 and OsDWARF gene expression was higher in d61-4 than in the wild type in both shoots and roots; however, the increase was higher in shoots than in roots (Fig. 7I). The increased expression of these BR biosynthetic genes in shoots relative to root expression may be correlated with the higher levels of CS in shoots (Table III). Taken together, these expression results suggest that the mild phenotype of d61-4 roots may be a result of compensation by the BR1 homologous genes OsBRL1 and OsBRL3, and that consequently the BR signal can be transduced in the roots, while the phenotype cannot be rescued by the small increase in OsBRL1 and OsBRL3 expression in the shoots.

We also compared the expression of these BR-related genes between the wild type and brd1-1. As seen in d61-4, the expression of the OsBRI1, D2, and OsDWARF genes was higher in the brd1-1 mutant; however, the increase of OsBRL1 and OsBRL3 was not observed either in shoots or in roots in brd1 (Fig. 7J).

These results suggest that BR-deficient phenotypes should be partly ameliorated by the increased BRI1 expression in brd1 shoots, but not in roots, because the increased expression of BRI1 or BRII homologs did not occur in roots.

DISCUSSION

In this study, we isolated eight alleles of the rice bri1 mutant, in addition to the already reported d61-1 and d61-2 alleles. Four of the 10 mutant alleles caused much more severe dwarf phenotypes than what was reported for d61-1 and d61-2 (Yamamuro et al., 2000). We analyzed in detail the null allele of rice bri1, d61-4, which caused the most severe phenotype among the 10 alleles, and discussed the role of BR and BR receptors in rice.
**Figure 7.** The phenotypic comparison between *d61-4* and *brd1-1* and the expression of BR-related genes in *d61-4* and *brd1-1* plants. A, The gross morphology of the shoots of 1-month-old *d61-4* (left) and *brd1-1* (right) plants (bar = 5 cm). B, The gross morphology of the roots of 2-month-old wild-type (left), *d61-4* (middle), and *brd1-1* (right) plants (bar = 5 cm). C to H, Longitudinal section of the P4 leaf primordia (C, E, and G) and root elongation zone (D, F, and H) of the wild-type (C and D), *d61-4* (E and F), and *brd1-1* (G and H) plants at 1 month after sowing (C–H, bar = 100 μm). I and J, The expression levels of *OsBRI1*, *OsBRL1*, *OsBRL3*, *D2*, *D11*, and *OsDWARF* were analyzed using RNA extracted from 6-week-old wild-type and *d61-4* plants (I), and wild-type and *brd1* plants (J). The mRNA level for each gene was quantified by semiquantitative reverse transcription-PCR (25 cycles for *OsBRI1*, *OsBRL1*, *OsBRL3*, and *OsDWARF*; 30 cycles for *D2* and *D11*). The expression of the *OsACT1* gene was used as a control (25 cycles). ep, Epidermis; sc, sclerenchyma; cor, cortex; S, shoot; R, root.
BR Signaling Is Regulated Differently in Shoots and Roots

In *d61-4*, we noticed a difference in the phenotypic severity between shoots and roots. A clear difference between shoots and roots in *d61-4* was in the accumulation of BRs. The level of CS in the mutant shoot was about 30-fold higher than that in the wild type, whereas there was only a 1.5-fold accumulation in the root (Table III). This corresponded to the high and low increases in the expression of BR biosynthetic genes, such as *D2*, *D11*, and *OsDWARF*, in shoots and roots, respectively, expression that was negatively regulated by BRs and BR signals (Hong et al., 2002, 2003; Tanabe et al., 2005; Fig. 7I). In *d61-4*, the functional homologs of *OsBRI1*, *OsBRL1*, and *OsBRL3* were preferentially expressed in the roots, and their expression was very low in shoots (Fig. 7I). These results suggest that, in roots of *d61-4*, the BRI1 homologs compensated for the lack of BR signal caused by the loss of BRI1 function. Interestingly, the pattern of phenotypic severity of shoots and roots in *brd1-1*, one of the most severe rice BR-deficient mutants (Hong et al., 2002), was opposite that seen in *d61-4* (Fig. 7, A and B). In fact, the shoot phenotype in *brd1-1* was much milder than that in *d61-4*, and the root phenotype in *brd1-1* was more affected than that in *d61-4*. This phenomenon could also be explained by the expression patterns of *OsBRI1*, *OsBRL1*, and *OsBRL3*, as follows. In *brd1-1*, the expression of *OsBRI1* was increased in shoots and partially compensated for the severe phenotype, whereas *OsBRI1* and *OsBRLs* were not up-regulated and there was no compensation in the roots. The reason for the increased expression of *OsBRL1* and *OsBRL3* in *d61-4* and the decreased expression in *brd1-1* is not clear. Perhaps there is an unknown BR signaling pathway in the roots distinct from that in the shoots.

Why does the expression of *OsBRL1* and *OsBRL3* in the roots not completely compensate for the abnormal root phenotype in *d61-4*? There are two possible answers to this question. The function of *OsBRL1* or *OsBRL3* may not completely overlap that of *OsBRI1*, and, consequently, the expression of *OsBRL1* and *OsBRL3* would not completely compensate for the loss of *OsBRI1* function. Alternatively, *OsBRI1* and its homologous genes could have different expression patterns in terms of tissue or cell specificity or both. At present, we cannot determine which answer is correct. In Arabidopsis, *AtBRL1* was expressed in roots and stems, and *AtBRL1* and *AtBRL3* were also preferentially expressed in vascular tissues. In rice, *OsBRL1* and *OsBRL3* were preferentially expressed in roots (Fig. 7), and *OsBRL3* was also expressed in embryos (data not shown). Such preferential expression of rice and Arabidopsis BRL1 and BRL3 leads us to speculate that three BR receptors, BRI1, BRL1, and BRL3, function in an organ- or tissues-specific manner both in monocot (rice) and dicot (Arabidopsis) plants. Even such case, it is no wonder that BRI1 plays a dominant and essential function in both plants and BRLs have a subordinate function. Why then do both rice and Arabidopsis BRL1 and BRL3 retain a similar structure and a similar expression pattern? Further analysis of the OsBRL genes is needed to clarify the understanding of BR signaling in rice roots.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Wild-type rice plants (*Oryza sativa* L. cv Taichung 65) and plants with mutant alleles of *d61* were grown in the field, a greenhouse at 30°C and 24°C (day/night), or a plant box containing half-strength Murashige and Skoog medium with 1.5% (w/v)Suc at 30°C.

Semiquantitative Reverse Transcription-PCR

Total RNA was isolated using the standard SDS-phenol method. The first strand of cDNA was synthesized from 2 μg of total RNA using an Omniscript RT kit (Qigene). The primer sequences used for analysis were 5′-CTCCTG-CACGCGTGAGGTTGAGCCGCTAAG-3′ and 5′-TACAGTTGAATTCCCCTT-3′ for *OsBRI1*, 5′-GGTGTCCGTGAGCCGCTAAG-3′ and 5′-TGAGTTGAATTCCCCTT-3′ for *OsBRL1*, and 5′-CCGGTGAGATACCAGACAAG-3′ and 5′-AGAGTTGTCCGAGCTGAGCCTC-3′ for *OsBRL3*. The primer sets used for the *OsDWARF*, *OsACT1*, *D2*, and *D11* gen analyses were described previously (Hong et al., 2002, 2003; Tanabe et al., 2005). The products amplified using these primers were separated on a 1.2% (w/v) agarose gel.

Histological Analysis and in Situ Hybridization

Tissues were fixed overnight at 4°C in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, dehydrated through a graded ethanol series followed by a 1:3 butanol series, and finally embedded in ParaPlast Plus (Oxford Labware) and sectioned to 8-μm thicknesses. For histological analysis, microtome sections were stained with Delafield’s hematoxylin. In situ hybridization with digoxigenin-labeled RNA was performed following the methods of Kouchi and Hata (1993). Digoxigenin-labeled RNA was produced from the cording region without the poly(A) of *OSH1*, *OsSCR*, *Ramy1A*, and histone H4.

Quantification of Endogenous BRs

Six-week-old wild-type and *d61-4* plants were separated into shoots and roots and lyophilized immediately at −80°C. To analyze the endogenous BRs, lyophilized samples were extracted twice with 250 mL of methanol/CHCl3 (4:1, v/v). BR purification and quantification were performed following the methods of Fujioka et al. (2002) and He et al. (2003).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *OsBRI1*, BAB86053; *OsBRL1*, BAD34326; *OsBRL2*, AAK52544; *OsBRL3*, BAD01717; *AtBRI1*, AAC49810; *AtBRL1*, AAB60331; *AtBRL3*, AAK52545; *AtD11*, AAD54959; *AtDF3*, AAD54960; *PsBRI1*, BAC99050; and *HvBRI1*, BAD06331.

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LITERATURE CITED


