Running title
Variation in Barley Brassinosteroid Genes

Corresponding author
Mats Hansson, Copenhagen Plant Science Center, University of Copenhagen, Thorvaldsensvej 40, room T249, DK-1871 Frederiksberg C, Copenhagen, Denmark, Phone +45 35338132, E-mail mats.hansson@plen.ku.dk

Research area
Primary: Genes, development and evolution
Secondary: Signaling and response
Title
Induced Variations in Brassinosteroid Genes Define Barley Height and Sturdiness, and Expand the “Green Revolution” Genetic Toolkit

Authors
Christoph Dockter a,2, Damian Gruszka b,2, Ilka Braumann a, Arnis Druka c, Ilze Druka c, Jerome Franckowiak d, Simon P. Gough a, Anna Janeczko e, Marzena Kurowska b, Joakim Lundqvist a, Udda Lundqvist f, Marek Marzec b, Izabela Matyszczak a, André H. Müller a,3, Jana Okleštíková g, Burkhard Schulz h, Shakhira Zakhрабекова a, and Mats Hansson a,4,*

Affiliations
a Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-1799 Copenhagen V, Denmark.
b Department of Genetics, Faculty of Biology and Environment Protection, University of Silesia, Jagiellonska 28, PL-40-032 Katowice, Poland.
c The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom.
d Department of Agriculture, Fishery and Forestry, Agri-Science Queensland, Hermitage Research Facility, Warwick, Queensland 4370, Australia.
e Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Krakow, Poland.
g Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University and Institute of Experimental Botany ASCR, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic.
h Purdue University, Horticulture Building 109, 625 Agricultural Mall Drive, West Lafayette, Indiana, 47907, USA.

One sentence summary
Historic barley short-culm mutants belonging to different mutant groups are deficient in brassinosteroid genes, which are attractive targets for development of lodging-resistant crop plants.
Footnotes

1 This work was made possible thanks to generous support from the Carlsberg Foundation, GUDP (Denmark, 34009-12-0522), DFG (Germany, DO1482/1-1), MNiSW (Poland, PBZ-MNiSW-2/3/2006/8, IP2011-016471), NSF (USA, CAREER-IOS 1054918), MEYS (Czech Republic, LK 21306), and European Commission (MC-ITN CropLife and ERA-PG BARCODE).

2 C.D. and D.G. contributed equally to this work.

3 Present address: Department of Structural Biology, Stanford University School of Medicine, Fairchild Science Building, D143, Stanford, California, 94305, USA.

4 Present address: Copenhagen Plant Science Center, University of Copenhagen, Thorvaldsensvej 40 room T249, DK-1871 Frederiksberg C, Copenhagen, Denmark.

* Address correspondence to mats.hansson@plen.ku.dk
ABSTRACT
Reduced plant height and culm robustness are quantitative characteristics important for assuring cereal crop yield and quality under adverse weather conditions. A very limited number of short-culm mutant alleles were introduced into commercial crop cultivars during the “Green Revolution”. We identified phenotypic traits, including sturdy culm, specific for deficiencies in brassinosteroid biosynthesis and signaling in semi-dwarf mutants of barley (*Hordeum vulgare* L.). This set of characteristic traits was explored to perform a phenotypic screen of near-isogenic short-culm mutant lines from the *brachytic, breviaristatum, dense spike, erectoides, semi-brachytic, semidwarf,* and *slender dwarf* mutant groups. *In silico* mapping of brassinosteroid-related genes in the barley genome in combination with sequencing of barley mutant lines assigned more than 20 historic mutants to three brassinosteroid-biosynthesis genes (Hv-*BRD*, Hv-*CPD*, Hv-*DIM*) and one brassinosteroid-signaling gene (Hv-*BRI1*). Analyses of F2- and M2-populations, allelic crosses, and modeling of non-synonymous amino-acid exchanges in protein crystal structures gave a further understanding of the control of barley plant architecture and sturdiness by brassinosteroid-related genes. Alternatives to the widely used but highly temperature-sensitive *uzu1.a* allele of Hv-*BRI1* represent potential genetic building blocks for breeding strategies with sturdy and climate-tolerant barley cultivars.
INTRODUCTION

The introduction of dwarfing genes to increase culm sturdiness of cereal crops was crucial for the first “Green Revolution” (Hedden, 2003). The culms of tall cereal crops were not strong enough to support the heavy spikes of high-yielding cultivars, especially under high-nitrogen conditions. As a result, plants fell over, a process known as lodging. This caused losses in yield and grain quality issues due to fungal infections, mycotoxin contamination, and pre-harvest germination (Rajkumara, 2008). Today, a second Green Revolution is on its way, to revolutionize the agricultural sector and to ensure food production for a growing world population. Concurrently, global climate change is expected to cause more frequent occurrences of extreme weather conditions, including thunderstorms with torrential rain and strong winds, thus promoting cereal culm breakage (Porter and Semenov, 2005; National Climate Assessment Development Advisory Committee, 2013). Accordingly, plant architectures that resist lodging remain a major crop-improvement goal and identification of genes that regulate culm length is required to enhance the genetic toolbox in order to facilitate efficient marker-assisted breeding.

The mutations and the corresponding genes that enabled the Green Revolution in wheat and rice have been identified (Hedden, 2003). They all relate to gibberellin metabolism and signal transduction. It is now known that other plant hormones such as brassinosteroids are also involved in the regulation of plant height. Knowledge of the molecular mechanisms underlying the effects of the two hormones on cell elongation and division has mainly come from studies in Arabidopsis (Bai et al., 2012). Mutant-based breeding strategies to fine-tune brassinosteroid metabolism and signaling pathways could improve lodging behavior in modern crops (Vriet et al., 2012) such as barley (Hordeum vulgare L.), which is the fourth most abundant cereal in both area and tonnage harvested (http://faostat.fao.org).

A short-culm phenotype in crops is often accompanied by other phenotypic changes. Depending on the penetrance of such pleiotropic characters, but also the parental background and different scientific traditions and expertise, short-culmed barley mutants were historically divided into groups, such as brh (for brachytic), ari (for breviaristatum), dsp (for dense spike), ert (for erectoides), uzu (for semi-brachytic), sdw (for semidwarf), or sld (for slender dwarf) (Franckowiak and Lundqvist, 2012). Subsequent mutant characterization was limited to intra-group screens and very few allelism tests between mutants from different groups have been
reported (Franckowiak and Lundqvist, 2012). Although the total number of short-culm barley mutants exceeds 500 (Franckowiak and Lundqvist, 2012), very few have been characterized at the DNA level (Helliwell et al., 2001; Jia et al., 2009; Chandler and Harding, 2013; Houston et al., 2013). One of the first identified haplotypes was “uzu barley” (Chono et al., 2003). The Uzu1 gene encodes the brassinosteroid hormone receptor and is orthologous to the BRI1 (for BRASSINOSTEROID-INSENSITIVE 1) gene of Arabidopsis, a crucial promoter of plant growth (Li and Chory, 1997). The uzu1.a allele has been used in East Asia for over a century and is presently distributed in winter barley cultivars in Japan, the Korean peninsula, and China (Saisho et al., 2004). Its agronomic importance comes from the short and sturdy culm that provides lodging resistance, and an upright plant architecture that tolerates dense planting.

Today, more than 50 different brassinosteroids have been identified in plants (Bajguz and Tretyn, 2003). Most are intermediates of the complex biosynthetic pathway (Shimada et al., 2001). Approximately nine genes code for the enzymes that participate in the biosynthetic pathway from episterol to brassinolide (Supplemental Figure 1 online). Brassinosteroid deficiency is caused by downregulation of these genes, but it can also be associated with brassinosteroid signaling. The first protein in the signaling network is the brassinosteroid receptor encoded by BRI1 (Li and Chory, 1997; Kim and Wang, 2010). In the present work, we show how to visually identify brassinosteroid-mutant barley plants and we describe more than 20 relevant mutations in four genes of the brassinosteroid biosynthesis and signaling pathways that can be used in marker-assisted breeding strategies.

RESULTS

Phenotypic Identification of Brassinosteroid Mutants in Barley

A large number of brh, ari, dsp, ert, uzu, sdw, and sld short-culm mutants have been induced in various barley cultivars, and many mutants within each group were found to be allelic (Franckowiak and Lundqvist, 2012). We hypothesized that mutants from different groups might be allelic too, but due to their different genetic backgrounds direct comparison of their mutant phenotypes was impossible. Recently, representative lines from these groups, along with many
other mutants, were crossed into the genetic background of the barley cultivar Bowman and
genotyped with up to 3,000 markers (Druka et al., 2011). By growing such near-isogenic lines in
parallel in the same environment, we were able to perform a comprehensive comparison of their
mutant phenotypes independently of their original genetic background or preconceptions derived
from previous historic classification (Figure 1).

The brassinosteroid receptor, encoded by \textit{Uzu1} (\textit{Hv-BRI1}), is known to regulate plant height in
barley (Chono et al., 2003). We used BW885, the near-isogenic line carrying the \textit{uzu1.a} allele
(Druka et al., 2011), as an ideotype for a barley brassinosteroid mutant. When grown at low
temperatures, the \textit{uzu1.a} mutant is a semi-dwarf with 80% of wild-type culm length (Figure 2).
The elongation of upper-stem internodes is particularly reduced while the stem diameter remains
unaltered. The overall plant architecture is more erect, with acute leaf-blade angles. Short-awned
spikes are compact with dense basal spikelets, and frequently with opposite spikelets in the tip
caused by irregular elongation of rachis internodes. Leaf margins and auricles of \textit{uzu1.a} have a
slightly undulating appearance, similar to the wavy leaf phenotype found in maize \textit{Wab1} mutants
(Hay and Hake, 2004). The same phenotypic detail, but to a more extreme extent, was found in
Bowman wild-type plants treated with propiconazole (Figure 2), a potent inhibitor of
brassinosteroid biosynthesis (Hartwig et al., 2012). In anatomical and microscopic analyses, we
found other phenotypic details (Supplemental Figure 2 online).

We used the unique combination of visible brassinosteroid-mutant characters once they were
established to perform a phenotypic screen of 160 Bowman near-isogenic lines with reduced
culm length. Sixteen lines fulfilled the brassinosteroid phenotype criteria. These mutants were
found in the \textit{brh}, \textit{uzu}, \textit{ari}, and \textit{ert} groups (Figure 3). We also tested the response of dark-grown
seedling leaves to exogenously applied brassinolide (Honda et al., 2003), in order to differentiate
potential brassinosteroid-signaling mutants from those with impaired brassinosteroid
biosynthesis. In this so-called leaf-unrolling test, the mutants were grown in darkness for six
days at 26°C and tightly rolled leaf segments were immersed in water or in a solution containing
the most active brassinosteroid, brassinolide, for 72 h. Mutants \textit{ari.256}, \textit{ert-ii.79}, and \textit{uzu1.a}
showed greatly reduced leaf-unrolling response to brassinolide (Figure 3D), suggesting defective
brassinosteroid signaling. In rice, a leaf lamina inclination assay has been successfully used to
identify brassinosteroid signaling mutants (Fujioka et al., 1998; Hong et al., 2003). We adopted this method to barley. Addition of brassinolide to the tip of barley seedlings resulted in an increased angle between the first leaf blade and culm in wild-type plants and those mutants that responded to exogenously applied brassinolide. Mutants insensitive in the leaf-unrolling assay also missed increased leaf inclination, an indication for a defect in brassinosteroid signaling (Supplemental Figure 3 online).

In order to test the robustness of the brassinosteroid-deficient characters for direct phenotypic screening, we studied 950 M2 plants of a chemically mutagenized doubled-haploid population of the barley line H930-36. Of 16 candidate plants with short-culm phenotypes, one showed the specific brassinosteroid-deficient characters. In the leaf-unrolling test, the mutant leaf segments showed reduced response to added brassinolide (Figure 4A-F). Thus, the mutant was grouped with ari.256, ert-ii.79, anduzu1.a, and selected for further analysis of the brassinosteroid-signaling gene, Hv-BRI1.

Connecting Brassinosteroid Biosynthesis Genes with Barley Mutant Loci

A rich variety of brassinosteroids are intermediates of the complex biosynthetic pathway (Supplemental Figure 1 online). After initial steps the pathway branches into an early or late C-6 oxidation route. Several enzymes have broad substrate specificity. Therefore, they catalyze multiple steps of the pathway. The final product is brassinolide, which is generated by conversion from castasterone. The oxidase catalyzing the final step has not been found in cereal grasses, like barley. In these plants castasterone was suggested to be the bioactive end product of the brassinosteroid biosynthetic pathway (Kim et al., 2008). We identified nine genes encoding enzymes of the pathway in the barley genome by homology searches between deduced polypeptide sequences of barley and Arabidopsis (Supplemental Figure 4 online). These were mapped on the barley genome in silico using the barley genome zipper (Mayer et al., 2011) and the barley physical map (International Barley Sequencing Consortium, 2012). The genomic locations were then compared with the mutant donor introgressions of the selected near-isogenic lines, which suggest that the mutants are alleles of three biosynthetic genes; Hv-BRD (for
BRASSINOSTEROID-6-OXIDASE), Hv-CPD (for CONSTITUTIVE PHOTOMORPHOGENIC DWARF), and Hv-DIM (for DIMINUTO), or of the brassinosteroid receptor gene Hv-BRI1 (Figure 5). This was tested by sequencing of the genes from available near-isogenic lines and historic barley mutants, and by performing allelic crosses.

Genetic Characterization of Hv-BRD Mutants

Hv-BRD, encoding a brassinosteroid-6-oxidase, is located in the telomeric region of the short arm of barley chromosome 2H (Figure 5, Supplemental Figure 5A online). The location of Hv-BRD is within the mutant donor introgressions of the near-isogenic lines ari-u.245, brh3.g, brh3.h, brh3.y, and ert-t.55. In the barley genome zipper, the common genetic donor interval of those lines contained 21 gene models but did not include the Hv-BRD ortholog of Brachypodium, rice, and sorghum. In EnsemblPlant (http://plants.ensembl.org), the same interval contained 68 gene models including Hv-BRD. Sequencing of Hv-BRD identified nonsense mutations in ari-u.245 (T1676A, Leu257*), brh3.g, and brh3.h (G2723A, Trp444*), and a point mutation in brh3.y (G2183A) causing a Gly353 to Asp modification in the dioxygen-binding site of Hv-BRD (Figure 6, Supplemental Figure 5B online, Dataset 1.1). Allelism between available brh3 and ert-t loci has been proven recently (Dahleen et al., 2005; Franckowiak and Lundqvist, 2012). In the present study, we additionally verified the allelic nature of ari-u.245 to the brh3 and ert-t groups by a cross between ari-u.245 and brh3.y. The F1 progeny, heterozygous for the parental mutations in Hv-BRD, clearly resembled the brassinosteroid-deficient phenotype (Figure 7A). We also identified mutations in brh3.i and ert-t.437, which have been reported as allelic mutants (Dahleen et al., 2005) but which are not represented in the collection of near-isogenic lines. The nonsense mutation in brh3.i was identical to brh3.g and brh3.h, and the point mutation in ert-t.437 was identical to brh3.y. Other genetic differences in Hv-BRD clearly indicate an independent history of ert-t.437 and brh3.y (Dataset 1.1). No mutation within the coding region of Hv-BRD could be found in BW324 (ert-t.55) or the original ert-t.55 mutant, even though the mutant was reported allelic to brh3 mutants (Dahleen et al., 2005) and to ert-t.437 (Franckowiak and Lundqvist, 2012). We therefore suggest that the ert-t.55 mutation is located outside the sequenced region of Hv-BRD, possibly in a regulatory element, or that the mutation has caused a
re-arrangement in the telomeric region of chromosome 2H, not detectable in our experimental set-up. Mutant ert-t.55 was induced by X-ray treatment (Dataset 1.1). BW093, the presumed near-isogenic line of brh3.i, had neither an introgression on chromosome 2H (Druka et al., 2011) nor harbored the brh3.i haplotype although it showed a short-culm phenotype (Dataset 1.1). In contrast to the original line brh3.i, BW093 was also missing the combination of brassinosteroid-mutant characters, indicating a diverse ancestry of the genotypes. The connection between mutations in Hv-BRD and the brassinosteroid-deficient phenotype, was further strengthened by an exact co-segregation of the phenotype with the brh3.g allele in an F2-mapping population. That is, 103 plants genotyped as homozygous brh3.g mutants had all a short awn length (range 4.5-8.1 cm, average ± standard deviation 6.8 ± 0.81 cm) in contrast to long awn length of 190 heterozygous mutants (10.5-14.6 cm, 12.6 ± 0.84 cm) and 90 wild-type plants (11.4-15.0 cm, 12.8 ± 0.90 cm).

**Genetic Characterization of Hv-CPD Mutants**

Hv-CPD, encoding the barley C-23α-hydroxylase CYP90A1, could not be localized on the physical map of the barley genome. Instead, the syntenic to Brachypodium, rice, and sorghum genomes (Mayer et al., 2011) was used to identify likely markers for Hv-CPD. Among mutants with a brassinosteroid-deficient phenotype, only brh13.p has an introgression region (Druka et al., 2011) co-localizing with the suggested position of Hv-CPD at 44.24 cM on chromosome 5H (Figure 5, Supplemental Figure 6A online). However, the introgression region of brh13.p is large and supposed to contain 1,380 gene models according to the barley genome zipper (Mayer et al., 2011) and 808 gene models according to EnsemblPlant (http://plants.ensembl.org) but only one brassinosteroid-biosynthesis gene, Hv-CPD. The near-isogenic line of brh18.ac was not included in the marker analysis by Druka et al. (Druka et al., 2011), but brh18.ac has previously been linked to brh13.p via the SSR markers Bmag0387 and Hv-LEU (Dahleen et al., 2005). The latter is anchored approximately 6 cM from Hv-CPD. We performed a cross between mutant lines brh13.p and brh18.ac. The F1 progeny, heterozygous for the parental mutations in Hv-CPD, clearly displayed a brassinosteroid-mutant phenotype, verifying that brh13.p and brh18.ac are allelic (Figure 7B). Sequencing of Hv-CPD showed brh13.p and brh18.ac to carry point
mutations (C2562T and C2807T, respectively) causing substitutions of highly conserved amino acid residues - Pro445 to Leu in *brh13.p* and Pro479 to Ser in *brh18.ac*. Pro445 and Pro479 are located within and close to the highly conserved heme binding site, respectively, in the C-terminal part of Hv-CPD (Figure 6, Supplemental Figure 6B online, Dataset 1.1). It was noted that 11 additional near-isogenic lines with a short-culm phenotype, but without other distinct brassinosteroid-mutant characters, have overlapping introgression regions with Hv-CPD (Dataset 1.1). Sequencing of Hv-CPD from these 11 lines revealed no mutations, which suggest that they are not deficient in Hv-CPD.

**Genetic Characterization of Hv-DIM Mutants**

The sequence of Hv-DIM, encoding the barley Δ^5^-sterol-Δ^24^-reductase DIMINUTO, corresponds directly to SNP marker 1_0547 in the telomere on the long arm of chromosome 7H. The six near-isogenic lines of *ari-o.40, brh.af, brh14.q, brh16.v, ert-u.56, and ert-zd.159* have a small, common genetic donor interval containing the Hv-DIM locus (Figure 5, Supplemental Figure 7A online). In addition, the six mutants show a brassinosteroid-deficient phenotype including a short culm mainly caused by a specific and extreme shortening of the second culm internode (Figure 3A). In the barley genome zipper (Mayer et al., 2011), the interval contained three gene models including the Hv-DIM ortholog of Brachypodium, rice, and sorghum. In EnsemblPlant (http://plants.ensembl.org), the same interval contained 27 gene models. Severe DNA changes were identified in the six mutants (Dataset 1.1). In *brh14.q*, induced by EMS treatment, a C628T substitution was detected resulting in the exchange Leu210 to Phe in the highly conserved FAD-binding site. Mutation *ert-zd.159* was found in the same domain (A530T, Glu177 to Val), while the mutant *ari-o.40* had a T1171A substitution resulting in a Trp391 to Arg exchange. Although being located in a domain of unknown function, residue Trp391 is highly conserved among plant species and also in comparison to the human ortholog of DIM, SELADIN-1 (Supplemental Figure 7B online) (Greeve et al., 2000). The deletions found in *brh16.v* (C3937 deleted, causing a frame shift followed by a premature stop codon), *ert-u.56* (exon 1 deleted, induced by X-rays) and *brh.af* (deletion of >12 kb, induced by neutrons) further verified Hv-DIM as the candidate gene. Crosses generated F1 progenies with identical phenotypes to their parental lines, verifying the
allelic character of the gene loci (Figure 7C). In addition to ari-o.40, six other alleles of ari-o are available. Hv-DIM mutations in ari-o.43 and ari-o.143 were identical to ari-o.40, whereas no mutations in Hv-DIM were found in ari-o.297, ari-o.301, ari-o.304, and ari-o.306 even though they showed a brassinosteroid-deficient phenotype. Crosses of ari-o.297, ari-o.301, ari-o.304, and ari-o.306 to ari-o.40 resulted in F1 progenies with a tall wild-type phenotype without brassinosteroid-mutant characters (Figure 7D). This confirmed that ari-o.297, ari-o.301, ari-o.304, and ari-o.306 are wrongly annotated alleles of Hv-DIM. Sequencing the latter four lines for Hv-CPD, Hv-BRD and Hv-BRI1 identified three mutants: non-synonymous point mutations in Hv-BRI1 in ari-o.297 (T158C, Phe53 to Ser; renamed zuu1.297) and ari-o.301 (T845A, Val282 to Asp; renamed zuu1.301), and a nonsense mutation in Hv-BRD in ari-o.304 (A2736T; Lys449*; renamed ari-u.304) (Figure 6). No mutation was found in ari-o.306 (renamed ari.306).

Genetic Characterization and Novel Alleles of Hv-BRI1

In addition to the zuu1 standard line BW885, the reported zuu1.a mutation (Chono et al., 2003) is also present in BW860 (sld1.a + zuu1.a) and BW912 (wst1.c + zuu1.a). These three near-isogenic lines delimit the Hv-BRI1 position to a shared interval covered by nine SNPs on chromosome 3H (Figure 5, Supplemental Figure 8A online). In EnsemblPlant (http://plants.ensembl.org), this interval contained 150 gene models and in the barley genome zipper 217 gene models, including the BRI1 ortholog of Brachypodium, rice, and sorghum. Sequencing of Hv-BRI1 from those lines revealed the expected single substitution A2612G (His857 to Arg), known as allele zuu1.a, which is found in North East Asian short-culm cultivars and landraces such as Akashinriki (Chono et al., 2003) (Dataset 1.1).

Hv-BRI1 is also located within the introgression regions of near-isogenic lines ari.256 and ert-ii.79 (Supplemental Figure 8A online), which—like BW885 (zuu1.a)—showed a brassinosteroid signaling-deficient phenotype in a leaf-unrolling test (Figure 3D). Sequencing of Hv-BRI1 revealed mutations in both ari.256 (A1733T; renamed zuu1.256) and ert-ii.79 (C1760A and C1761A) (Figure 6, Supplemental Figure 8B online, Dataset 1.1). The amino acid residues affected by the mutations in zuu1.256 (Arg564 to Trp) and ert-ii.79 (Thr573 to Lys) are located...
in the region encoding the steroid-binding island domain (Figure 8A-B). The introduction of the charged Lys573 to the hydrophobic active site surrounding residues, destroying charge neutrality, is expected to prevent brassinosteroid binding. In both cases, steric clashes with neighboring residues are likely to occur (see inserts in Figure 8B) and as a consequence the steroid binding and the general positioning of the brassinosteroid-binding site may be affected. Alternatively, the change in the surface polarity (Figure 8C-D) in the putative docking-site of BAK1 and other SERK-like co-receptors (yellow circle in Figure 8D) may simply hinder the BRII-SERK interaction, although less severe than in Arabidopsis bri1-102 (AtThr750 to Ile), which is a strong dwarf mutant (Supplemental Figure 8B online) (Hothorn et al., 2011; She et al., 2011; Sun et al., 2013). By using the Arabidopsis BAK1/SERK3 (AT4G33430) sequence as probe we identified at least five SERK-like barley genes [MLOC_19580, AK252995 (identical to MLOC_80630 and MLOC_59982), MLOC_8229, MLOC_6922 and MLOC_79186] in available barley sequence databases (IPK barley BLAST server, http://webblast.ipk-gatersleben.de/). Their specific functions in barley are not known yet but they are likely to form a BRII/SERK heterodimer by docking to the brassinosteroid binding island domain in the BRII ectodomain (marked area in Figure 8C) (Sun et al., 2013).

More than 30 additional near-isogenic lines with a short-culm or spike morphology phenotype, but without the unique combination of brassinosteroid-mutant characters, have overlapping introgression regions with Hv-BRII. Five out of eight mutants induced in Steptoe carried a 4-bp and a 1-bp deletion in the 3’ UTR and two out of four mutants induced in Akashinriki-derived lines carried the same deletions plus three nucleotide substitutions that caused two non-synonymous changes of amino acid residues (Ala79 to Phe and Leu1028 to Val) in the BRII N- and C-terminal domain, respectively (Akashinriki II-allele, (Chono et al., 2003)). None of those haplotypes displayed an obvious brassinosteroid phenotype (Dataset 1.1), which suggests that they are not deficient in Hv-BRII. Haplotypes 6 and 7 were found in the wrongly annotated ari-o alleles uzu1.297 and uzu1.301 (Figure 7D). The amino acid exchange found in uzu1.297 (Phe53 to Ser, Figure 8A) is located within the C-lobe of the N-terminal cap (Hothorn et al., 2011). The exchange to Ser53 may destabilize the structure of this domain causing endoplasmic reticulum retention of BRII (Hong et al., 2008). The exchange of the nonpolar Val282 residue to the negatively charged Asp in uzu1.301 will result in the formation of a salt bridge between Asp282
and Lys302 (α-C distance 4.46 Å), which may destabilize the structure of the folded protein due to a loss of solvation energy of Lys302, which is solvent exposed (Bosshard et al., 2004). Alternatively, the exchange of the nonpolar Val282 to the negatively charged Asp may inhibit the superhelical arrangement (Ile-Pro spine) in LRR12 and thus cause a repositioning of the upstream LRRs and the N-terminal cap (Hothorn et al., 2011; Santiago et al., 2013).

Interestingly, the ert-ii.79 mutation was identified earlier in the uzu1 mutant 093AR (uzu1.b) (Gruszka et al., 2011). To exclude a common ancestry between 093AR and ert-ii.79, we genotyped the original accessions using 383 AFLP markers. We found a high level of polymorphism (7.6%) throughout the genomes, indicating independent mutagenic events. The phenotypic characteristics of uzu1.b could be seen in all three genetic backgrounds (Figure 9), supporting the robustness of the brassinosteroid-deficient characters for direct phenotypic screening of barley mutant collections.

Sequencing of Hv-BRI1 from the brassinosteroid-insensitive mutant isolated among the 950 M2 plants of a chemically mutagenized population derived from the doubled-haploid line H930-36, revealed a novel Hv-BRI1 allele (a single-nucleotide G2171A substitution), which we named uzu1.c (Figure 6, Supplemental Figure 8B online, Dataset 1.1). Co-segregation of genotype and phenotype in M3 plants and allelism tests with both uzu1.a and uzu1.b verified the new uzu1 haplotype (Figure 4G and H). Substitution of the semi-conserved Arg710 to Lys in close proximity to the BRI1 transmembrane-binding domain causes a mild brassinosteroid phenotype. No other mutations were found in Hv-BRI1 among the remaining 949 M2 plants. The successful identification of the Hv-BRI1 mutant in a chemically induced mutant population and the clear brassinosteroid phenotypes seen in crosses and in different genetic backgrounds (Figure 4, Figure 7, Figure 9) strongly suggest that brassinosteroid phenotypes are identifiable in mutant populations of most barley cultivars.

**Temperature sensitivity of uzu1.a**
The *uzu1.a* allele, widely distributed in North East Asian winter barley cultivars, reduces lodging (Chono et al., 2003; Saisho et al., 2004) but shows sensitivity to elevated temperatures. We compared the near-isogenic line of *uzu1.a* with other brassinosteroid signaling and biosynthesis mutants grown at 14°C and 26°C. At 14°C, all mutants had an erect phenotype with height reduction ranging from strong (*brh3.h*, 61% of wild-type length) to weak semi-dwarfism (*uzu1.256*, 85%) (Figure 10). The average culm length of *uzu1.a* was 72% of the wild-type length. At 26°C, *uzu1.a* showed extreme dwarfing (29% of wild-type length) in contrast to the other mutants (65–91%). When moved from 26°C to 14°C, already developed tillers remained short but new tillers grew taller (Figure 10). We conclude that the temperature-sensitive phenotype is not associated with brassinosteroid or Hv-*BRI1* mutations in general, but specific for the *uzu1.a* allele. We also measured the level of castasterone, the suggested end-product of the brassinosteroid biosynthetic pathway in monocots (Kim et al., 2008). The highest level was found in *uzu1.a* grown at 26°C and in *ert-ii.79*, which are the signaling mutants with the strongest phenotype. This finding correlates well with the high levels of castasterone and brassinolide found in the Arabidopsis mutant *bri1-4* (Noguchi et al., 1999). Barley lines mutated in Hv-*BRD*, Hv-*CPD*, or Hv-*DIM* are deficient in brassinosteroid biosynthesis prior the formation of castasterone and had therefore, as expected, the lowest castasterone levels (Table I).

**DISCUSSION**

**The Group of Brassinosteroid-deficient Barley**

The use of nitrogen fertilizers has an enormous impact on global cereal production and was a key component of the Green Revolution. Initially, the culms were not strong enough to support the heavy spikes of fertilized plants, which resulted in lodging and overall loss in yield. It was therefore necessary to combine the use of fertilizers with breeding of short-culm cultivars, which are less prone to lodging due to their compact and sturdy culms (Hedden, 2003). So far, only a few genes have been explored in such short-culm cultivars—*Rht1* (for *Reduced height 1*), *Rht2*, and *Rht8* in wheat (Daoura et al., 2005), *sd1* (for *semidwarf 1*) in rice (Nagano et al., 2005), and *sdw1* (for *semidwarf 1/denso*) in barley (Hellewell et al., 2000). The known *Rht* gene products
belong to the DELLA subgroup of the GRAS protein family, which are thought to function as transcriptional regulators and interfere with the signal transduction pathway of the gibberellin growth hormone (Pysh et al., 1999). In contrast, sd1 and sdw1/denso are involved in the production of gibberellin since they encode gibberellin 20-oxidases of the biosynthetic pathway (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002; Jia et al., 2009).

Plant height and cell elongation are also regulated by brassinosteroids (Vriet et al., 2012). The access to a collection of 160 short-culm mutants in a near-isogenic background and with roughly mapped mutant gene locations (Druka et al., 2011), in combination with in silico gene mapping using the latest barley genome resources (Mayer et al., 2011; International Barley Sequencing Consortium, 2012), enabled us to effectively discover brassinosteroid mutants by a reverse approach of phenotyping followed by genotyping. Unexpectedly, the identified semi-dwarf gene loci do not belong to the same barley complementation group but were described as brachytic, semi-brachytic, breviaristatum, and erectoides barley. The history of those mutants might explain this misleading pre-grouping. The original mutations were induced and isolated in independent programs all over the world over a time period of several decades. Thus, mutagenic events were described and grouped by different barley researchers. An aggravating factor was that the plant material used for mutagenesis was dependent on national cultivar preferences and, over time, was often changed. Thus, even though the isolated brassinosteroid barley mutants share the same characteristics, the connection between those original mutations was less obvious since the different parental backgrounds contribute other, distracting characters such as 2- versus 6-row spikes. Only the unification of the genetic background in the near-isogenic Bowman collection (Druka et al., 2011) helped to overcome this problem.

The short culm of brassinosteroid and gibberellin mutants is a trait of agronomic interest since it provides resistance to lodging. The erect and upright growth is unique to brassinosteroid mutants only and distinguishes them from gibberellin mutants. The erect leaves enhance light capture for photosynthesis, serve as nitrogen reservoir for grain filling and enable denser planting with a higher leaf surface per area of land (Sakamoto et al., 2006). It was shown that rice mutants deficient in brassinosteroid biosynthesis enhanced grain yield under conditions of dense planting
(Sakamoto et al., 2006). Thus, brassinosteroid mutants provide additional traits for plant breeding which are not found in gibberellin mutants.

Interestingly, we only found mutations in three out of approximately nine genes involved in brassinosteroid biosynthesis starting from episterol (Supplemental Figure 1 online). The genes Hv-DIM, Hv-CPD, and Hv-BRD encode enzymes from different parts of the pathway. Mutations in the three genes show almost identical visible phenotypes, which suggest that loss of function of any other brassinosteroid biosynthesis gene should result in a similar plant architecture. However, it was found that a knock-out of DWF4 in rice causes mild semi-dwarfism only (Sakamoto et al., 2006). Thus, it is possible that barley Hv-DWF4 mutants were never isolated as short-culm mutants and therefore not included in the mutant collections explored in this study. Similar, loss of function of a CYP90-like gene in rice resulted in severe dwarfism (Hong et al., 2003). As severe barley dwarfs are not included in the collection of near-isogenic lines, it is possible that no suitable candidate plant for Hv-CYP90D1 was present in our screen.

Also, the existence of gene duplications in the barley genome or gene families with overlapping functions might explain why brassinosteroid biosynthesis mutants were only found in three genes. For example, DWF4 appears to be represented by two very similar DWF4-like genes at almost identical positions on chromosome 4H at 60 cM in the physical map of barley (International Barley Sequencing Consortium, 2012). It is possible that only a double mutant might cause a brassinosteroid phenotype. Both Hv-DWF4 genes overlap with the introgression regions of five near-isogenic lines with short-culm phenotypes, but without the unique combination of brassinosteroid features. Sequencing of Hv-DWF4 from these lines did not reveal any mutations. Thus, it is likely that these lines are deficient in one or several genes linked to Hv-DWF4, but not involved in brassinosteroid biosynthesis or signaling.

Future studies on barley mutants deficient in other brassinosteroid-related genes will show whether we should re-group barley semi-dwarfs and introduce the “group of brassinosteroid-deficient barley” defined by the unique combination of phenotypic characters and genotypes described in this study. Screening for genetic polymorphism in known brassinosteroid-related
genes in for example TILLING populations would reveal the phenotypic variation, which is not covered in the present study due to the pre-set selection for short-culm mutants.

**Induced Mutations in Barley**

In our extensive screen for brassinosteroid mutants, we noted four interesting cases of identical mutations. In the first case, the mutants were induced by identical chemical treatment (brh3.g, brh3.h, brh3.i), in the second case by different treatments (ari-o.40, -o.43, -o.143). In each case, the mutants were induced in the same mutagenesis program, and thus we cannot exclude a mix-up of mutant accessions after the mutagenic treatment as it was suggested earlier for alleles of barley loci mat-a and trdl (Houston et al., 2012; Zakhrabekova et al., 2012). Two other cases, however, strongly point towards an independent ancestry of identical mutations. Mutants uzul.b (093AR) and ert-ii.79 were induced in independent programs in different decades and countries. Here, we tested the genetic background and found a high level of polymorphism throughout the mutant genomes, strongly supporting their independent ancestry. In the other case, brh3.y and ert-t.437, silent mutations within Hv-BRD, in addition to the loss-of-function mutation, are clear evidences of an independent ancestry (Dataset 1.1). Both cases invite speculations concerning mutation hotspots in barley, preferentially selected due to the strong brassinosteroid-mutant phenotype. Modern analytical techniques such as exome capture and genotyping-by-sequencing will help to clarify this finding and other cases in the near future.

**Lodging Resistant Barley in a Changing Climate**

The barley uzul.a mutant is known to have alterations in brassinosteroid signaling due to a non-synonymous amino-acid exchange in the BRI1 kinase domain (Chono et al., 2003). Although the uzul.a gene modification has a long history in barley breeding, it remained exclusively in barley winter cultivars in Northeast Asia (Saisho et al., 2004). As shown in the present study, the uzul.a heat-sensitive reduction in plant height, severe in two-row spring barley grown in warmer climates, might be a reason why this haplotype never spread globally during the Green
Revolution. It is obvious that other alleles of Hv-*BRI1*, especially *uzu1.256* with its milder phenotype and without the pronounced temperature sensitivity of *uzu1.a*, should be considered as more reliable alternatives for culm-length control in barley breeding. Interestingly, the *uzu1.a* mutation causes a change (His857 to Arg) in a kinase surface domain (Bojar et al., 2014) that is not involved in ATP binding; nor does it interfere with the phosphorylation of the activation loop or with the position of the putative inhibitor BKI1 and SERK co-receptor binding site (Figure 8E-F). This is in agreement with the mild brassinosteroid phenotype we found in *uzu1.a* grown in moderate climate regimes. Mutations interfering with ATP binding, phosphorylation, or SERK interaction are known to cause drastic phenotypes in Arabidopsis and rice (Supplemental Figure 8B online) while mutations *uzu1.a* (at 14°C), *d61-10*, and *bri1-301*, all located within the same peripheral domain, result in mild phenotypes only (Figure 8E-F, Supplemental Figure 8B online). Here, exchanges of amino-acid residues might not disturb the general structure of the kinase domain but instead interactions with other proteins of the signaling cascade, such as CDG or BSKs. However, as the temperature sensitivity is specific for *uzu1.a* only, it is very likely that the dynamics of BRI1 is affected by the modification of His857. Such dynamic conformational changes might involve temporal stabilization by the cation-π interaction between His857 and Phe917 in the underlying α-helix (Figure 8E-F). A highly charged Arg857 might push the equilibrium to the conformational state where the protein is stabilized in its inactive state via the cation-π interaction. Interestingly, it has been shown that elevated temperatures stabilize cation-π interactions (Prajapati et al., 2006). A temperature-dependent inactivation of BRI1 would be consistent with the temperature-sensitive dwarfism found in *uzu1.a*. We note that His-Phe or His-Tyr pairs are often arranged in very similar positions (His-[X_{57-59}]-Phe/Tyr) in many kinases, including plant receptor-like kinases (Dataset 1.2).

The global temperature is currently rising and it has also been postulated that the climate may become more extreme with weather conditions that promote cereal lodging (Porter and Semenov, 2005; National Climate Assessment Development Advisory Committee, 2013). Accordingly, it would be desirable to have access to an increased repertoire of lodging-tolerance genes that also work under higher temperatures. The more than 20 temperature insensitive mutations identified in this study can be transferred to elite cultivars by recurrent backcrosses. However, this is time-consuming and may bring unwanted variants through linked genes. An alternative approach is to
screen for novel mutations directly induced in elite cultivars that already have favorable agronomic and grain-quality attributes. This can be done when the gene of interest is known. The visual phenotyping provides a fast and inexpensive method of identifying brassinosteroid mutants and thereby efficiently reducing the number of candidate genes to be analyzed in order to reveal the molecular cause of a short and sturdy culm. An alternative approach is to create mutations through targeted mutagenesis and genome editing. However, in barley these techniques are still in their infancy (Wendt et al., 2013) and although it would be possible to create mutations without leaving any traces of foreign DNA in the resulting breeding lines, most countries regard such plants as genetically modified or legal decisions are pending. Regardless the outcome of such decisions, the public opinion has to show a positive acceptance to gene editing before it will be of economic importance. In view of these obstacles, we postulate that the more than 20 identified mutations and the visual identification of brassinosteroid-deficient short-culm phenotypes in induced mapping populations will remain attractive for many years. We propose that the phenotypic screening can be performed in any genetic background. We are currently screening a large number of uncharacterized short-culm mutants (including severe dwarfs) in order to identify mutations in other genes involved in brassinosteroid signaling and biosynthesis that were not revealed in this study.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions.** Barley (*Hordeum vulgare* L.) cultivar Bowman and other parental cultivars, Bowman near-isogenic mutant lines (Druka et al., 2011) and original mutant lines (Dataset 1.1), F1 plants derived from allelism crosses, and an F2-mapping population from a cross between BW091 (*brh3.g*) × Bowman were grown in a greenhouse at 18°C under 16 h light/8 h dark cycles. Temperature-dependent experiments were performed in a growth chamber at either 14°C or 26°C under 16 h light/8 h dark cycles. Light intensity was set to a photon flux of 300 μmol m⁻² sec⁻¹.

For propiconazole treatments, barley seeds of the cultivar Bowman were imbibed and germinated on filter paper drenched with the respective propiconazole dilutions in water. Seeds were transplanted in soil after radicle emergence. For propiconazole treatments, barley plants
were grown under greenhouse conditions at 26°C (day) and 18°C (night). Single plants were
grown in 20 cm wide pots in 1:1 (v/v) mixtures of Turface Athletics MVP (Profile Products,
Buffalo Grove, IL) and Pro-Mix-BX with micorise (Premier Horticulture, Quakertown, PA).
Plants were fertilized with 200 ppm Miracle-Gro Excel (Scotts, Marysville, OH) adjusted to pH
6.0 following the manufacturer’s recommendations. The watering solution was supplemented
with 500 µM propiconazole solution using the commercial fungicide formulation Banner Maxx®
(Syngenta, Greensboro, NC). Propiconazole treatments were applied during each watering cycle
via soil drench. At full development of spikes, morphometric data were gathered and
photographs taken.
Plant seeds are either available from the Nordic Genetic Resource Center (NordGen), Alnarp,
Sweden (www.nordgen.org) or from the National Small Grains Collection (NSGC), Aberdeen,
ID, USA (www.ars.usda.gov). Seeds of mutant *brh.af* were kindly provided by A. Kleinhofs.

**Allelism Crosses.** Allelism tests were performed through crosses between barley accessions
carrying mutations in identical brassinosteroid-related genes. Plants were grown in a greenhouse
as described above, emasculated, and pollinated within 3 days. Bowman near-isogenic lines
(Druka et al., 2011) were used for allelism tests concerning Hv-*BRD*, Hv-*DIM* and Hv-*CPD*:
BW031 (*ari-u.245*) × BW094 (*brh3.y*), BW053 (*ari-o.40*) × BW072 (*brh.af*), BW053 (*ari-o.40*)
× BW085 (*brh14.q*), BW053 (*ari-o.40*) × BW325 (*ert-u.56*), BW053 (*ari-o.40*) × BW333 (*ert-
zd.159*), BW333 (*ert-zd.159*) × BW087 (*brh16.v*), and BW084 (*brh13.p*) × BW089 (*brh18.ac*).
Additionally, we crossed the original mutant accession *ari-o.40* with *ari-o.297*, *ari-o.301*, *ari-
o.304* and *ari-o.306*. In the case of Hv-*BRI1*, mutant *uzu1.c* (*R710K*) was crossed to both BW885
(*uzu1.a*) and 093AR (*uzu1.b*). F₁ progenies of all crosses were genotyped to confirm successful
crossings, and grown to maturity for phenotypic analysis. Allelism tests between other mutants
identified in this study are described elsewhere (Dahleen et al., 2005; Franckowiak and
Lundqvist, 2012).

**Leaf-Segment Unrolling and Leaf Lamina Inclination Assay.** Leaf-segment unrolling tests
were performed as described in (Chono et al., 2003; Gruszka et al., 2011). The rate of leaf-
segment unrolling was calculated according to (Wada et al., 1985). For leaf lamina inclination
tests plants were grown in vermiculite until the first leaf protruded 2 cm above the coleoptile.
One μl of 2 mg/ml brassinolide (24-epi-brassinolide, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solved in 96% ethanol was added to the tip of the barley seedlings. The angle between the first and the second leaf was measured when the second leaf emerged.

**Quantification of Castasterone Levels.** Isolation and identification of the brassinosteroid biosynthetic intermediate castasterone was performed according to a modified protocol described in (Swaczynová et al., 2007; Janeczko et al., 2010). All plants were grown for 14 days at 17°C, except BW885, which was also grown at 26°C. For each genotype, measurements were performed in three replicates. The leaf material (2 g of fresh weight) was homogenized in 20 mL 80% (v/v) cold methanol. After centrifugation, the supernatant was enriched in deuterium-labeled brassinosteroids (internal standards) and passed through a Strata X column (Phenomenex, Torrance, CA). The bound fraction was eluted with acetonitrile and evaporated to dryness. Brassinosteroids were determined by ultra-high performance liquid chromatography with tandem mass spectrometry.

**In Silico Gene Mapping.** Coding DNA sequences and protein sequences of Arabidopsis genes STE1 (AT3G02580), DWF5 (AT1G50430), DIM/DWF1 (AT3G19820), DET2 (AT2G38050), CYP90B1/DWF4 (AT3G50660), CYP90C1/ROT3 (AT4G36380), CYP90D1 (AT3G13730), CYP90A1/CPD (AT5G05690), CYP85A/BR6ox/BRD 1 (AT5G38970), or BRD2 (AT3G30180) and rice CYP724B1/DWF11 (LOC_Os04g39430) were used as probes to identify full-length barley gene and peptide sequences via the barley BLAST server of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (http://webblast.ipk-gatersleben.de/). Inferred and anchored positions of barley ‘high-confidence’ genes were extracted from the barley genome zipper (Mayer et al., 2011) and the barley genome map (International Barley Sequencing Consortium, 2012), respectively. Barley and Arabidopsis peptide sequences were aligned using the software Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

**Resequencing of Hv-BRD, Hv-CPD, Hv-DIM, Hv-DWF4, and Hv-BRII.** Genomic DNA was extracted from green leaf material using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Green leaves of an F2-
mapping population were sampled in 96-well format and genomic DNA was isolated using a Freedom EVO 200 robot (Tecan Group, Switzerland). PCR amplifications of AK372445 (Hv-BRD), MLOC_10658 (Hv-CPD), MLOC_52405 (Hv-DIM), AK355174 (Hv-DWF4), and MLOC_5176 (Hv-BRII) were performed according to the manufacturer’s protocol (REDExtract-N-Amp PCR ReadyMix; Sigma-Aldrich).

PCRs for Hv-BRD fragments 1, 2, and 5 were performed for 42 cycles (initial denaturation at 94°C/3 min followed by 3 cycles of 94°C/45 s, 61.5°C/45 s, and 72°C/90 s for extension; followed by 3 cycles of 94°C/45 s, 59.5°C/45 s, and 72°C/90 s for extension; followed by 36 cycles of 94°C/45 s, primer-pair-dependent annealing temperature/45 s, and 72°C/90 s for extension; with a final extension step of 72°C/10 min). PCRs for Hv-BRD fragments 3 and 4 were performed for 30 cycles (initial denaturation at 94°C/3 min followed by 30 cycles of 94°C/45 s, 54.5°C/45 s, and 72°C/105 s for extension, with a final extension step of 72°C/10 min). PCRs for Hv-BRII were performed using the protocol published in (Gruszka et al., 2011). PCRs for Hv-CPD were performed for 37 cycles (initial denaturation at 94°C/3 min followed by 37 cycles of 94°C/45 s, 54.5°C/45 s, and 72°C/90 s for extension, with a final extension step of 72°C/10 min). PCRs for Hv-DIM were performed for 40 cycles (initial denaturation at 94°C/3 min followed by 40 cycles of 94°C/30 s, 59°C/30 s, and 72°C/60–90 s for extension, with a final extension step of 72°C/10 min). PCRs for Hv-DWF4 were performed for 42 cycles (initial denaturation at 94°C/3 min followed by 3 cycles of 94°C/45 s, 58°C/45 s and 72°C/90 s for extension; followed by 3 cycles of 94°C/45 s, 56°C/45 s, and 72°C/90 s for extension; followed by 36 cycles of 94°C/45 s, 54.5°C/45 s, and 72°C/90 s for extension, with a final extension step of 72°C/10 min). Primer sequences including specific annealing temperatures used for PCR amplifications are listed and described in Dataset 1.3.

PCR products were resolved by agarose gel electrophoresis (GenAgaroseLE; GENAXXON Bioscience GmbH, Germany) and visualized after ethidium bromide staining. PCR products were purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel GmbH & Co. AG, Germany) according to the manufacturer’s instructions. Purified PCR products were sequenced by StarSEQ (Mainz, Germany).

**Molecular Analysis of uzu1.b Mutants ert-ii.79 and 093AR.** Genomic DNA of two uzu1.b alleles, ert-ii.79 and 093AR, and of their respective parents, cultivar Bonus and cultivar Aramir,
was isolated from green leaf material using the ‘micro C-TAB’ protocol described in (Gruszka et al., 2011). For this analysis, AFLP marker technique was applied. Two parental varieties were compared with each other. Similarly AFLP band patterns were compared between the mutants and additionally, mutants were reciprocally compared with the parental varieties. During the analysis, on average, more than 380 AFLP bands were scored for each comparison.

**Mutagenesis and TILLING Screen of a Barley M2 Generation.** The doubled-haploid line H930-36 was developed from the F1 generation of a cross between barley cultivar Klages and variety Mata. H930-36 was mutagenized by chemical treatment with N-methyl-N-nitrosourea at a dose 1.5 mM/3 h. 950 individual plants of the M2 generation were grown in the field and phenotyped at maturity. Leaf material of individual plants was harvested, dried in Silica Gel Type III (Sigma-Aldrich) and ground with an electric grinder (Retsch, Haan, Germany). DNA was extracted using the ‘micro C-TAB’ protocol (Gruszka et al., 2011) and genotypes were analyzed using the TILLING approach described in (Kurowska et al., 2012). Two fragments of the Hv-*BRI1*, spanning the gene region 1639-2994 bp (Supplemental Figure 8B online), were analyzed for sequence alterations.

**Anatomical and Microscopic Analysis.** For histological assays of 2-mm root segments of 5-day-old barley seedlings, conventional fixation, substitution, and embedding procedures were performed as described elsewhere (Marzec et al., 2013). Semi-thin sections (~2 μm thick) were stained for 2 min with 1% (w/v) methylene blue/1% (w/v) Azur II in 1% (w/v) aqueous borax at 60°C before light microscopic examination with a Zeiss Axiovert 135 microscope. In order to analyze the root architecture, one seed per plastic tube (length 50 cm, diameter 40 mm) was sown in vermiculite. Roots of 18-day-old plants were analyzed using a standard Epson® scanner and WinRhizo® software (Regent Instruments Inc., Canada).

**Accession Numbers**
Gene sequences reported in this paper have been deposited in the NCBI GenBank under the accession numbers KF318307 (Hv-*DIM*), KF318308 (Hv-*BRD*) and KF360233 (Hv-*CPD*)

**Supplemental Data**
The following materials are available in the online version of this article.

**Supplemental Figure 1.** The Brassinosteroid Biosynthetic Pathway as Found in Arabidopsis.

**Supplemental Figure 2.** Anatomical and Microscopic Analysis of Seedlings of Bowman Near-Isogenic Lines BW031 (ari-u.245), BW084 (brh13.p), BW333 (ert-zd.159), and BW312 (ert-ii.79).

**Supplemental Figure 3.** Leaf-Inclination Assay of Seedlings of Bowman Near-Isogenic Lines BW031 (ari-u.245), BW084 (brh13.p), BW312 (ert-ii.79), and BW333 (ert-zd.159).

**Supplemental Figure 4.** Alignments of Brassinosteroid-Biosynthesis Enzymes from Arabidopsis and Barley.

**Supplemental Figure 5.** In Silico Mapping and Detailed Analysis of Conserved Amino Acid Residues of Hv-BRD (Hordeum vulgare BRASSINOSTEROID-6-OXIDASE).

**Supplemental Figure 6.** In Silico Mapping and Detailed Analysis of Conserved Amino Acid Residues of Hv-CPD (Hordeum vulgare CONSTITUTIVE PHOTOMORPHOGENIC DWARF).

**Supplemental Figure 7.** In Silico Mapping and Detailed Analysis of Conserved Amino Acid Residues of Hv-DIM (Hordeum vulgare DIMINUTO).

**Supplemental Figure 8.** In Silico Mapping and Detailed Analysis of Conserved Amino Acid Residues of Hv-BRI1 (Hordeum vulgare BRASSINOSTEROID-INSENSITIVE 1).

**Supplemental Dataset 1.**

**Dataset 1.1.** Overview and Description of Analyzed Barley Mutant Accessions and Parental Cultivars.

**Dataset 1.2.** Examples of Kinases with a Cation-Pi His-[X_{57-59}]-Phe/Tyr Motif.

**Dataset 1.3.** Primers Used for Amplification of the Hv-BRD, Hv-BRI1, Hv-CPD, Hv-DIM and Hv-DWF4 Genomic DNA Sequences.
ACKNOWLEDGEMENTS

We thank L. Faldborg, C. Augsburg, C. Riis, O. Novák, E. Glodowska, E. Wierus for technical assistance and NordGen, NSGC and A. Kleinhofs for providing seeds.
LITERATURE CITED

Brassinosteroid, gibberellin and phytochrome impinge on a common transcription 

Bajguz A, Tretyn A (2003) The chemical characteristic and distribution of brassinosteroids in 
plants. Phytochemistry 62: 1027-1046

Bishop GJ, Yokota T (2001) Plants steroid hormones, brassinosteroids: current highlights of 
molecular aspects on their synthesis/metabolism, transport, perception and response. 
Plant Cell Physiol 42: 114-120

the phosphorylated BRI1 kinase domain and implications for brassinosteroid signal 
initiation. Plant J 78: 31-43

Bosshard HR, Marti DN, Jelesarov I (2004) Protein stabilization by salt bridges: concepts, 
experimental approaches and clarification of some misunderstandings. J Mol Recognit 
17: 1-16

Chandler PM, Harding CA (2013) 'Overgrowth' mutants in barley and wheat: new alleles and 

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

mapping of genes determining semidwarfism in barley. J Hered 96: 654-662

agronomic traits in common wheat (Triticum aestivum L.) and QTL analysis on its linked 
traits. Field Crops Res 156: 22-29


Kurowska M, Labocha-Pawłowska A, Gnilza D, Maluszynski M, Szarejko I (2012) Molecular analysis of point mutations in a barley genome exposed to MNU and gamma rays. Mutat Res 738: 52-70


National Climate Assessment Development Advisory Committee (2013) http://ncadac.globalchange.gov/


**FIGURE LEGENDS**

**Figure 1.** Main Barley Complementation Groups in the Bowman Near-Isogenic Line Collection, which Contain Mutants with a Short-Culm Phenotype.
Gross morphology of representatives of the barley mutant groups *brh* (for *brachytic*), *ari* (for *breviaristatum*), *dsp* (for *dense spike*), *ert* (for *erectoides*), *uzu* (for *semi-brachytic*), *sdw* (for *semidwarf*), and *sld* (for *slender dwarf*) compared to cultivar Bowman. Scale bar: 10 cm.

**Figure 2.** The Brassinosteroid Phenotype of the Bowman Near-Isogenic Line BW885 (*uzu1.a*) Compared to Wild-Type Bowman (Bow).
(A) Gross morphology and overall plant architecture.
(B) and (C) Awns and spikes are short with reduced rachis-internode length at the base and tip.
(D) and (E) Leaves are erect with undulating leaf margins. The latter phenotype was also found in the wild-type cultivar Bowman (Bow) grown in the presence of the brassinosteroid-biosynthetic inhibitor propiconazole (PCZ).
Scale bars: 10 cm.

**Figure 3.** Phenotypic Details of Mutants in the Collection of Bowman Near-Isogenic Lines with an *uzu1.a*-Like Plant Architecture.
(A) Reduced culm length due to short upper internodes.
(B) Irregular rachis internode length and short awns.
(C) Acute leaf angles and undulating leaf margins.
(D) Insensitivity to exogenously applied brassinolide (*uzu1.a,uzu1.256* and *ert-ii.79*) in a leaf-unrolling bioassay is indicative of defects in brassinosteroid signaling. Mutant *uzu1.256* was renamed from its previous name *ari.256*.
Scale bar in panel (B): 10 cm.

**Figure 4.** Phenotypic Characterization of the Hv-*BRI1* Allele *uzu1.c* Compared to the Double-Haploid Line H930-36.
(A) Gross morphology and overall plant architecture with reduced culm length.
(B) Acute leaf angles.
(C) Awns and spikes are short with reduced rachis-internode length.
(D) Undulated leaf margins.
(E) Reduced leaf elongation under dark growing conditions.
(F) Reduced sensitivity to exogenously applied brassinolide (BL) in a leaf-unrolling bioassay.
(G) and (H) Gross morphology of Hv-\textit{BRI1} alleles \textit{uzu1.a}, \textit{uzu1.b}, \textit{uzu1.c} and their respective F\textsubscript{1} progeny of allelism crosses.

Scale bars: 10 cm.

**Figure 5.** In Silico Mapping of Brassinosteroid Biosynthesis Genes and Receptor Gene Hv-\textit{BRI1} Revealed Mutant Candidates in the Collection of Bowman Near-Isogenic Lines.

MLOC-gene positions on barley chromosomes (gray) according to the barley genome map (International Barley Sequencing Consortium, 2012); mutant-donor introgressions (black) according to Druka \textit{et al.} (Druka et al., 2011). Loci Hv-\textit{DET2} and Hv-\textit{CPD} are not anchored in the barley genome map, but were localized in the barley genome zipper (Mayer et al., 2011).

**Figure 6.** Structures of Analyzed Brassinosteroid Genes.

(A) Hv-\textit{BRD} (for \textit{Hordeum vulgare BRASSINOSTEROID-6-OXIDASE}).
(B) Hv-\textit{CPD} (for \textit{Hordeum vulgare CONSTITUTIVE PHOTOMORPHOGENIC DWARF}).
(C) Hv-\textit{DIM} (for \textit{Hordeum vulgare DIMINUTO}).
(D) Hv-\textit{BRI1} (for \textit{Hordeum vulgare BRASSINOSTEROID-INSENSITIVE 1}).

The location and number of exons (E) and the identity of mutations at the gene and peptide levels (three letter code, italic) are indicated. Dotted lines in (C) represent deleted gene fragments in the respective mutant accessions.

**Figure 7.** Allelism Crosses.

(A) Gross morphology of putative Hv-\textit{BRD} mutants BW031 (\textit{ari-u.245}), BW094 (\textit{brh3.y}) and the F\textsubscript{1} progeny of a cross BW031 × BW094.

(B) Gross morphology of putative Hv-\textit{CPD} mutants BW084 (\textit{brh13.p}), BW089 (\textit{brh18.ac}) and the F\textsubscript{1} progeny of a cross BW084 × BW089.
(C) Gross morphology of putative Hv-DIM mutants BW053 (ari-o.40), BW072 (brh.af), BW085 (brhl4.q), BW087 (brhl6.v), BW325 (urt-u.56), and BW333 (ert-zd.159) and their respective F₁ progeny of allelism crosses.

(D) Gross morphology of wild type cultivar Bonus and cultivar Kristina, original ari-o alleles and their respective F₁ progeny of allelism crosses. Scale bars: 10 cm.

**Figure 8.** Structural Basis of Brassinosteroid Perception and Signaling in Barley Hv-BRI1 Mutants.

(A, B) and (C, D) X-ray structure of the Arabidopsis BRI1 brassinosteroid-binding domain (Protein Data Bank accession code 3RGZ) (Hothorn et al., 2011; She et al., 2011) displayed as ribbon and hydrophobicity model, respectively. Leucine-rich repeats (LRR) missing in monocots are shown in white, the brassinosteroid-binding site (island domain) in yellow, and the bound steroid hormone brassinolide in red.

(E and F) X-ray structure of the Arabidopsis BRI1 kinase domain (Protein Data Bank accession code 4OAB). The two views are rotated 90° relative to each other. BRI1 in complex with ATP (red), the BRI1 activation loop with phosphorylation sites (in yellow) and the proposed protein-docking platform for the BRI1 inhibitor BKI or the SERK co-receptors (circled) are highlighted (Bojar et al., 2014).

The numbering of amino acid residues refers to the barley peptide sequence shown in Supplemental Figure 8B online where corresponding residue positions in Arabidopsis At-BRI1 or rice Os-BRI1 are indicated. Protein structures were visualized using the software Chimera (Pettersen et al., 2004).

**Figure 9.** Phenotypic Characterization of uzu1.b Lines ert-ii.79 (uzu1.b in Bonus), 093AR (uzu1.b in Aramir), and BW312 (uzu1.b in Bowman).

(A) Gross morphology and general semi-dwarf phenotype.

(B) General spike phenotype with short awns, irregular rachis internodes, and elongated basal rachis internodes (a character not so pronounced in a Bowman genetic background).

(C) Erect leaf architecture.

(D) Undulating leaf margins of mutants compared to their respective parental cultivars. Scale bars in A and B: 10 and 5 cm: respectively.
Figure 10. Temperature-Responsive Phenotypes of Brassinosteroid Mutants in the Collection of Bowman Near-Isogenic Lines Compared to Wild Type Bowman.

(A) Culm length of plants grown at 14°C (dark-colored bars) or 26°C (light-colored bars).

(B) Hv-BRII mutants grown at 26°C. At high temperatures, elongation of zuu1.a culms is strongly reduced compared to Bowman and other brassinosteroid mutants.

(C) and (D) At 26°C, zuu1.a leaf margins are strongly undulating and internodes fail to elongate.

(E) Height reduction is partly neutralized by moving plants from warm temperature (26°C) to cool temperature (14°C). Red squares and blue stars mark tillers developed at 26°C and 14°C, respectively.

Scale bars: 10 cm.
Table I. Level of Castasterone in Barley Near-Isogenic Lines Carrying Mutations in the Brassinosteroid Biosynthesis Genes Hv-CPD, Hv-BRD and Hv-DIM, or the Hv-BRI1 Gene Encoding the Brassinosteroid Receptor.

<table>
<thead>
<tr>
<th>Near-isogenic line</th>
<th>Genotype</th>
<th>Castasterone [pmol/g leaf]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average ± std</td>
</tr>
<tr>
<td>Bowman</td>
<td>Wild type</td>
<td>2.68 ± 0.40</td>
</tr>
<tr>
<td>BW084</td>
<td>brh13.p (Hv-CPD)</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>BW091</td>
<td>brh3.g (Hv-BRD)</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>BW333</td>
<td>ert-zd.159 (Hv-DIM)</td>
<td>0.84 ± 0.29</td>
</tr>
<tr>
<td>BW033</td>
<td>uzu1.256 (Hv-BRI1)</td>
<td>4.52 ± 0.53</td>
</tr>
<tr>
<td>BW312</td>
<td>ert-ii.79 (Hv-BRI1)</td>
<td>9.39 ± 1.76</td>
</tr>
<tr>
<td>BW885</td>
<td>uzu1.a (Hv-BRI1)</td>
<td>7.43 ± 0.21</td>
</tr>
<tr>
<td>BW885 (26°C)</td>
<td>uzu1.a (Hv-BRI1)</td>
<td>10.31 ± 0.18</td>
</tr>
</tbody>
</table>