Title

Brachytic 1 of Barley (*Hordeum vulgare* L.) Encodes the α Subunit of Heterotrimeric G Protein

Author names and Affiliations

Ai Ito^{1*}, Ayumi Yasuda^{1*}, Kosuke Yamaoka¹, Minase Ueda¹, Akira Nakayama¹, Suguru Takatsuto² and Ichiro Honda¹

¹Department of Biotechnology, Maebashi Institute of Technology, Maebashi, Gunma 371-0816, Japan

²Department of Chemistry, Joetsu University of Education, Joetsu, Niigata 943-8512, Japan

*These authors contributed equally to this paper

Corresponding author

Ichiro Honda

Tel +81-27-265-7367, Fax +81-27-265-3837, E-mail: ihonda@maebashi-it.ac.jp

Summary

Physiological and molecular biological analysis of the dwarf barley (*Hordeum vulgare* L.) mutant *brachytic 1* (*brh1*) was conducted. The root responses of *brh1* to brassinolide were weaker than those of wild type, but the responses of leaf segments of dark-grown plants were not. Responses of *brh1* to gibberellin A₃ were similar to or slightly stronger than those of wild type. Endogenous levels of these hormones in young seedlings were not clearly different between *brh1* and wild type. Skotomorphogeneses of *brh1* were similar to those of wild type. Some of these physiological characteristics of *brh1* resemble those of the dwarf rice (*Oryza sativa* L.) mutant *daikoku* (*dwarf1*; *d1*), whose dwarfism is caused by a mutation in the heterotrimeric G protein α (G α) subunit. A database search indicated that the barley G α gene is located near the locus where *Brh1* has already been genetically mapped. Sequences of the G α gene and cDNAs of five *brh1* alleles contained substitutions and a deletion that lead to the production of abnormal G α proteins. These results indicate that the phenotype of *brh1*, similarly to that of *d1*, is caused by mutations in an orthologous gene encoding G α .

Keywords

brachytic 1; brassinosteroid; *daikoku*; dwarf; gibberellin; G protein alpha

Abbreviations

BL, brassinolide; BR, brassinosteroid; *brh1*, *brachytic 1*; CS, castasterone; *d1*, *dwarf1* (*daikoku*); FW, fresh weight; G α , heterotrimeric GTP binding protein α subunit; GC-MS, gas chromatograph – mass spectrometry; GC-SIM, gas chromatograph – selective ion monitoring; GAx, Gibberellin Ax

Introduction

In cereals such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), mature ears at the top of the plants become heavy, which makes plants prone to lodging and reduces grain yield and quality. Dwarf or short-culm genes, which reduce plant height and may help to improve resistance to lodging, are well studied and are used in breeding. Wheat *RhtB1/D1* and rice *sd1* lead to semi-dwarf phenotypes because of defects in a negative regulator of plant hormone gibberellin (GA) signalling (*rhtB1/D1*) and in an enzyme of GA biosynthesis (*SD1*); they have been introduced into wheat and rice cultivars worldwide and have contributed to the increase in yield known as the 'Green Revolution' (Peng et al., 1999; Sasaki et al., 2002).

Dwarf or short-culm mutants are also useful for clarifying the mechanisms of plant architecture development. In barley, more than 300 such mutants have been isolated; allelism tests, backcrossing, and GA responses have been used to characterize these mutants (Franckowiak and Pecio, 1992). Those mutants were classified and some of them assigned to 86 gene loci, namely 18 *brachytic* (*brh*), 21 *breviaristatum* (*ari*), 4 *dense spike* (*dsp*), 29 *erectoides* (*ert*), 1 *uzu* (*uzu*), 5 *semidwarf* (*sdw*), 8 *slender dwarf* (*sld*) and others (Franckowiak et al., 2015). Although many gene loci have been mapped, only *uzu* and *sdw1* have been identified at the DNA level (Chono et al., 2003; Jia et al., 2009) before complete genome sequences of three barley cultivars (Barke, Bowman and Morex) were published in 2012 (The International Barley Genome Sequencing Consortium, 2012). Dockter et al. (2014) recently screened barley mutants of the brassinosteroid (BR) biosynthesis genes *brassinosteroid-6-oxidase* (*HvBRD*), *constitutive photomorphogenic dwarf* (*HvCPD*), and *diminute* (*HvDIM*); more than 20 mutants from *brh3*, *brh13*, *brh14*, *brh16*, and *brh18* were assigned to these three genes. DNA analyses of barley dwarf mutants without registration of gene loci mentioned above (Franckowiak et al., 2015) have been conducted, but the number of such studies is still limited (Chandler et al., 2002; Chandler and Harding, 2013; Gruszka et al., 2016; Helliwell et al., 2001).

The dwarf mutant of barley *brachytic1* (*brh1*) was first reported about 80 years ago (Powers, 1936; Swenson, 1940). Serial numbers have been assigned to 729 barley genetic stocks (BGS) of various phenotypes including dwarf (Franckowiak et al., 2015), and *brh1* is listed as BGS 1, suggesting that this is one of the traditionally known barley

mutants. A recessive *brh1* plant has short leaves, culms, spikes, awns, and kernel (Franckowiak and Lundqvist, 2013). Many *brh1* alleles were isolated and backcrossed to cv. Bowman, and various lines carrying *brh1* alleles in the Bowman background were established (Franckowiak and Lundqvist, 2013). Introduction of *brh1* highly increased lodging resistance without significant yield reduction (Dahleen et al., 2005). Genetic studies indicated that *Brh1* is located on chromosome 7HS, and its position is 7H bin 01 near the border with 7H bin 02 (Dahleen et al., 2005; Druka et al., 2011; Li et al., 2002); however, the gene underlying the *brh1* phenotype remained unknown.

The double mutant *brh1/uzu* shows a severe dwarf phenotype, and a physiological relationship between *Brh1* and *Uzu* has been suggested (Takahashi and Hayashi, 1956). Chono et al. (2003) reported that *Uzu* is a gene orthologous to *BR11* in Arabidopsis (Li and Chory, 1997) and *D61* in rice (Yamamuro et al., 2000), which encodes a BR receptor.

In this study, we examined the physiological responses of *brh1* to BR and GA, the endogenous levels of both groups of hormones, and skotomorphogeneses. Some of the physiological characteristics of *brh1* resembled those of a traditionally known dwarf mutant of rice, *daikoku* (*dwarf1*; *d1*) (Akemin, 1925; Kadam, 1937), that has a mutation in the heterotrimeric G protein α subunit ($G\alpha$) (Ashikari et al., 1999; Fujisawa et al., 1999). As the chromosomal location of the $G\alpha$ gene in barley fits well the position of *Brh1*, we analyzed the $G\alpha$ gene as a good candidate for *Brh1*. We report here that barley *brh1* carries mutations in the $G\alpha$ gene, similar to rice *d1*.

Materials and Methods

Plant materials

Five near-isogenic lines of barley carrying *brh1* (*brh1.a*, GSHO1820; *ari-i.38*, GSHO1821; *brh1.e*, GSHO1822; *brh1.t*, GSHO1823; *brh1.x*, GSHO1824; Franckowiak and Lundqvist, 2013) backcrossed to cv. Bowman were used. A *uzu* near-isogenic line backcrossed to Bowman (GSHO1963) was also used to experiments on skotomorphogeneses. All seeds were provided by the National Small Grain Collection of USDA (<http://www.ars.usda.gov/>) and propagated in the field near the Maebashi Institute of Technology.

Responses to plant hormones and skotomorphogeneses

Responses of root and leaf segments from dark-grown plants responses to brassinolide (BL) were examined according to Chono et al. (2003).

Skotomorphogeneses were examined as described by Saisho et al. (2004). To assess responses to GA, 15 seeds were sown in a plastic box (15 × 5 × 10 cm) containing commercial soil mixture and grown in a growth chamber (12 h light, ca. 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 25°C; 12 h dark, 20°C). Gibberellin A₃ (GA₃) solution (10 ml) was applied to the soil surface 7 and 11 days after sowing. Plant height and first leaf sheath length were measured 3 days after the second application.

Analysis of endogenous GAs and BRs

For GA analysis, levels of bioactive GA₁ and the biosynthetic precursors GA₂₀ and GA₁₉ were analysed. Seeds of Bowman and GSHO1820 were shown into a commercial soil mixture in a basket (32 cm × 50 cm × 6 cm) and grown in a greenhouse for 11 days (mean temperature, 21.7°C). Seedlings excluding roots were collected, homogenized in methanol, and filtered. After addition of 100 ng each of [²H₂]-GA₁, GA₁₉ and GA₂₀ (Olchemim, Olomouc, Czech Republic), the extracts were evaporated to dryness and further purified according to Shiraiwa et al. (2011). Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Selected Ion Monitoring (SIM) analyses were carried out in a mass spectrometer (JMS-GCmate, JEOL, Tokyo, Japan) equipped with a gas chromatograph (6890A, Agilent Technology, Santa Clara, CA) fitted with a capillary column (DB-1, 0.25 mm i.d. × 15 m, 0.25 μm film thickness, Agilent Technology). In GC-MS identification, mass spectra and retention time on GC of putative GAs and ²H₂-GAs were compared with those of authentic standards. The content of identified GAs was calculated from peak area ratios between ²H₀ and ²H₂ molecular ions of each GA examined by GC/SIM.

For BR analysis, levels of bioactive BR, castasterone (CS) and its biosynthetic precursor 6-deoxocastasterone (6-deoxoCS) were analysed. Both lines were sown as above and incubated for 10 days in the growth chamber (12 h light, ca. 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 25°C; 12 h in the dark, 20°C). Methanol extracts were prepared as above, 100 ng each

of [$^2\text{H}_6$]-CS and 6-deoxoCS were added to each extract, BRs were purified according to Chono et al. (2003) and analysed by GC-MS and GC-SIM as for GAs.

Retrieval of nucleotide sequences

A sequence of G α mRNA gene of barley (GenBank accession no. AF267485.2; coding sequence 1152 bp) was used as a query in searches on the IPK barley BLAST server (<http://webblast.ipk-gatersleben.de/barley/>). Sequences of the following BR signalling-related rice genes were retrieved from the Rice Annotation Project database (<http://rapdb.dna.affrc.go.jp/>): *OsBAK1* (Os08g0174700), *OsBRL1* (Os09g0293500), *OsBRL3* (Os08g0342300), *OsGSK1* (Os01g0205700) and *OsBZR1* (Os07g0580500). These sequences were used to detect chromosomal locations of their orthologous barley genes.

Sequence analysis

Seedlings were grown in the dark for 6 days (for DNA isolation) or 12 days (for RNA isolation) at 20°C. Total genomic DNA was extracted with a buffer containing 0.2 M Tris-HCl (pH 7.5), 0.25 M NaCl, 25 mM EDTA and 0.5% (w/v) SDS. Total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). cDNA was synthesised with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). PCR was performed with the primers listed in Supplementary Tables S1 for genomic DNA and S2 for cDNA. PCR was initiated by heating to 95°C for 2 min, followed by 32 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s. Amplified PCR products of the expected length were purified with a Nucleospin Gel and PCR Clean-up Kit (Macherey–Nagel, Düren, Germany). Purified PCR products were directly sequenced with a Big Dye Terminator Cycle sequencing kit and an ABI PRISM 3730XL Genetic Analyzer (both from Applied Biosystems, Foster City, CA). The PCR product amplified from cDNA of GSHO1822 was inserted into pGEM-T easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* strain DH5 α . The plasmid was purified by using a Wizard Plus SV miniprep DNA purification system (Promega) and sequenced. Sequences were analysed with the free software ApE.

Results

Hormonal responses and skotomorphogenesis

In many plant species, including barley, high BL concentrations inhibit root development and induce root curling (Chono et al., 2003). The root length gradually decreased with increasing BL concentrations in Bowman but not in a *brh1* line GSHO1820 (Fig. 1A–C), but plant height was not affected in both lines (Fig. 1D). Under continuous light or darkness, root growth was inhibited by BL in Bowman, but not in any of the *brh1* lines (GSHO1820–GSHO1824; Supplementary Fig. S1).

GSHO1820 showed slightly higher leaf unrolling responses than Bowman in 10^{-4} and 10^{-3} ppm BL treatments (Fig. 2A), similar to our previous report (Honda et al., 2003). The response of GSHO1821 was slightly weaker than those of the other *brh1* lines at 10^{-4} ppm BL, but did not clearly differ at 10^{-3} or 10^{-2} ppm BL (Supplementary Fig. S2A).

Plant height (Fig. 2B) and leaf sheath length (Fig. 2C) gradually increased with the increase in GA₃ in both Bowman and GSHO1820. At 10 ppm GA₃, plant height relative to untreated controls was 1.09 in Bowman and 1.37 in GSHO1820, and relative first leaf sheath length was 1.28 in Bowman and 1.30 in GSHO1820, indicating that the relative responses of GSHO1820 were similar to or slightly higher than those of Bowman. Responses of other *brh1* lines were similar (Supplementary Fig. S2B, C).

The internodes of dark-grown Bowman and GSHO1820 plants elongated similarly, but those of GSHO1963 (*uzu*) did not elongate (Fig. 3). Internode elongations in the other *brh1* lines were also similar to those in Bowman (Supplementary Fig. S3). Plant height of all 5 *brh1* lines was similar and significantly lower than that of Bowman (Supplementary Fig. S4).

Endogenous hormone levels

The levels of GA₁ and GA₁₉ were slightly lower in GSHO1820 (GA₁, 0.32 ng/g fresh weight (FW); GA₁₉, 28.7 ng/g FW) than in Bowman (GA₁, 0.48 ng/g FW; GA₁₉, 36.6 ng/g FW). The level of GA₂₀ was slightly higher in GSHO1820 (0.75 ng/g FW) than in Bowman (0.64 ng/g FW). The level of CS was slightly lower in GSHO1820 (0.058 ng/g FW) than in Bowman (0.080 ng/g FW), whereas that of 6-deoxoCS was slightly higher (0.47 and 0.28 ng/g FW, respectively) (Table 1).

Elucidation of the Brh1 gene and analysis of genomic DNA

Neither of the 5 barley orthologues of rice genes related to BR signalling fit the reported genomic location of *Brh1*, whereas the position of barley $G\alpha$ gene AF257485.2 located on chromosome 7H at 9.065 cM was close to that one of *Brh1* (Dahleen et al., 2005; Druka et al., 2011; Li et al., 2002). Therefore, we investigated the genomic region of AF267485.2 in more detail. On the IPK barley BLAST server, a 5195-bp Barke_contig_319927 (plus strand) contained the entire sequence of AF267485.2 and included 13 exons and 12 introns (3780 bp, positions 935–4714 in the contig). The corresponding sequence of Bowman in this region was divided into 3 contigs (Bowman_contig_924199, 214154 and 16039; with an 85-bp gap between contig_214154 and 16039); the only nucleotide substitution (A to C at position 1366 of Barke_contig_319927) was located in the putative intron 2 of the $G\alpha$ gene. As no clear PCR amplification products were obtained for the whole gene, it was divided into 4 regions covering all exons and the neighbouring introns and amplified. The corresponding PCR products were purified and the sequences were analysed.

As compared to the sequence of Barke_contig_319927, a 2-bp deletion resulted in a frame shift in exon 13, and stop codon was not detected within the analysed sequence of GSHO1820. In GSHO1824, an A to T substitution was identified in exon 13, leading to a premature stop codon. In GSHO1821 and GSHO1823, 1-bp changes (AG to AA in GSHO1821 and to TG in GSHO1823) were found at the putative end of the splicing site in intron 8 (just before exon 9). In GSHO1822, a 1-bp change (AG to AA) was found at the putative end of the splicing site in intron 4 (just before exon 5). In addition, a 1-bp change that led to an amino acid change (Leu 102 to Pro) was found in all *brh1* lines except GSHO1822 (Fig. 4A). The sequences of Bowman we determined did not differ from those on the IPK barley BLAST server. Silent mutations found in exons of the five *brh1* lines are listed in Supplementary Table S3.

cDNA analysis

In addition to the sequence AF267495.2, a full-length cDNA sequence of barley $G\alpha$ (GenBank accession no. AK249881.1; clone FLbaf50i02 from cv. Haruna Nijo) has

been reported (Sato et al., 2009). A full-length coding sequence of Gα (MLOC_67224.3; 1179 bp, without stop codon) is also registered on the IPK barley BLAST server; this sequence has a 30-bp extension before the first ATG codon of AF267485.2. Based on the AK249881.1 sequence, primer pairs covering MLOC_67224.3 were designed and used to amplify products from the 5 *brh1* lines and Bowman. PCR products were obtained from Bowman (expected length, 1328 bp) and all *brh1* lines; that of GSHO1822 was slightly longer than the others (Fig. 5). In all *brh1* lines and Bowman, the 30-bp added sequence was identical to that of MLOC_67224.3. In GSHO1820 and GSHO1824, the same changes in exon 13 were found as in their genome sequences (Fig. 4B). In GSHO1821 and GSHO1823, 11-bp insertions between exons 8 and 9 were found. These sequences were identical to those of the end of intron 8 and the sequences just before them were AG, suggesting production of mis-spliced mRNAs in both lines (Supplementary Fig. S5A). In GSHO1822, a mixture of sequences between exons 4 and 5 indicated the presence of multiple splicing variants. Purified PCR products from GSHO1822 were then cloned and the sequences of five clones were examined. In clones 2–4, 74-bp sequences at the end of intron 4 remained; 23 bp from the end of intron 3 (clone 1), 22 bp from the end of intron 5 (clone 3) and the entire intron 12 (clone 5) also remained. The entire intron 4 remained in clone 5, and the first 16 bp of exon 5 were deleted together with intron 4 in clone 1 (Supplementary Figs. S5B, S6). All these spliced sequences were terminated by AG, indicating that mis-splicing occurred at various positions of the mRNA in GSHO1822. Mis-splicing may lead to premature stop codons and production of abnormal proteins in GSHO1821, GSHO1822 and GSHO1823 (Fig. 4B, Supplementary Fig. S6).

Discussion

Physiological characteristics of brh1

Root growth inhibition by high concentrations of BL is a well-known response in many plants including rice and barley; this effect is not observed in rice *d61* and barley *uzu*, which are defective in the BR receptor (Chono et al., 2003; Yamamuro et al., 2000). The root growth of Gα mutants of Arabidopsis (*gpa1*; Ullah et al., 2002) and rice (*d1*; (Oki et al., 2009a; Wang et al., 2006) is also not inhibited by BR treatment. Root growth

of Bowman but not *brh1* lines was clearly inhibited by 0.1 ppm BL (Fig. 1, Supplementary Fig. S1); therefore, *brh1* roots are less sensitive to BL than Bowman roots. This root response of *brh1* suggested that *Brh1* encodes a component of BR signalling such as Gα.

Leaf unrolling test is very sensitive and specific for the detection of response of barley and wheat to BRs (Honda et al., 2003; Wada et al., 1985); it corresponds to the lamina inclination test in rice. In this test, both *d61* and *uzu* did not respond to BRs (Chono et al., 2003; Yamamuro et al., 2000). Honda et al. (2003) reported that *brh1* lines showed a slightly higher response than Bowman in this assay, and this finding was re-confirmed in this study (Fig. 2A, Supplementary Fig. S2A). In rice, Wang et al. (2006) reported that the response was weaker in *d1* line *DK22* (Nipponbare background) than in the wild type. However, in that study, which used 10^{-6} M 24-epibrassinolide (24-epiBL), the lamina angles were ca. 140° (treated *d1*), 180° (treated wild type), 45° (untreated *d1*) and 110° (untreated wild type). A possible interpretation of these results may be that *d1* has similar or higher sensitivity than wild type. Oki et al. (2009a, 2009b) reported weak response of another *d1* line *T65d1* (Taichun 65 background) to 24-epiBL in this test, in clear contrast to the report of Wang et al. (2006).

In our GA application experiments, leaf sheath of *brh1* lines elongated similarly or slightly more than those of the wild type (Fig. 2B, C, Supplementary Fig. S2B, C), in agreement with a previous report that shoot length of the dwarf mutant *ari-i.38*, whose gene was introduced to GSHO1821, treated with 10 ppm GA₃ was 229% of the untreated control, whereas that of the parental line was 204% (Börner, 1996). Rice *d1* previously has been recognized as a GA-related mutant. Ueguchi-Tanaka et al. (2000) reported that in the aleurone cells of *d1*, α-amylase induction was lower than that of the wild type. GA sensitivity of second leaf sheath elongation in *d1* was similar to that of the wild type, but the response of internode elongation to GA was much lower in *d1*. On the basis of these results, they proposed that two Gα signalling pathways, only one of which is involved in GA signalling, exist in rice.

Morphology of dark-grown plants (skotomorphogenesis) is another criterion to determine whether a mutant is BR-sensitive. Internodes of wild-type rice and barley

grown in the dark elongate well, while those of *d61* and *uzu* do not (Saisho et al., 2004; Yamamuro et al., 2000). In our study, internodes of all *brh1* lines and Bowman elongated similarly (Fig. 3, Supplementary Fig. S3). Wang et al. (2006) reported that the internode of *d1 DK22* elongated well, but Oki et al. (2009a) reported that the internode of T65*d1* did not elongate, probably due to the difference in the recurrent parents of these lines. Arabidopsis *gpa1* mutant grown in the dark is partially de-etiolated (Ullah, 2001); internode elongation in dark-grown rice and barley was interpreted as similar to the etiolation response of hypocotyls in dicot plants such as Arabidopsis. Therefore, leaf unrolling response to BRs and skotomorphogenesis of barley *brh1* were similar to those of rice *d1 DK22* reported by Wang et al. (2006).

Our results indicate that the *brh1* mutation has no significant effects on endogenous levels of GAs and BRs in 10 to 11-day-old seedlings. The accumulation of GA₁ and GA₂₀ in the GA signalling mutant *RhtB1* (Webb et al., 1998), and CS and the biosynthetic precursors in the BR signalling mutants *d61* (Yamamuro et al. 2000) and *uzu* (Chono et al. 2003) suggests that *brh1* is different in this respect. Ueguchi-Tanaka et al. (2000) reported that the levels of GA₁ and their biosynthetic precursors were higher in the second and third internodes of *d1* than in those of the wild type. Oki et al. (2009a) reported that the levels of CS and their biosynthetic precursors in young florets did not differ between *d1* and the wild type. There are no other reports on endogenous GAs and BRs in *d1*. Although the results on effects of GAs on *d1* differed from our results for *brh1*, this difference can be disregarded because of different plant ages, and tissues analysed.

The longitudinal length of the *d1* seeds is shorter than that of wild type, but the lateral length is not (Oki et al., 2009a, 2009b); reduction of the longitudinal length of *brh1* seeds has also been reported (Franckowiak and Lundqvist, 2013). Dwarfism of *d1* and *brh1* is mainly caused by the reduction in cell number, not cell elongation (Swenson, 1940; Ueguchi-Tanaka et al., 2000).

Some physiological traits of *brh1* resembled those of *d1*, the phenotype of which is caused by a mutation in *Gα* (Ashikari et al., 1999; Fujisawa et al., 1999). Barley *Gα* gene was located on chromosome 7H close to *Brh1*, the position of which has already been reported (Dahleen et al., 2005; Druka et al., 2011; Li et al., 2002), which suggested

G α as a good candidate for *Brh1*.

As both barley *brh1* (Powers, 1936; Swenson, 1940) and rice *d1* (Akemin, 1925; Kadam, 1937) are the traditionally known dwarf mutants, the finding of their physiological resemblance was surprising to us.

Sequence of G α and its role in barley

G α is a heterotrimeric G protein subunit coupled to the $\beta\gamma$ dimer. G α is activated by G protein-coupled receptors that sense extracellular signals such as hormones, neurotransmitters, light and flavours, and transmit the signals to various intracellular signalling pathways. Many G α genes are known in animals, but only one G α gene exists in Arabidopsis and rice (Urano et al., 2013). Loss-of function mutants of G α are useful to clarify the physiological role of G α in plants; however, such mutants are available only in Arabidopsis (*gpa1*; Ullah et al. 2001), rice (*d1*; Ashikari et al. 1999, Fujisawa et al. 1999) and maize (*ct-2*; Bommert et al., 2013).

The sequences AF267485.2 and MLOC_67224.3 are identical except for the first 30 bp in MLOC_67224.3 starting with an ATG triplet, which are absent in AF267485.2. In rice, a similar 30-bp sequence starting with an ATG triplet upstream of the ATG was reported; the shorter sequence was proposed as G α in rice based on sequence similarity to those of Arabidopsis and tomato (Ishikawa et al., 1995). Here we also consider the shorter sequence, AF267485.2, encoding a polypeptide of 383 amino acids, as G α in barley. Nucleotide sequence identity between rice G α (GenBank accession no. L28001.1) and barley G α (AF267485.2) is 85% and protein sequence identity is 88%; both G α genes consist of 13 exons and 12 introns. Four *brh1* lines (except GSHO1822) have a point mutation (305T to C) that leads to an amino acid alteration (Leu102 to Pro) in G α (Fig. 4A). This point mutation was also found in the full-length cDNA sequence of G α (AK249881.1) from barley cv. Haruna Nijo (Sato et al., 2009). The culm length of Haruna Nijo (ca. 90 cm; Ministry of Agriculture, Forestry and Fisheries, 1985) is approximately similar to Bowman under normal growing conditions (Dahleen et al., 2005; Honda et al., 2003), and the awn length is similar to that of Bowman, but that of *brh1* is clearly shorter (Supplementary Fig. S7). Thus, this amino acid alteration may not affect the function of G α in plant morphology.

All five *brh1* alleles had various mutations that led to abnormal proteins. Only 11 C-terminal amino acids were changed and some amino acids were added in GSHO1820, and 20 C-terminal amino acids were deleted in GSHO1824 in comparison with Bowman. The predicted G α proteins were much shorter in GSHO1821, GSHO1822 and GSHO1823 than in Bowman (Fig. 4B). However, physiological responses and agronomic traits did not clearly differ among the five *brh1* lines (Figs. 1–3, Supplementary Figs. S1–S4). These results may indicate that all five *brh1* lines are loss-of-function mutants of G α . In rice, Oki et al. (2009b) examined 10 *d1* alleles and reported that *d1-4*, having one amino acid change in the first GTP binding site of G α , showed mild phenotype but the other 9 alleles showed severe phenotypes.

In animals, the C-terminal region of G α binds to the G protein–coupled receptor (Noel et al., 1993). In rice *d1* and Arabidopsis *gpa1*, mutations in C-terminal regions have not been identified, but in maize the *ct2-Ref* mutant with a clearly dwarf phenotype a 126-bp insertion in exon 14 (last exon) of G α was reported (Bommert et al. 2013). In barley, the C-terminal region of G α may be functionally important, because a short C-terminal deletion in GSHO1824 and a C-terminal modification in GSHO1820 (replacement and extension) led to similar biological responses to those of the other 3 *brh1* lines, which have large deletions at the C-termini.

To clarify the relationship between G α and BR receptor, double mutants *gpa1-2/bri1-5* in Arabidopsis (Gao et al., 2008) and *T65d1/d61-7* in rice (Oki et al., 2009a) have been developed and analysed. In Arabidopsis, the plant height of the double mutant was about half that of *bri1-5*, suggesting that *gpa1* enhances dwarfism of *bri1-5*. In rice, the plant height of the double mutant was smaller than that of *d61-7*, but seed length and weight did not differ between the double mutant and *T65d1*. Oki et al. (2009c) also analysed the transgenic plants derived from *T65d1* and *T65d1/d61-7*, in which a constitutively active form of G α was introduced, and they proposed that G α mainly functions separately from the BR receptor cascade and enhances some of the BR responses via an unidentified signalling pathway. In barley, Takahashi and Hayashi (1956) reported that stem length of the double mutant *brh1/uzu* is about one-seventh of that of each single mutant. Clearly stronger effects in double mutants were also observed for coleoptile and awn length, but ear and grain length was only slightly

affected. These differences may indicate that BR receptor and G α may act in coordination in some processes and independently in others.

Information available on G α signalling in animals vastly exceeds that available in plants (Trusov and Botella, 2016; Urano et al., 2013). Although traditionally known, *brh1* have never been introduced into practical breeding programs. In addition to the 5 *brh1* lines examined here, other lines of *brh1* were also reported and are available to researchers (Franckowiak and Lundqvist 2013). Further analyses of *brh1* using various alleles and studies to find additional *brh1* alleles may provide new information about the role of G α in plants and utilization of the *brh1*.

Conclusion

In this study, we demonstrated that the *Brh1* gene encodes barley G α . Surprisingly, the two most traditional barley and rice dwarf mutants carry mutations in orthologous genes, G α . Future studies based on our findings may help to clarify the physiological role of G α in plants as well as utilize *brh1*.

Acknowledgement

We are indebted to the National Small Grain Collection of USDA for providing the Bowman and *brh1* backcross lines, which were established and donated by Dr. J. D. Franckowiak. We thank Dr. Makiko Chono, Dr. Yasuhiro Suzuki and Mr. Takahiro Sekiwa for providing the plant materials used in this work. We also thank all the members of our laboratory and colleagues in the field for plant cultivation. This work was partly supported by the priority research fund of the Maebashi Institute of Technology.

References

- Akemin, M., 1925. Genetics of rice dwarf. Rep. Japanese Assos. Adv. Sci. 1, 308–314 (in Japanese).
- Ashikari, M., Wu, J., Yano, M., Sasaki, T., Yoshimura, A., 1999. Rice gibberellin-insensitive dwarf mutant gene *Dwarf 1* encodes the α -subunit of GTP-binding protein. Proc. Natl. Acad. Sci. U. S. A. 96, 10284–10289.

- Bommert, P., Je, B. Il, Goldshmidt, A., Jackson, D., 2013. The maize *Gα* gene *COMPACT PLANT2* functions in *CLAVATA* signalling to control shoot meristem size. *Nature* 502, 555–558.
- Börner, A., 1996. GA response in semidwarf barley. *Barley Genet. Newsl.* 25, 24–26.
- Chandler, P.M., Harding, C.A., 2013. ‘Overgrowth’ mutants in barley and wheat: New alleles and phenotypes of the ‘Green Revolution’ *DELLA* gene. *J. Exp. Bot.* 64, 1603–1613.
- Chandler, P.M., Marion-Poll, A., Ellis, M., Gubler, F., 2002. Mutants at the *Slender1* locus of barley cv Himalaya. Molecular and physiological characterization. *Plant Physiol.* 129, 181–190.
- Chono, M., Honda, I., Zeniya, H., Yoneyama, K., Saisho, D., Takeda, K., Takatsuto, S., Hoshino, T., Watanabe, Y., 2003. A semidwarf phenotype of barley uzu results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. *Plant Physiol.* 133, 1209–1219.
- Dahleen, L.S., Vander Wal, L.J., Franckowiak, J.D., 2005. Characterization and molecular mapping of genes determining semidwarfism in barley. *J. Hered.* 96, 654–662.
- Dockter, C., Gruszka, D., Braumann, I., Druka, A., Druka, I., Franckowiak, J., Gough, S.P., Janeczko, A., Kurowska, M., Lundqvist, J., Lundqvist, U., Marzec, M., Matyszczyk, I., Muller, A.H., Oklestkova, J., Schulz, B., Zakhrebekova, S., Hansson, M., 2014. Induced variations in brassinosteroid genes define barley height and sturdiness, and expand the green revolution genetic toolkit. *Plant Physiol.* 166, 1912–1927.
- Druka, A., Franckowiak, J., Lundqvist, U., Bonar, N., Alexander, J., Houston, K., Radovic, S., Shahinnia, F., Vendramin, V., Morgante, M., Stein, N., Waugh, R., 2011. Genetic dissection of barley morphology and development. *Plant Physiol.* 155, 617–627.
- Franckowiak, J.D., Kleinhofs, A., Lundqvist, U., 2015. Descriptions of barley genetic stocks for 2015. *Barley Genet. Newsl.* 45, 32–79.
- Franckowiak, J.D., Lundqvist, U., 2013. Descriptions of barley genetic stocks for 2013. *Barley Genet. Newsl.* 43, 1–223.
- Franckowiak, J.D., Pecio, A., 1992. Coordinator’s report: Semidwarf genes: A listing of

- genetic stocks. *Barley Genet. Newsl.* 22, 116–126.
- Fujisawa, Y., Kato, T., Ohki, S., Ishikawa, A., Kitano, H., Sasaki, T., Asahi, T., Iwasaki, Y., 1999. Suppression of the heterotrimeric G protein causes abnormal morphology, including dwarfism, in rice. *Proc. Natl. Acad. Sci. U. S. A.* 96, 7575–7580.
- Gao, Y., Wang, S., Asami, T., Chen, J.-G., 2008. Loss-of-function mutations in the *Arabidopsis* heterotrimeric G-protein α subunit enhance the developmental defects of brassinosteroid signaling and biosynthesis mutants. *Plant Cell Physiol.* 49, 1013–1024.
- Gruszka, D., Gorniak, M., Glodowska, E., Wierus, E., Oklestkova, J., Janeczko, A., Maluszynski, M., Szarejko, I., 2016. A reverse-genetics mutational analysis of the barley *HvDWARF* gene results in identification of a series of alleles and mutants with short stature of various degree and disturbance in BR biosynthesis allowing a new insight into the process. *Int. J. Mol. Sci.* 17.
- Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S., Peacock, W.J., 2001. The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proc. Natl. Acad. Sci. U. S. A.* 98, 2065–2070.
- Honda, I., Zeniya, H., Yoneyama, K., Chono, M., Kaneko, S., Watanabe, Y., 2003. *Uzu* mutation in barley (*Hordeum vulgare* L.) reduces the leaf unrolling response to brassinolide. *Biosci. Biotechnol. Biochem.* 67, 1194–1197.
- Ishikawa, A., Tsubouchi, H., Iwasaki, Y., Asahi, T., 1995. Molecular cloning and characterization of a cDNA for the α subunit of a G protein from rice. *Plant Cell Physiol.* 36, 353–359.
- Jia, Q.J., Zhang, J.J., Westcott, S., Zhang, X.Q., Bellgard, M., Lance, R., Li, C.D., 2009. GA-20 oxidase as a candidate for the semidwarf gene *sdw1/denso* in barley. *Funct. Integr. Genomics* 9, 255–262.
- Kadam, B.S., 1937. Genes for dwarfing in rice. *Nature* 139, 1070.
- Li, J., Chory, J., 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90, 929–938.
- Li, M., Pan, Y., Li, A.-S., Kudrna, D., Kleinhofs, A., Ming, L., Yi, P., An-Sheng, L., Kudrna, D., Kleinhofs, A., 2002. Fine mapping of a semi-dwarf gene *brachytic 1* in barley. *Acta Genet. Sin.* 29, 634–637.
- Ministry of Agriculture, Forestry and Fisheries. 1985. Haruna Nijyo, In: New variety of

- wheat and barley, 378–382 (in Japanese).
- Noel, J.P., Hamm, H.E., Sigler, P.B., 1993. The 2.2 Å crystal structure of transducin- α complexed with GTP γ S. *Nature* 366, 654–663.
- Oki, K., Inaba, N., Kitagawa, K., Fujioka, S., Kitano, H., Fujisawa, Y., Kato, H., Iwasaki, Y., 2009a. Function of the α subunit of rice heterotrimeric G protein in brassinosteroid signaling. *Plant Cell Physiol.* 50, 161–172.
- Oki, K., Inaba, N., Kitano, H., Takahashi, S., Fujisawa, Y., Kato, H., Iwasaki, Y., 2009b. Study of novel d1 alleles, defective mutants of the α subunit of heterotrimeric G-protein in rice. *Genes Genet. Syst.* 84, 35–42.
- Oki, K., Kitagawa, K., Fujisawa, Y., Kato, H., Iwasaki, Y., 2009c. Function of α subunit of heterotrimeric G protein in brassinosteroid response of rice plants. *Plant Signal. Behav.* 4, 126–128.
- Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, a J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D., Harberd, N.P., JinRong, P., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, a J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D., Harberd, N.P., 1999. ‘Green revolution’ genes encode mutant gibberellin response modulators. *Nature* 400, 256–261.
- Powers, L., 1936. The Nature of the interaction of genes affecting four quantitative characters in a cross between *Hordeum deficiens* and *Hodeum vulgare*. *Genetics* 21, 398–420.
- Saisho, D., Tanno, K., Chono, M., Honda, I., Kitano, H., Takeda, K., 2004. Spontaneous brassinolide-insensitive barley mutants ‘uzu’ adapted to east asia. *Breed. Sci.* 54, 409–416.
- Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., Khush, G.S., Kitano, H., Matsuoka, M., 2002. A mutant gibberellin-synthesis gene in rice. *Nature* 416, 701–702.
- Sato, K., Shin-I, T., Seki, M., Shinozaki, K., Yoshida, H., Takeda, K., Yamazaki, Y., Conte, M., Kohara, Y., 2009. Development of 5006 full-length cDNAs in barley: A tool for accessing cereal genomics resources. *DNA Res.* 16, 81–89.
- Shiraiwa, N., Kikuchi, K., Honda, I., Shigyo, M., Yamazaki, H., Tanaka, D., Tanabe, K.,

- Itai, A., 2011. Characterization of endogenous gibberellins and molecular cloning of a putative gibberellin 3-oxidase gene in bunching onion. *J. Am. Soc. Hortic. Sci.* 136, 382–388.
- Swenson, S.P., 1940. Genetic and cytologic studies of a brachytic mutation in barley. *J. Agric. Res.* 60, 687–714.
- Takahashi, R., Hayashi, J., 1956. Genetic studies of barley crosses involving two brachytic genes. *Japanese J. Genet.* 31, 250–258.
- The International Barley Genome Sequencing Consortium, 2012. A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491, 711–716.
- Trusov, Y., Botella, J.R., 2016. Plant G-proteins come of age: breaking the bond with animal models. *Front. Chem.* 4, 24. doi:10.3389/fchem.2016.00024
- Ueguchi-Tanaka, M., Fujisawa, Y., Kobayashi, M., Ashikari, M., Iwasaki, Y., Kitano, H., Matsuoka, M., 2000. Rice dwarf mutant *d1*, which is defective in the α subunit of the heterotrimeric G protein, affects gibberellin signal transduction. *Proc. Natl. Acad. Sci. U. S. A.* 97, 11638–11643.
- Ullah, H., Chen, J.-G., Young, J.C., Im, K.-H., Sussman, M.R., Jones, A.M., 2001. Modulation of Cell Proliferation by Heterotrimeric G Protein in Arabidopsis. *Science*. 292, 2066–2069.
- Ullah, H., Chen, J.G., Wang, S.C., Jones, A.M., 2002. Role of a heterotrimeric G protein in regulation of Arabidopsis seed germination. *Plant Physiol.* 129, 897–907.
- Urano, D., Chen, J.-G., Botella, J.R., Jones, A.M., 2013. Heterotrimeric G protein signalling in the plant kingdom. *Open Biol.* 3, 120186.
- Wada, K., Kondo, H., Marumo, S., 1985. A simple bioassay for a wheat leaf-unrolling test. *Agric. Bol. Chem.* 49, 2249–2251.
- Wang, L., Xu, Y.-Y., Ma, Q.-B., Li, D., Xu, Z.-H., Chong, K., 2006. Heterotrimeric G protein α subunit is involved in rice brassinosteroid response. *Cell Res.* 16, 916–922.
- Webb, S.E., Appleford, N.E.J., Gaskin, P., Lenton, J.R., 1998. Gibberellins in internodes and ears of wheat containing different dwarfing alleles. *Phytochemistry* 47, 671–677.
- Yamamuro, C., Ihara, Y., Wu, X., Noguchi, T., Fujioka, S., Takatsuto, S., Ashikari, M.,

Kitano, H., Matsuoka, M., 2000. Loss of function of a rice *brassinosteroid insensitive1* homolog prevents internode elongation and bending of the lamina joint. *Plant Cell* 12, 1591–1606.

Table 1. Endogenous GA and BR levels in Bowman and *brh1* (GSHO1820)

	Bowman			GSHO1820		
	Exp. 1	Exp. 2	Mean	Exp. 1	Exp. 2	Mean
GA						
GA ₁₉	30.3	41.9	36.6	20.7	36.6	28.7
GA ₂₀	0.66	0.61	0.64	0.74	0.76	0.75
GA ₁	0.59	0.37	0.48	0.32	0.32	0.32
BR						
6-deoxoCS	0.17	0.39	0.28	0.46	0.49	0.47
CS	0.076	0.083	0.080	0.046	0.070	0.058

Hormone levels are indicated in nanograms per gram fresh weight. The results from two independent experiments are shown.

Figure Legend

Fig. 1. Effects of brassinolide (BL) treatment. (A, B) Morphology of Bowman (A) and the *brh1* near-isogenic line GSHO1820 (B) treated with BL. Scale bars = 10 cm. (C, D) Effects of BL on root length (C) and plant height (D). Error bars indicate standard error ($n = 30$).

Fig. 2. Responses of Bowman and GSHO1820 to BL and GA₃. (A) Leaf unrolling in response to BL treatment; $n = 10$. (B, C) Responses to GA₃ treatment: changes in plant height and (B) first leaf sheath length (C); $n = 15$. Values next to the symbols indicate ratios to control at 10 ppm GA₃. Error bars indicate standard error.

Fig. 3. Skotomorphogenesis of *brachytic1* mutant. Plants were grown in soil in the dark for 11 days at 25°C. Arrows indicate coleoptile tops, arrowheads indicate internodes, scale bar = 5 cm.

Fig. 4. Positions of mutations in the $G\alpha$ gene in five *brh1* lines. (A) Genomic sequence (Barke_contig_319927; 5915 bp). Boxes, $G\alpha$ exons (predicted from GenBank accession No. AF267485.2). Vertical arrows indicate alterations of the $G\alpha$ protein sequence. Mutations: c., position in the coding sequence; p., position in the protein sequence; X, stop codon. (B) Protein sequence (383 aa) predicted from cDNA. Shaded box, receptor-binding region; black arrows above the diagram, GTP-binding site; white arrow, CTX-binding site; arrows from boxes with line numbers, position of the same sequence to Bowman are terminated; dotted arrows, sequences different from those of Bowman. The length of the protein in GSHO1820 is unknown (no stop codon was found within the analyzed region). In GSHO1822, multiple variants were observed and only most abundant one is shown. The Leu102Pro substitution resulting from SNPs in GSHO1820, 21, 23 and 24 is not shown.

Fig. 5. PCR products amplified on cDNAs of $G\alpha$ prepared from Bowman and five *brh1* lines. The sequences of primers are listed in Supplementary Table S2. Triangle indicates the expected products.

Fig. 1

Fig. 2

Fig. 3.

Fig. 4.

Fig.5.

Marker Bowman GSHO1820 GSHO1821 GSHO1822 GSHO1823 GSHO1824