Morphological Alteration Caused by Brassinosteroid Insensitivity Increases the Biomass and Grain Production of Rice

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The rice (Oryza sativa) dwarf mutant d61 phenotype is caused by loss of function of a rice BRASSINOSTEROID INSENSITIVE1 ortholog, OsBRII1. We have identified nine d61 alleles, the weakest of which, d61-7, confers agronomically important traits such as semidwarf stature and erect leaves. Because erect-leaf habit is considered to increase light capture for photosynthesis, we compared the biomass and grain production of wild-type and d61-7 rice. The biomass of wild type was 38% higher than that of d61-7 at harvest under conventional planting density because of the dwarfism of d61-7. However, the biomass of d61-7 was 35% higher than that of wild type at high planting density. The grain yield of wild type reached a maximum at mid-density, but the yield of d61-7 continued to increase with planting density. These results indicate that d61-7 produces biomass more effectively than wild type, and consequently more effectively assimilates the biomass in reproductive organ development at high planting density. However, the small grain size of d61-7 counts any increase in grain yield, leading to the same grain yield as that of wild type even at high density. We therefore produced transgenic rice with partial suppression of endogenous OsBRII1 expression to obtain the erect-leaf phenotype without grain changes. The estimated grain yield of these transformants was about 30% higher than that of wild type at high density. These results demonstrate the feasibility of generating erect-leaf plants by modifying the expression of the brassinosteroid receptor gene in transgenic rice plants.

Dwarfism is one of the most valuable traits in crop breeding. The major factor enabling the Green Revolution was the introduction of high-yielding semidwarf cultivars of wheat (Triticum aestivum) and rice (Oryza sativa) in combination with the application of large amounts of nitrogen fertilizer (Khush, 1999). Nitrogen fertilization is essential to increasing grain yield, but it also promotes leaf and stem elongation, resulting in an overall increase in plant height. Tall plants are easily flattened by wind and rain and, consequently, dramatic yield losses occur. The semidwarf cultivars of wheat and rice are more resistant to damage by wind and rain (lodging resistant), and the reduction in plant height improves harvest index (grain/grain plus straw) and enhances biomass production (Khush, 1999).

Because of their agronomic importance, dwarf mutants have been extensively characterized in many plant species. The phytohormone gibberellin (GA) is one of the important factors associated with dwarf phenotype. It is noteworthy that both Green Revolution genes—wheat Reduced height1 (Rht1) and rice semidwarf1 (sd1)—are involved in GA signaling and GA biosynthesis, respectively (Peng et al., 1999; Ashikari et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). Characterization of the Green Revolution genes has taught that controlling GA metabolism or perception is the best target for producing high-yielding semidwarf cultivars by traditional crop breeding.

Recently, another important target for producing high-yielding semidwarf cultivars was identified in barley (Hordeum vulgare). As semidwarf barley accessions carrying the uzu gene showed lodging resistance, the uzu gene has been introduced into all hull-less barley now cultivated in Japan (Saisho et al., 2004). The uzu phenotype is brassinosteroid (BR) insensitive (Honda et al., 2003), as caused by the missense mutation of an ortholog of Arabidopsis (Arabidopsis thaliana) BRASSINOSTEROID INSENSITIVE1 (BR1), HsBR1.
(Chono et al., 2003; Saisho et al., 2004). This is evidence that a BR-related mutation is a feasible target for producing high-yielding semidwarf cultivars.

Arabidopsis BRI1 encodes a Leu-rich-repeat receptor-like kinase that functions as a BR receptor (Li and Chory, 1997; He et al., 2000; Wang et al., 2001; Kinoshita et al., 2005). BR is a polyhydroxylated steroid phytohormone that is involved in cell and stem elongation, dark-adapted morphogenesis (skotomorphogenesis), responses to environmental stress, and tracheary element differentiation (Clouse and Sasse, 1998; Sasse, 2003). BRI1 orthologs have been identified in many plant species, including rice, tomato (Lycopersicon esculentum Mill.), pea (Pisum sativum), and cotton (Gossypium hirsutum; Yamamuro et al., 2000; Montoya et al., 2002; Nomura et al., 2003; Sun et al., 2004).

In contrast to the barley BRI1 mutants, the loss-of-function mutants of a rice BRI1 ortholog (OsBRI1), namely d61, show a range of phenotypes (Yamamuro et al., 2000; Nakamura et al., 2006). Although severe alleles d61-3 and d61-4 produce severe dwarfism and malformed leaves with tortuous leaf blades, weak alleles d61-1 and d61-2 produce agronomically useful traits such as semidwarf stature, erect leaves, and elongated neck internodes. It is considered that erect leaves improve light capture for photosynthesis and nitrogen use by grain and lead to a higher leaf area index (one-side leaf area per unit of land area) in dense plantings, all of which increase yield (Sinclair and Sheehy, 1999). Unfortunately, however, morphological alterations in d61-1 and d61-2 lines have also been observed in reproductive organs and the grain yield is decreased in these mutants.

In this article, we analyze the morphological traits and yield components of paddy field-grown d61-7, the weakest allele of d61 we have obtained, under different planting conditions. We also generated transgenic rice with suppressed OsBRI1 expression, which showed erect-leaf phenotype without any defects in reproductive organ development. We discuss the feasibility of reducing BR signaling to improve crop production by genetic manipulation of the BR receptor in rice.

**RESULTS**

**Characterization of d61 Weak Alleles**

The previously identified spontaneous mutants d61-1 and d61-2 show dwarf phenotype; d61-2 is the shorter of the two (Fig. 1, A–C). In wild-type plants, the leaf blade bends away from the vertical axis of the leaf sheath toward the abaxial side (Fig. 1D). In contrast, almost all of the leaves of d61-1 and d61-2 are erect (Fig. 1E). Although the panicle length is not different among wild type, d61-1, and d61-2 (Fig. 1F),
identified seven lines as containing novel BR response of these candidates in detail and finally selected over 100 dwarf mutants as candidates. We analyzed the phenotype and irradiation, we selected more than 100 dwarf mutants. Therefore, we performed a large-scale screening for semidwarf phenotypes with erect leaves (Fig. 1F). Among all five weak alleles, d61-7 showed the weakest dwarf phenotype. Interestingly, the panicle length was significantly increased in d61-7 relative to wild type (Fig. 1F), resulting in increased grain number per panicle (Fig. 1G). The heading date (flowering time) of d61-7 was 5 d later than that of wild type, but days from heading date to harvest time (maturity) of d61-7 were the same as wild type. On the basis of these results, we selected this allele, d61-7, for further analysis of grain production in paddy fields.

Figure 2. Comparison of biomass production between wild type and d61-7 at different planting densities. Black bars correspond to vegetative organs (culm, leaf sheath, and leaf blade); white bars correspond to reproductive organs (panicle). Error bars represent SE from the mean of five plants. Stages I, II, and III indicate heading date, 20 d after heading, and harvest time, respectively.

Progression of Aerial Biomass of d61-7 Plants Grown in a Paddy Field

Figure 2 shows the progression of the dry matter weight of aerial parts (culm, leaf sheath, leaf blade, and panicle) at different planting densities. The vegetative biomass of wild-type and d61-7 plants (black bars) decreased from stage I (heading) to stage II (20 d after heading) at every density. This decrease might be caused by the translocation of reserved substance from leaves to developing panicles. At stage III (harvest), both wild-type and d61-7 plants showed recovery, but the trend of recovery differed between them. At the conventional planting density (22.2 plants m\(^{-2}\)), the recovery of wild type from stage II to stage III was obvious, amounting to 40%, but d61-7 showed little or no increase. At higher densities, in contrast, wild type showed little or no increase (0% at 44.4 plants m\(^{-2}\) and 10% at 66.7 plants m\(^{-2}\)), whereas the vegetative biomass of d61-7 increased by 11% at middensity and 45% at high density. Consequently, the vegetative biomass of d61-7 at stage III was 35% higher than that of wild type at high density.

In wild type at conventional density, the reproductive (panicle) biomass (Fig. 2, white bars) increased markedly from stage II to stage III, but decreased by 7% at middensity and by 12% at high density. In contrast, in d61-7, although increased only slightly at conventional density, it increased by 10% at middensity and 20% at high density. These results strongly suggest that d61-7 with erect leaves produces biomass more efficiently than wild type from stage II to stage III.

<table>
<thead>
<tr>
<th>Line</th>
<th>Density</th>
<th>Panicle No.</th>
<th>Total Grain No.</th>
<th>Fertile Grain No.</th>
<th>Fertility</th>
<th>Grain Weight (1,000 grains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>22.2</td>
<td>13.0 ± 0.6 a</td>
<td>118.1 ± 7.0 bcd</td>
<td>111.8 ± 7.1 ab</td>
<td>0.95 ± 0.004 a</td>
<td>26.7 ± 0.2 a</td>
</tr>
<tr>
<td>d61-7</td>
<td>22.2</td>
<td>11.8 ± 0.2 a</td>
<td>146.2 ± 2.9 a</td>
<td>121.9 ± 2.2 a</td>
<td>0.83 ± 0.001 b</td>
<td>21.0 ± 0.4 b</td>
</tr>
<tr>
<td>d61-7</td>
<td>44.4</td>
<td>8.2 ± 0.3 b</td>
<td>105.7 ± 5.2 cd</td>
<td>98.8 ± 5.1 bc</td>
<td>0.93 ± 0.022 a</td>
<td>26.9 ± 0.1 a</td>
</tr>
<tr>
<td>Wild type</td>
<td>66.7</td>
<td>5.5 ± 0.7 c</td>
<td>95.3 ± 2.9 d</td>
<td>88.1 ± 1.4 c</td>
<td>0.93 ± 0.000 a</td>
<td>27.9 ± 0.5 a</td>
</tr>
<tr>
<td>d61-7</td>
<td>66.7</td>
<td>5.5 ± 0.0 c</td>
<td>130.3 ± 0.2 ab</td>
<td>111.1 ± 1.7 ab</td>
<td>0.85 ± 0.012 b</td>
<td>21.9 ± 0.4 b</td>
</tr>
</tbody>
</table>

Table 1. Morphological characterization of wild type and d61-7 grown in the paddy field

Data represent the mean ± s.e. of four plants in each plot with two replications. Letters denote statistically significant differences according to the Tukey-Kramer honestly significant different test (P < 0.05).
at higher densities, and consequently more effectively assimilates the biomass in reproductive organ development (see “Discussion”).

Yield Performance of d61-7 in the Paddy Field

Table I shows the yield components of wild type and d61-7 at each planting density. At all densities, the panicle number per plant of d61-7 and wild type were almost the same, whereas the total grain number per panicle of d61-7 was larger (approximately 30%) than that of wild type ($P < 0.05$). In contrast, the fertility of d61-7 was less than that of wild type, so the number of fertile grains of d61-7 was 6% to 25% larger than that of wild type, depending on the planting condition. Furthermore, the grain weight of d61-7 was less than that of wild type (Table I) because of the small grain size of d61-7 (Table II). BR-deficient mutants such as d2 and d11 also produce small grains (Hong et al., 2003; Tanabe et al., 2005), indicating that BR affects the grain size of rice.

We compared the relationship between planting density and grain yield per area between wild type and d61-7 (Fig. 3A). The grain yield of wild type reached a maximum at middensity. In contrast, the grain yield of d61-7 continued to increase with planting density, although it was about 80% of that of wild type at conventional density. Consequently, the grain yield of d61-7 per area was the same as that of wild type at high density.

The rate of decrease of grain yield per plant of d61-7 was less than that of wild type (Fig. 3B). This supports the idea that the grain yield of d61-7 did not peak at middensity because its erect leaves provided more assimilate than wild type for panicle development at higher density. Similar results were also obtained from a rice BR-deficient mutant, osdwarf4-1, which showed erect leaves without small grain phenotype (Sakamoto et al., 2006). These results strongly suggest that the erect-leaf phenotype of BR-related mutants improves biomass production at high densities. However, the small grain of d61-7 counters the increase in crop yield, resulting in the same or less yield than that of wild type even at high densities.

OsBRII Knock-Down Plants

To test the ability of the d61 mutation to increase crop production, we have intensively looked for novel alleles showing weaker phenotype with erect leaves but not small grain, but could not find any. Therefore, we tried a transgenic approach to generate a favorable phenotype by partially suppressing endogenous OsBRII expression.

To obtain OsBRII knock-down (KD) plants, we introduced antisense OsBRII cDNA. Most transgenic plants showed more severe phenotype than d61-1, and the rest showed similar phenotype, but we could not find any plants showing milder phenotype at the second (T₀) or later generations (data not shown). Thus, we abandoned this approach and used a cosuppression strategy to repress endogenous OsBRII expression. In this strategy, we constitutively expressed a truncated sense OsBRII cDNA construct (OsBRII-KD; Fig. 4A) under the control of the rice actin promoter (McElroy et al., 1991). Primary transformants (T₁) showed a range of phenotypes. The height of most was <20 cm and their gross morphology was indistinguishable from that of severe d61 alleles (data not shown), but some plants showed similar or milder phenotype than d61-7. We selected two lines for further analysis because their phenotype resembled that of wild type except for the erect leaves. They carried a single copy of the introduced OsBRII-KD gene. We self pollinated these two lines to obtain inbred plants (BKD11 and BKD22). At the T₅ stage, we obtained

<table>
<thead>
<tr>
<th>Line</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7.09 ± 0.18</td>
<td>3.36 ± 0.11</td>
<td>2.17 ± 0.10</td>
</tr>
<tr>
<td>d61-7</td>
<td>6.37 ± 0.20</td>
<td>3.22 ± 0.07</td>
<td>2.14 ± 0.00</td>
</tr>
</tbody>
</table>

**, Significant at the 1% level; NS, not significant.
lines whose progeny ubiquitously carried a single copy of the transgene per haploid.

Both BKD11 and BKD22 plants produced erect leaves (Fig. 4B). In these transformants, endogenous OsBRI1 expression was well, but not completely, suppressed (Fig. 4C). The plant height and culm length of both were not significantly different from those of wild type (Fig. 5A). Although weak d61 alleles, including d61-7, fail to elongate the second internode, BKD11 and BKD22 did not show this defect (Fig. 5B). In contrast, the degree of bending between leaf blade and leaf sheath was decreased in the transgenic lines relative to wild type (Fig. 5C). About 90% of wild-type plants had a third-leaf bending angle of 16° to 25°, and no plants had leaves with an angle <15°; by contrast, about 80% of BKD11 and BKD22 plants had a third-leaf bending angle <10° (Fig. 5C, top). The erect-leaf phenotype was seen not only in lower leaves that expanded at the vegetative stage, but also in upper leaves that expanded at the reproductive stage, including the flag leaf (Fig. 5C, bottom).

Neither panicle length (Fig. 5D) nor grain morphology (Fig. 5E) of the two transgenic lines was significantly different from those of wild type. The yield components and grain yield of wild type, BKD11, and BKD22 were evaluated in pot experiments in a transgenic glasshouse (Table III). No statistically significant difference was observed in the yield components among wild type, BKD11, and BKD22, although the panicle number per plant and grain weight were slightly increased in the transgenic lines. These results indicate that the grain production of both BKD11 and BKD22 resembled that of wild type, although the transgenic plants produced erect leaves. The grain yield per plant calculated from the yield components showed that the yield potentials of BKD11 and BKD22 are slightly higher than that of wild type (Table III). Because the rate of decrease of grain yield per plant under high densities was lower in mutants with erect leaves than in wild type, as described above, the yield potential should be higher in BKD11 and BKD22 than in wild type (see “Discussion”).

**DISCUSSION**

BR-deficient or -insensitive rice mutants show dwarf phenotype and malformed leaves, stems, and flowers. For instance, plants bearing the null alleles of d61 cannot grow more than 5 cm even 3 months after sowing and develop severely malformed leaves with tortuous blades (Nakamura et al., 2006). So far, we have collected many rice BR-related mutants and examined their phenotypic characters (Hong et al., 2002, 2003, 2005; Tanabe et al., 2005; Nakamura et al., 2006; Sakamoto et al., 2006). They show a range of phenotypes, including severe dwarfism, deficient leaf sheath development, and tortuous leaf blades. Erect leaf is the only common phenotype among all BR-related mutants, indicating that this is the most sensitive of the BR-related phenotypes.

Even though the photosynthetic capacity of lower leaves is lower than that of upper leaves, the contribution of lower leaves to photosynthesis is still significant in rice (Horton, 2000). Because light shading by upper leaves prevents effective photosynthesis by lower leaves, the grain yield of wild type reaches a ceiling at middensity (Fig. 3). In contrast, erect leaves allow greater penetration of light to lower leaves and avoid the yield ceiling (Sinclair and Sheehy, 1999). Indeed, the grain yield of d61-7 was increased even at high density (Fig. 3). These field experiments suggest that the erect-leaf phenotype caused by the d61 mutation could be used to produce rice cultivars that yield well at high planting densities.

However, the erect-leaf phenotype caused by BR-related mutations has not been utilized in traditional rice breeding, probably because most BR-related mutants, including d61-7, have small grain or decreased fertility, both of which are unfavorable for crop breeding (Hong et al., 2003, 2005; Tanabe et al., 2005). Unfortunately, we could not obtain weaker alleles of d61 than d61-7, although we have screened more than 1,000 semidwarf mutants. We therefore generated...
transgenic rice plants in which OsBRI1 expression was suppressed and extensively screened them to obtain plants with very mild BR-related phenotype. We found two transgenic lines showing a suitable phenotype (BKD11 and BKD22; Fig. 4B). Interestingly, these plants did not show a semidwarf or dwarf phenotype. Consequently, the gross morphology of BKD11 and BKD22 was indistinguishable from that of wild type except for the erect leaves. To produce these transgenic plants, we used a ubiquitous promoter, rice actin promoter, to suppress OsBRI1 expression in leaves (McElroy et al., 1991). This indicates that quantitative regulation of OsBRI1 expression is sufficient to produce erect-leaf plants without any other abnormal phenotypes. In other words, partial suppression of OsBRI1 function will induce erect-leaf phenotype but no other abnormal phenotypes, and therefore it should still be possible to find such mutants, even though we have failed to do so in this study.

Because of restrictions on the cultivation of transgenic plants in the field, we could not plant our transgenic lines in a paddy field. Thus, we estimated the yield potential of BKD11 and BKD22 on the assumption that the rate of decrease of grain yield per plant of the transgenic plants is the same as that of d61-7. The grain yield per plant of d61-7 at middensity was 57% of that at conventional density, and at high density was 78% of that at middensity. Similar results were obtained with a BR-deficient rice mutant, osdwarf4-1 (data not shown). If the negative increment of yield per plant from conventional to high density is equal in d61-7 and BKD11, the grain yield of BKD11 can be calculated as 35% larger than that of wild type at high density (BKD11, 12.29 t ha⁻¹; wild type, 9.13 t ha⁻¹; Table IV). Similarly, the grain yield of BKD22 was approximately 26% higher, indicating that the combination of erect-leaf plants with dense planting can increase grain production without extra fertilizer application.

Although further studies are needed to confirm that weakly OsBRI1-deficient plants such as BKD11 and BKD22 have higher grain yield at high planting density in paddy fields, our results suggest the feasibility of generating erect-leaf plants without defects in reproductive development by modifying the expression of the BR receptor gene. This will make it possible to introduce a single dominant transgene that would increase the grain yield at high density without the need for a conventional, long-term breeding program and the negative environmental effects caused by fertilizers.

Table III. **Morphological characterization of wild-type and OsBRI1-KD transgenic rice**

Data represent the mean ± SD of 11 plants in each line.

<table>
<thead>
<tr>
<th>Line</th>
<th>Panicle No.</th>
<th>Total Grain No.</th>
<th>Fertility (g 1,000 grains⁻¹)</th>
<th>Grain Weight (g)</th>
<th>Grain Yield (g plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plant⁻¹</td>
<td>panicle⁻¹</td>
<td>plant⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>10.1 ± 2.2</td>
<td>123.8 ± 16.5</td>
<td>0.91 ± 0.06</td>
<td>27.8 ± 0.9</td>
<td>31.6</td>
</tr>
<tr>
<td>BKD11</td>
<td>12.0 ± 1.3</td>
<td>120.4 ± 19.1</td>
<td>0.85 ± 0.08</td>
<td>28.0 ± 1.2</td>
<td>34.3</td>
</tr>
<tr>
<td>BKD22</td>
<td>11.4 ± 2.1</td>
<td>118.2 ± 23.4</td>
<td>0.85 ± 0.07</td>
<td>28.2 ± 1.9</td>
<td>32.2</td>
</tr>
</tbody>
</table>
Materials and Methods

Plant Materials and Field Experiments

The field trial of wild-type rice (Oryza sativa L. cv Taichung 65) and the db1-7 mutant derived from Taichung 65 was performed at the experimental farm of Nagoya University (Togo town, Aichi, Japan; latitude 35°06′ north; longitude 137°05′ east). Thirty-eight-day-old seedlings were transplanted into a paddy field at one plant per hill with a spacing of 30 × 15 cm (22.2 plants m⁻²). Each treatment had two replications in randomized blocks, and each plot size was 2.1 × 2.5 m. Each plot was divided into two parts, one for measurement of the aerial biomass, the other for yield assessment. Field management followed normal agricultural practices. A total of 80 kg ha⁻¹ of nitrogen fertilizer was applied in three splits (40 kg ha⁻¹ before heading, and 20 kg ha⁻¹ at 2 weeks after heading). To monitor dry weight accumulation, we destructively sampled five representative plants from each plot. Plant samples were air dried for 2 months. The aerial parts of cells in the leaves and stem were harvested from each plot. Grain number and fertility of all panicles were measured. Grain weight was estimated from the grain number in 5 g of grain.

Statistical analysis was performed with a commercially available statistical package, JMP version 5.1 (SAS Institute Japan).

Transgenic Analysis

To generate an OsBRI1-KD construct, a partial OsBRI1 cDNA containing the juxtamembrane region, kinase domain, and C-terminal region was amplified by PCR and inserted between the rice actin promoter and the gene for the extracellular domain of the receptor kinase BRI1. 

Table IV. Estimation of yield potential of wild-type and OsBRI1-KD transgenic rice

<table>
<thead>
<tr>
<th>Line</th>
<th>Density</th>
<th>Potential Grain Yield</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>8.65</td>
<td>6.82</td>
</tr>
<tr>
<td>BDK11</td>
<td>22.2</td>
<td>9.40</td>
</tr>
<tr>
<td>BDK22</td>
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<tr>
<td>Wild type</td>
<td>4.44</td>
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<tr>
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<td>10.47</td>
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<tr>
<td>BDK22</td>
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<td>8.82</td>
</tr>
<tr>
<td>Wild type</td>
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<td>9.13</td>
</tr>
<tr>
<td>BDK11</td>
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</tr>
<tr>
<td>BDK22</td>
<td>66.7</td>
<td>11.52</td>
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Literature Cited


