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ORIGINAL PAPER

Semi-dwarf barley (*Hordeum vulgare* L.) *brh2* and *ari-l* mutants are deficient in a U-box E3 ubiquitin ligase

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Abstract

Lodging is the process where crop plants fall over and lie on the ground due to strong winds and heavy precipitation. This problem reduces yield and increases the risk of fungal infections and pre-harvest germination. In order to avoid lodging, plant breeders utilize short-culm mutants, which often have a robust culm that can support the weight of a heavy spike. In barley (*Hordeum vulgare* L.), thousands of short-culm mutants have been isolated in breeding programs around the world. Our long-term goal is to reveal the genetic network underlying culm length, with the objective to provide an enlarged repertoire of genes and alleles suitable for future breeding of lodging resistant barley. In the present work we studied a group of allelic *brh2* and *ari-l* mutants, which have a relatively strong semi-dwarf phenotype and are phenotypically similar to previously identified mutants deficient in brassinosteroid signalling or metabolism. The *Brh2* gene is located in the centromeric region of chromosome 4H and we applied a candidate gene approach to identify the gene. *Brh2* is orthologous to *TUD1* in rice (*Orysa sativa* L.), which encodes a U-box E3 ubiquitin ligase. We identified one missense mutation, one nonsense mutation and four deletions of the complete *Brh2* gene. The mutants could respond to exogenously applied brassinolide, which suggests that the apparent brassinosteroid deficient phenotype of barley *brh2* and *ari-l* mutants is related to brassinosteroid metabolism rather than signalling.

Keywords *Ari-l* · *Brh2* · Heterotrimeric G protein · Lodging · *TUD1*

Abbreviations

ari	*Breviaristatum*
brh	*Brachytic*
ert	*Erectoides*
GDP	Guanosine-5′-diphosphate
GTP	Guanosine-5′-triphosphate
RGS	Regulator-of-G-protein-signalling
sdw	*Semi-dwarf*
uzu	*Semi-brachytic*

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Introduction

The introduction of nitrogen fertilizers has had an enormous impact on agricultural production but it also brought a challenge to the plant breeders since the culms of the cereal crop cultivars could not support the heavy spikes of fertilized plants and therefore the plants were prone to lodging. The use of growth regulators has been one approach to combat the problem with lodging. Another approach has been to explore semi-dwarf mutants, which are often more resistant to lodging due to their short and sturdy culm. Uzu-type barley (*Hordeum vulgare* L.) was one of the first short-culm mutants to be used (Takahashi 1955). In the 1930s, 70% of the barley grown in Japan was of uzu-type and 70 years later the *uzu1.a* allele had been introduced in almost all Japanese hull-less barley cultivars (Saisho et al.

2004). It is now known that *uzu1.a* has a point mutation in the *HvBRI1* gene encoding the brassinosteroid receptor (Chono et al. 2003; Dockter et al. 2014). While *uzu1.a* is a spontaneous mutation isolated in Japanese land races, *ert-k.32* was induced by X-ray treatment of the Swedish cultivar Bonus in 1947. Mutant *ert-k.32* was released in 1958 as the cultivar Pallas, which was one of the first cereal crop cultivars developed from induced mutants. The *Ert-k* gene has still not been identified at DNA level (Skov Kristensen et al. 2016). Another very successful short-culm cultivar has been the Scottish malting cultivar Golden Promise, which was isolated after γ-ray mutagenesis of cultivar Maythorpe in 1956 (Ahloowalia et al. 2004; Foster 2001; Wendt et al. 2016). The most successful locus has however been *Sdw1* (*HvGA20ox2*) encoding gibberellin 20-oxidase (Xu et al. 2017). The first reported allele was *sdw1.c* (originally named denso), a spontaneous mutation found in the cultivar Abed Denso from the Abed breeding station in Denmark (Haahr and von Wettstein 1976; Vestergaard et al. 1949). The *sdw1.d* allele was obtained by X-ray treatment of the cultivar Valticky and released as the cultivar Diamant in 1965 (Bouma 1967; Haahr and von Wettstein 1976). The *sdw1.d* allele is widespread in many modern barley elite cultivars grown today (Xu et al. 2017). The success of *sdw1.d* is probably due to less apparent pleiotropic effects of the mutation, since short-culm mutants often have unwanted traits like short roots, small and rounded kernels and short spikes. However, also *sdw1* mutant alleles are associated with negative pleotropic effects on yield and potentially malting quality (Hellewell et al. 2000). Lodging can be expected to become an increasing problem since global climate warming is supposed to increase the number of days with strong winds and thunderstorms (Porter and Semenov 2005). In the view of increased problems with lodging and that fact that *sdw1.d* tend to dominate the short-culm barley elite cultivars (Xu et al. 2017), it is of relevance to identify and characterize the gene network regulating plant architecture and thereby lodging resistance, which can be mobilized in future plant-breeding activities.

The short-culm alleles used in the today's barley elite cultivars are the result of massive efforts in mutant breeding that has generated thousands of short-culm mutants of which many are stored at various seedbanks. These mutants can be explored to dissect the molecular mechanisms regulating plant architecture. The mutants are mainly found in the mutant groups named *brachytic* (*brh*), *semi-brachytic* (*uzu*), *erectoides* (*ert*), *breviaristatum* (*ari*), *dense spike* (*dsp*), *curly dwarf* (*cud*), *semi-dwarf* (*sdw*) and *slender dwarf* (*sld*). Various mutations from these groups have been described at DNA level and associated with nine genes encoding proteins, which obviously are regulators of plant architecture (Table 1). More than 20 mutants are deficient in brassinosteroid signalling or biosynthesis. We have previously described an ideotype of brassinosteroid mutants in barley based on *uzu1.a* causing a His857Arg modification in the kinase domain of the brassinosteroid receptor (Dockter et al. 2014). This ideotype helped us to identify three other genes encoding key enzymes of the brassinosteroid biosynthetic pathway; *HvBRD1, HvCPD, HvDIM* (Dockter et al. 2014). Other mutants showed strong similarities to the brassinosteroid ideotype but turned out to be deficient in *HvD1* and *HvDEP1* encoding the α- and γ-subunit, respectively, of a heterotrimeric G protein (Table 1) (Braumann et al. 2017; Wendt et al. 2016). In addition to an α- and a γ-subunit, heterotrimeric G proteins are also composed of a β-subunit (Assmann 2002; Oki et al. 2009). Heterotrimeric G proteins are mostly known from mammalian systems where there are several genes encoding α (Gα), β (Gβ) and γ (Gγ) subunits. In mammalian systems, binding of a ligand to the G-protein-coupled receptor causes a rapid exchange of GDP to GTP bound by the Gα subunit, which promotes a dissociation of activated Gα-GTP from the Gβγ heterodimer (Temple and Jones 2007). Activated Gα-GTP and/or Gβγ interact with the effector proteins. A different system for G-protein signalling is found in plants. There is only one Gα, one Gβ and three Gγ encoding genes in Arabidopsis, and one Gα, one Gβ and four Gγ genes in rice (*Oryza sativa* L.) (Kato et al. 2004; Temple and Jones 2007; Utsunomiya et al. 2011). The Arabidopsis Gα subunit spontaneously binds GTP in vitro (Johnston et al. 2007) but its ability to stimulate effector molecules is inhibited by its association to a membrane bound Regulator-of-G-protein-signalling (RGS) protein (Urano et al. 2013). Upon binding of a ligand to the RGS protein, the Gα-GTP subunit is released and can interact with effector molecules.

In the present study we focus on mutants in the barley *Brh2* locus since they all show strong similarity to the brassinosteroid deficient ideotype. Mutants in the *Brh2* locus were isolated more than 50 years ago as short-culm or short-awn mutants (Kucera et al. 1975; Tsuchiya 1962) and *Brh2* is located in the centromeric region of chromosome 4H (Druka et al. 2011). We identify *Brh2* as an orthologue to rice *TUD1* encoding a U-box E3 ubiquitin ligase and describe seven allelic barley mutants at the DNA level. In rice, the *TUD1* encoded U-box E3 ubiquitin ligase has been shown to interact with the *D1* encoded α-subunit of the heterotrimeric G protein (Hu et al. 2013).

Materials and methods

Plant materials and growth conditions

Barley (*Hordeum vulgare* L.) cultivars Bonus, Foma, Bowman, Kristina, Svanhals as well as mutants *brh2.b, ari-l.3, ari-l.132, ari-l.135, ari-l.145, ari-l.214, ari-l.237, ari-l.257*,

Table 1 Overview of identified barley short-culm genes and their corresponding mutants

Gene name	Mutants	Gene product	Pathway	References
HvGA20ox2	*sdw1.a, sdw1.c, sdw1.d*	Gibberellin 20-oxidase	Gibberellin biosynthesis	Xu et al. (2017)
HvBRI1	*ert-ii.79, uzu1.a, uzu1.b, uzu1.c, uzu1.256, uzu1.297, uzu1.301*	Brassinosteroid receptor	Brassinosteroid signaling	Chono et al. (2003), Dockter et al. (2014), Gruszka et al. (2011)
HvBRD	*ari-u.245, ari-u.304, brh3.g, brh3.h, brh3.i, brh3.y, ert-t.437*	Brassinosteroid-6-oxidase	Brassinosteroid biosynthesis	Dockter et al. (2014)
HvCPD	*brh13.p, brh18.ac*	C-23α-hydroxylase cytochrome P450	Brassinosteroid biosynthesis	Dockter et al. (2014)
HvDIM	*ari-o.40, ari-o.43, ari-o.143, brh.af, brh14.g, brh16.v, ert-u.56, ert-zd.159*	Δ^5-sterol-Δ^{24}-reductase	Brassinosteroid biosynthesis	Dockter et al. (2014)
HvAP2	*Ert-r.52, Ert-r.67, Ert-r.329, Ert-r.453, Zeo1.a, Zeo1.b, Zeo1.c, Zeo2.c, Zeo2.d, Zeo2.h, Zeo2.j, Zeo2.av, Zeo2.ax*	Transcription factor containing two AP2 DNA-binding domains and a miR172-binding site	Unknown	Houston et al. (2013)
HvERECTA	*ert-m.34, ert-m.35, ert-m.40, ert-m.42, ert-m.54, ert-m.64, ert-m.87, ert-m.107, ert-m.115, ert-m.130, ert-m.144, ert-m.168, ert-m.169, ert-m.330, ert-m.363, ert-m.426*	Leucine-rich repeat receptor-like kinase	Unknown	Zakhrabekova et al. (2015)
HvD1	*ari-i.38, ari-m.12, ari-m.28, ari-m.141, ari-m.177, ari-m.251, ari-m.269, brh1.a, brh1.aa, brh1.ae, brh1.c, brh1.e, brh1.f, brh1.t, brh1.x*	Heterotrimeric G protein α-subunit	Unknown	Braumann et al. (2017), Ito et al. (2017)
HvDEP1	*ari-e.1, ari-e.30, ari-e.39, ari-e.119, ari-e.156, ari-e.166, ari-e.GP*	Heterotrimeric G protein γ-subunit	Unknown	Wendt et al. (2016)

Table 2 Description of barley *brh2* and *ari-l* mutants

Mutant	Mother cultivar	Mutagen	Year of isolation	Accession number
brh2.b	Svanhals	X-rays	1962 or earlier	GSHO 573
ari-l.3	Bonus	X-rays	1955	NGB 115848
ari-l.132	Foma	Ethylene imine	1960	NGB 115942
ari-l.135	Foma	Ethyl methanesulfonate	1960	NGB 115945
ari-l.145	Foma	Ethyl methanesulfonate	1960	NGB 115956
ari-l.214	Foma	Ethyl methanesulfonate	1965	NGB 116023
ari-l.237	Foma	*N*-ethyl-*N*-nitroso-*N*′-nitroguanidine	1966	NGB 116047
ari-l.257	Kristina	Ethyl methanesulfonate	1968	NGB 116066
BW050 (*ari-l.3*)	Near-isogenic line of *ari-l.3* created in cultivar Bowman			NGB 20458
BW090 (*brh2.b*)	Near-isogenic line of *brh2.b* created in cultivar Bowman			NGB 20496

BW050 (*ari-l.3* near-isogenic line in cultivar Bowman background) and BW090 (*brh2.b*) (Table 2) were grown in greenhouse at 18 °C under a cycle of 16-h light/8-h dark. Light intensity was set to a photon flux of 300 µmol m^{-2} s^{-1}. The plant material was obtained from the Nordic Genetic Resource Center (Alnarp, Sweden; http://www.nordgen.org) except for the original *brh2.b* mutant which was obtained from the National Small Grains Collection (https://www.ars.usda.gov/pacific-west-area/aberdeen-id/small-grains-and-potato-germplasm-research/docs/national-small-grains-collection/).

DNA techniques

Genomic DNA was isolated from leaf segments by using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich). Leaf segments of 0.5 cm were transferred to 96-well plates for genomic DNA extraction. Then, 40 µl of extraction solution was added to the plant samples and incubated at 95 °C for 10 min, followed by addition of 40 µl of dilution solution. PCR amplifications were performed according to the manufacturer's protocol by using REDExtract-N-Amp PCR ReadyMix, which contains JumpStart Taq antibody for specific hot-start amplification. For 25 µl PCR reactions, 10 µl REDExtract-N-Amp PCR ReadyMix was mixed with 10.5 µl water, 1.25 µl of each forward and reverse primers (10 µM) and 2 µl of genomic DNA. Primers were designed to generate four overlapping fragments of barley *Brh2* (Table 3). The primers were based on the sequence of bowman_contig_65106 containing the gene MLOC_79322 (International Barley Genome Sequencing et al. 2012), which we now identified as the *Brh2* gene. PCRs were performed for 37 cycles (initial denaturation at 94 °C/2 min followed by 37 cycles of 94 °C/45 s, 61 °C/45 s, and 72 °C/40 s for extension, with a final extension step of 72 °C/5 min). Amplified PCR products were purified for sequencing using the Nucleospin Gel and PCR Clean-Up kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instruction. DNA sequencing was performed by StarSEQ GmbH, Mainz, Germany using the primers described in Table 3. PCR fragments, instead of PCR fragments cloned into plasmid vectors, were sequenced in order to avoid fidelity problems of the polymerase. Each found mutation was covered by two overlapping PCR fragments amplified from different pairs of PCR primers. DNA and protein sequences were analyzed with BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the Phytozome BLAST tool (http://www.phytozome.net), the IPK Barley BLAST Server (http://webblast.ipk-gatersleben.de/barley_ibsc/) and BARLEX—The Barley Genome Explorer (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::). Multiple polypeptide sequence alignments were performed by the Multiple Sequence Alignment program "ClustalW" http://www.ebi.ac.uk/Tools/msa/clustalw2/.

Leaf-unrolling and leaf lamina inclination bioassays

In leaf-unrolling bioassays the plants were grown in darkness for 9 days at 16 °C. A 2 cm tightly rolled leaf segment was cut from each etiolated seedling and immersed in water or in a solution containing 200 nM 24-epi-brassinolide (Sigma-Aldrich). The width of the leaf segment was measured at 0 and 72 h. Also the full width (forced open) of the leaf segment was measured. All operations were performed in the dark or under green dim light (Dockter et al. 2014; Honda et al. 2003).

In leaf lamina inclination bioassays (Dockter et al. 2014; Fujioka et al. 1998; Hong et al. 2003), 1 µl of 2 mg ml^{-1} 24-epi-brassinolide solved in 96% (v/v) ethanol was added to the tip of barley seedlings just emerging from germination in vermiculite. Ethanol was added to control plants. The angle between the first leaf and the second leaf was observed when the second leaf obtained a similar size as the first leaf.

Table 3 Description of oligonucleotides used as primers for amplification and sequencing of *Brh2*

Primer	Sequence	Combined with primer	Length of fragment	Position of fragment in sequence
WU1For	AGGTAGCCGCAGAACCCCAG	WU1Rev	834	27–860
WU1Rev	GAGGTCAGCAACGACACGCC			
WU3For	GCGTGTCGTTGCTGACCTCG	WU3Rev	612	842–1453
WU3Rev	ACTCCTCGGGCACCATCCTG			
WU5For	TCATCTGCCCGATCTCCCTG	WU6Rev	546	452–997
WU6Rev	TGAGCATGTCCACCACGAGG			
WU9For	TGAAGGATTGCCCGAGGACG	WU10Rev	557	1334–1890
WU10Rev	GGTGGTGCTTATGCTGCCGC			

The position of fragment refers to the sequence in Fig. 4

Results

Barley *brh2.b* was isolated after X-ray treatment of the cultivar Svanhals (Tsuchiya 1962). It was later shown to be allelic to *ari-l.3* (Dahleen et al. 2005), which is one of seven allelic *ari-l* mutants (Kucera et al. 1975) (http://www.nordgen.org/bgs). The other six *ari-l* mutant lines are named *ari-l.132, ari-l.135, ari-l.145, ari-l.214, ari-l.237* and *ari-l.257* (Table 2). Near-isogenic lines have been created for *brh2.b* and *ari-l.3* through six recurrent backcrosses to the cultivar Bowman (Druka et al. 2011). The near-isogenic lines are named BW090 and BW050, respectively.

Barley *brh2* and *ari-l* mutants show a relatively strong semi-dwarf phenotype (Fig. 1; Tables 4, 5). Plant height and awn length are reduced to approximately 2/3 and 1/4 normal length, respectively. In addition, grain yield is lower since especially thousand-grain-weight is reduced (Table 4). Notably, *brh2* and *ari-l* mutants show many of the brassinosteroid-deficient characteristics previously described in barley mutants deficient in brassinosteroid signalling or biosynthesis (Dockter et al. 2014). This includes the semi-dwarf appearance, undulated leaf margins, an erect growth habit and short awns (Fig. 1). Due to the brassinosteroid-deficient characters we performed leaf-unrolling bioassays (Dockter et al. 2014; Honda et al. 2003) with BW050 (*ari-l.3*), BW090 (*brh2.b*) and Bowman. In this assay etiolated leaf segments are immersed in 200 nM brassinolide. Leaves of brassinosteroid receptor mutants are expected to remain rolled, whereas plants with an intact receptor open up, i.e. unrolling. The present experiment showed a clear unrolling of BW050 (*ari-l.3*), BW090 (*brh2.b*) and Bowman (Fig. 2). We also performed leaf lamina inclination assays (Dockter et al. 2014; Fujioka et al. 1998; Hong et al. 2003). In these assays brassinolide solubilized in ethanol, or just ethanol as negative control, is added to the tip of a newly germinating barley seedling. After approximately 2 weeks the barley plant has developed two leaves. In seedlings treated with brassinolide the first and second leaves become well separated from each other. In contrast, the first and second leaves remain close together in plants treated with ethanol or in brassinosteroid receptor mutants treated with brassinolide (Dockter et al. 2014). We observed that BW050 (*ari-l.3*), BW090 (*brh2.b*) and Bowman show a clear response to exogenous brassinolide in a leaf lamina inclination assay (Fig. 3). Thus, *brh2* and *ari-l* mutants are sensitive to

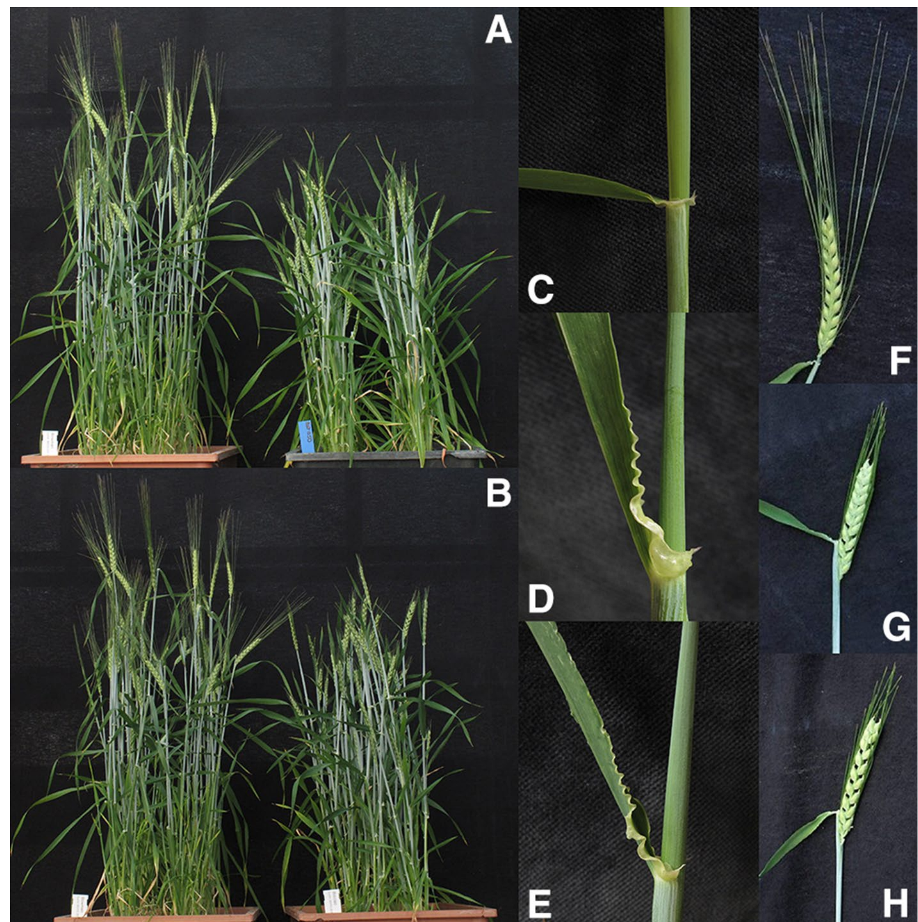

Fig. 1 Phenotypes of barley *ari-l.3* and *brh2.b* mutant lines. **a** The near-isogenic line BW050 (*ari-l.3*) to the right compared to Bowman on the left. **b** BW090 (*brh2.b*) to the right compared to Bowman on the left. **c–e** Leaves of BW050 (**d**) and BW090 (**e**) have undulating leaf margins and are more upright than leaves of Bowman (**c**). **f–h** Awns of BW050 (**g**) and BW090 (**h**) are much shorter compared to awns of Bowman (**f**)

Table 4 Phenotypic field data of *brh2* and *ari-l* mutants grown at Fyn, Denmark, 2012

	Bowman	BW050 (*ari-l.3*)	BW090 (*brh2.b*)	Foma	*ari-l.132*	*ari-l.135*	*ari-l.145*	*ari-l.214*	*ari-l.237*	Bonus	*ari-l.3*	Kristina	*ari-l.257*	Svanhals	*brh2.b*
No. of plants measured	19	19	19	13	18	17	15	16	17	19	17	15	19	15	18
Culm length [cm]	94.3 ± 5.1	56.8 ± 2.2	59.1 ± 2.3	93.2 ± 3.6	60.0 ± 2.8	63.3 ± 5.2	59.2 ± 4.9	57.9 ± 4.2	59.2 ± 3.6	93.6 ± 3.8	62.2 ± 2.3	86.0 ± 4.8	51.8 ± 4.0	114.8 ± 10.7	75.5 ± 4.4
p-value		< 0.001	< 0.001		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		< 0.001		< 0.001		< 0.001
Spike length [cm]	8.7 ± 0.60	7.8 ± 0.45	8.0 ± 0.82	9.5 ± 0.90	11.2 ± 0.75	9.1 ± 1.3	8.2 ± 0.78	8.0 ± 0.79	7.8 ± 0.81	10.8 ± 1.0	10.8 ± 0.85	9.7 ± 0.73	7.8 ± 0.49	8.6 ± 0.74	7.3 ± 0.31
p-value		< 0.001	0.003		< 0.001	> 0.05	< 0.001	< 0.001	< 0.001		> 0.05		< 0.001		< 0.001
Ratio length (spike + awn)/spike	2.4 ± 0.12	1.4 ± 0.10	1.3 ± 0.08	2.3 ± 0.15	1.2 ± 0.04	1.5 ± 0.13	1.3 ± 0.11	1.3 ± 0.07	1.4 ± 0.06	2.0 ± 0.10	1.2 ± 0.06	2.4 ± 0.14	1.7 ± 0.14	2.5 ± 0.21	1.4 ± 0.06
p-value		< 0.001	< 0.001		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		< 0.001		< 0.001		< 0.001
Number of grains per spike	21.3 ± 1.2	18.4 ± 2.3	19.1 ± 1.4	29.2 ± 1.4	29.9 ± 2.1	29.1 ± 3.8	25.5 ± 4	28.7 ± 3.2	24.8 ± 3.8	30.7 ± 1.6	30.6 ± 3.0	29.7 ± 2.1	24.8 ± 4.8	31.2 ± 2.5	27.2 ± 2.4
p-value		< 0.001	< 0.001		> 0.05	> 0.05	0.003	> 0.05	< 0.001		> 0.05		< 0.001		< 0.001
Thousand grain weight [g]	48.9 ± 3.1	37.4 ± 7.2	32.3 ± 2.6	47.9 ± 3.9	33.4 ± 2.8	24.4 ± 7.2	27.2 ± 5.3	32.6 ± 2.9	27.7 ± 7.6	48.2 ± 2.8	33.6 ± 2.1	48.7 ± 2.9	28.6 ± 3.0	53.5 ± 4.3	40.8 ± 2.4
p-value		< 0.001	< 0.001		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		< 0.001		< 0.001		< 0.001

In each plant the tallest tiller has been measured. The table shows average ± standard deviation. The p-value (unpaired Student's t-test) was obtained in comparisons between the mutant lines and the respective mother cultivar shown to the left of the mutant. It should be noted that no mutation was found in *Brh2* in the mutant line *ari-l.257* compared to its mother cultivar Kristina

Table 5 Phenotypic data of *brh2* and *ari-l* mutants grown in 2 L pots in greenhouse at Lund University, Sweden, 2015

	Bowman	BW050 (*ari-l.3*)	BW090 (*brh2.b*)	Bonus	*ari-l.3*	Svanhals	*brh2.b*
Culm length [cm]	63.8 ± 3.0	37.7 ± 2.5	39.7 ± 2.9	61.2 ± 4.5	38.4 ± 2.8	68.3 ± 4.1	41.9 ± 2.6
p-value		< 0.001	< 0.001		< 0.001		< 0.001
Spike length [cm]	8.4 ± 0.89	8.1 ± 0.55	7.7 ± 0.56	9.2 ± 0.87	8.9 ± 0.99	8.9 ± 0.82	8.0 ± 0.74
p-value		> 0.05	> 0.05		> 0.05		> 0.05
Ratio length (spike + awn)/spike	2.5 ± 0.21	1.3 ± 0.18	1.4 ± 0.14	2.3 ± 0.16	1.2 ± 0.11	2.6 ± 0.22	1.3 ± 0.11
p-value		< 0.001	< 0.001		< 0.001		< 0.001
Number of grains per spike	20.8 ± 1.7	19.5 ± 1.7	20.1 ± 1.5	29.1 ± 1.9	28.6 ± 1.3	28.7 ± 1.5	28.6 ± 1.2
p-value		> 0.05	> 0.05		> 0.05		> 0.05

Ten plants were analysed of each genotype. In each plant the tallest tiller has been measured. The table shows average ± standard deviation. The p-value (unpaired Student's t-test) was obtained in comparisons between the mutant lines and the respective mother cultivar shown to the left of the mutant

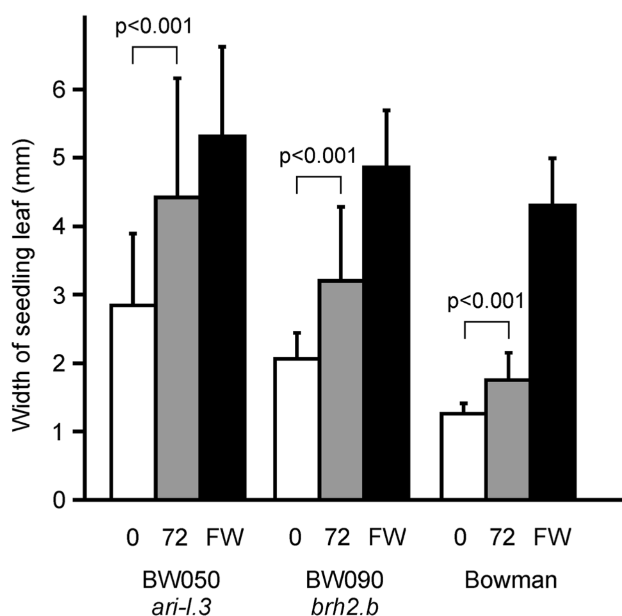

Fig. 2 Sensitivity to exogenously applied 24-epi-brassinolide as determined by a leaf-unrolling assay. Twenty barley seeds were germinated and grown in constant darkness until etiolated seedlings of approximately 15 cm were obtained. A two cm central leaf segment was cut from each leaf and submerged in 200 nM 24-epi-brassinolide. The width of each leaf was measured when newly cut (white bars show average, lines show standard deviation) and after 72 h (grey bars). The full width (FW, leaf segment forced open) was also measured (black bars). Both mutants and the wild type cultivar Bowman unroll as a response to exogenously added brassinolide, which indicate that they contain a functional brassinosteroid signaling pathway. The p-values were calculated from a paired Student's t-test

exogenously added brassinolide indicating no deficiencies in the brassinosteroid signalling pathway. Instead, our results suggest that the apparent brassinosteroid-deficient phenotype might be due to reduced brassinosteroid synthesis or increased brassinosteroid degradation since they behave like Bowman and brassinosteroid biosynthetic mutants, which respond to exogenous brassinolide in leaf-unrolling and leaf lamina inclination assays (Dockter et al. 2014).

The *Brh2* locus has previously been mapped to barley chromosome 4H, 1.5 cM proximal to *Gsh3* (Takahashi et al. 1971). Analysis of near-isogenic lines BW050 (*ari-l.3*) and BW090 (*brh2.b*) provided further details on the chromosomal location of *Brh2* (Druka et al. 2011). The introgression regions of BW050 (*ari-l.3*) and BW090 (*brh2.b*) are defined by SNP markers 2_1374 and 2_1332 on barley chromosome 4H (Druka et al. 2011). These markers correspond to positions 28.4 cM (bp 21,432,445) and 77.31 cM (bp 585,756,569) in the barley physical map (Mascher et al. 2017). The large genomic region of approximately 564 Mbp contains 2342 high-confidential gene models (Mascher et al. 2017). Considering the fact that BW050 and BW090 have been generated through six recurrent backcrosses and that the introgression regions is still 564 Mbp compared to the size of chromosome 4H, which is approximately 650 Mbp, we excluded a mapping approach as an efficient method to identify the genetic identity of the *Brh2* locus. Instead we employed a candidate gene approach to identify the gene deficient in *brh2* and *ari-l* mutants. During a manual inspection of the annotations of the 2342 genes in the 564 Mbp region we immediately noted the presence of two candidate genes of relevance for a short-culm phenotype; *HvDWF4* (HORVU4Hr1G065440) encoding a cytochrome P450 enzyme catalysing a hydroxylation at the 22-C position of the steroid in the brassinosteroid biosynthetic pathway (Choe et al. 1998), as well as *HvTUD1* (HORVU4Hr1G066070, located between bp 550,510,764–550,513,664 on chromosome 4H at 59.89 cM (Mascher et al. 2017)) encoding a polypeptide which is 92% identical to *OsTUD1* (Os03g13010) encoding a U-box E3 ubiquitin ligase in rice (Hu et al. 2013). Both *DWF4* and *TUD1* are associated with dwarfism in plants (Choe et al. 1998; Hu et al. 2013). DNA sequencing of the *HvDWF4* gene from BW050 (*ari-l.3*) and BW090 (*brh2.b*) revealed no mutations (Dockter

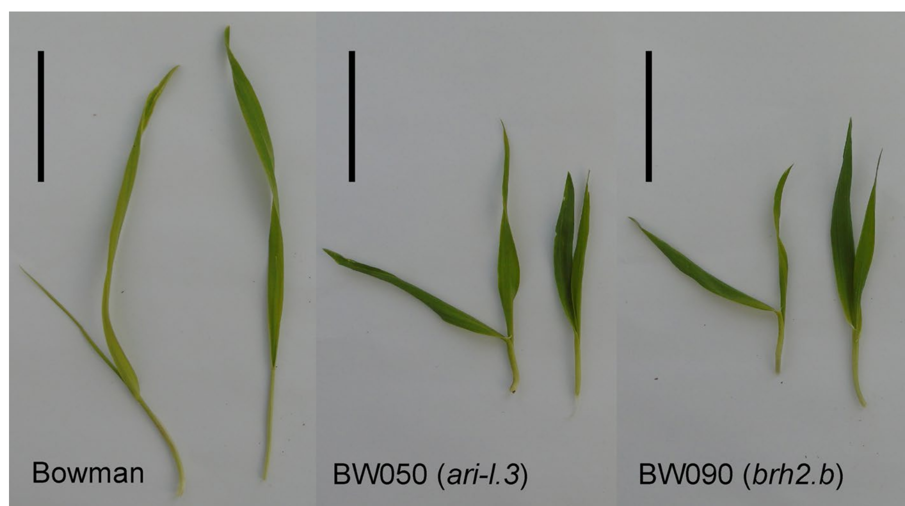

Fig. 3 Leaf lamina inclination assay performed on barley cultivar Bowman (wild type), BW050 (*ari-l.3*) and BW090 (*brh2.b*). The left plant in each photo was treated with 24-epi-brassinolide dissolved in 96% (v/v) ethanol. The control plant to the right in each photo was treated with 96% ethanol. The wide angle between the first and second leaf in each plant treated with brassinosteroid compared to the small angle in the control plants just treated with ethanol demonstrates that Bowman, BW050 (*ari-l.3*) and BW090 (*brh2.b*) can respond to exogenously added brassinosteroid and suggests that they have a functional brassinosteroid signaling pathway. Black bar; 5 cm

et al. 2014). In contrast, we found mutations in *HvTUD1* in BW050, BW090 and six of the seven *ari-l* mutant lines (Fig. 4). C-to-T substitutions were found in the three mutants *ari-l.135, ari-l.145* and *ari-l.214*. In *ari-l.135* this causes an exchange of Ser-382 to Phe in the armadillo-like fold of the HvTUD1 polypeptide. This Ser residue is modified to Leu in the rice mutant *tud1-2* (Hu et al. 2013). Lines *ari-l.145* and *ari-l.214* carry identical nonsense mutations resulting in a truncated polypeptide of 111 amino-acid residues, compared to the full-length protein of 460 residues. No fragments of *HvTUD1* could be amplified in *brh2.b, ari-l.3, ari-l.132, ari-l.237*, BW050 (*ari-l.3*) or BW090 (*brh2.b*) suggesting that these mutants carry deletions of the entire *HvTUD1* gene. No mutation could be found in *ari-l.257*. Instead, mutant *ari-l.257* and its mother cultivar Kristina showed three differences compared to the consensus sequence of cultivar Foma (Fig. 4); an A-to-C substitution 88 bp proximal to the ATG start codon, a silent C-to-T substitution in the coding region, and an insertion of a G 86 bp downstream of the TGA stop codon. Since the differences are found in both *ari-l.257* and Kristina, we do not expect them to affect the *HvTUD1* gene product in a negative way. Thus, the mutation causing the semi-dwarf phenotype in *ari-l.257* is in an unknown gene different from *HvTUD1* and the mutant should therefore be named *ari-257*. We have previously identified genes deficient in historic mutants obtained from various seedbank collections and occasionally experienced that accessions have been mixed over the decades (Mueller et al. 2012; Zakhrabekova et al. 2012, 2015. The identification of severe mutations in *HvTUD1* in 9 out of 10 available accessions strongly support *HvTUD1* as the gene responsible for the short-culm phenotype of the *brh2* and *ari-l* mutants.

Discussion

It was recently found that the *TUD1* gene product in rice encodes a U-box E3 ubiquitin ligase (Hu et al. 2013). Five allelic rice *tud1* mutants showed a semi-dwarf phenotype similar to rice *d1* mutants deficient in the gene encoding the Gα subunit of the heterotrimeric G protein. Mutants in *TUD1* and *D1* are characterized by a short second internode, erect leaves and photomorphogenic growth in darkness. Hu et al. (Hu et al. 2013) showed in rice that the Gα subunit encoded by *D1* physically interacts with the E3 ubiquitin ligase encoded by *TUD1* and suggested that TUD1 functions as an activator of brassinosteroid signalling.

Ubiquitination is a post-translational modification of proteins and one of the most prominent mechanisms that regulates protein degradation to modulate protein levels in eukaryotic cells (Ciechanover 2005). This also includes proteins involved in signalling and metabolism of plant hormones such as auxin, gibberellic acid, jasmonic acid, ethylene, abscisic acid and brassinosteroids (Hu et al. 2013; Vierstra 2009). Ubiquitin is a polypeptide of 76 amino-acid residues. It becomes covalently attached to target proteins through the action of three enzymes; an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase (Bae and Kim 2014). The target specificity is determined by the E3 ubiquitin ligases, which binds to their specific substrate proteins. There are many genes

Fig. 4 The barley *HvTUD1* gene as determined in the cultivar Foma encoding a U-box E3 ubiquitin ligase. The genomic DNA sequence shown in the figure consists of only one exon. Below the DNA sequence is the polypeptide sequence of 460 amino-acid residues. The postulated U-box domain and a domain with an armadillo-like fold are marked in light grey (amino-acid residues 65-139) and dark grey (171-440), respectively (Groves and Barford 1999; Hu et al. 2013; Vogelmann et al. 2014). Identical amino-acid residues between barley and rice TUD1 are marked in bold and non-identical residues are marked in italic. The *ari-l.145* and *ari-l.214* mutants have identical nonsense mutations resulting in a truncated polypeptide of 111 amino-acid residues. The *ari-l.135* point mutation results in exchange of Ser-382 to Phe in the armadillo-like fold domain. The mutants *brh2.b, ari-l.3, ari-l.132* and *ari-l.237* are probably large deletions since no fragments of *HvTUD1* could be amplified by PCR. No mutation could be found in *ari-l.257*. Three SNPs could be detected in *ari-l.257* and the mother cultivar Kristina. The consensus sequence shown in the figure is identical to that of MLOC_79322 (International Barley Genome Sequencing et al. 2012)

```
   1 CTCGCTCGCCTCCCCTTTTGACCGGTAGGTAGCCGCAGAACCCCAGAAATCCAAGAAAAG   60

  61 CAGCGGGTCAAGAAAGAGGGAAAGCAAGCGAGGAGGTCGATGATGATGGCGATAAGTCGC  120

                                        C in Kristina and ari-l.257
 121 CCCATGATTCACCGCATCATTCCGTCTCCTGCGCGACTGTGCTAAAAGGGCCTCGCCGTC  180

 181 CGTGTACTCTTTCGGCGAGCGGTGCTTCCTTGGGCAGACGGTCAGGCCAAGACCACCGCT  240

 241 GCAATGCCGCAGTACCAGGAGCTGCCCTGCGGCGGGCAGGTGCTCGACATCGACACCGCG  300
   1    M  P  Q  Y  Q  E  L  P  C  G  G  Q  V  L  D  I  D  T  A   19

 301 CTCAAGGACGGCATCCTGGGGTGCGGCCCGGAGCCCGGGGACGGGGCGCTCGGGGACGGA  360
  20 L  K  D  G  I  L  G  C  G  P  E  P  G  D  G  A  L  G  D  G   39

 361 GGTAAGCAGCCGGTGGAGCTGCGGAAGATGATGGACGAGCTGGACGCGGCCGGGGACGCC  420
  40 G  K  Q  P  V  E  L  R  K  M  M  D  E  L  D  A  A  G  D  A   59

 421 GGCGGCGGGGACGAGGTCGTGCCGGCCGTCTTCATCTGCCCGATCTCCCTGGAGCCCATG  480
  60 G  G  G  D  E  V  V  P  A  V  F  I  C  P  I  S  L  E  P  M   79

 481 GTGGATCCGGTCACGCTCTGCACCGGCCAGACGTACGAGCGCGCCAACATCTCCCGGTGG  540
  80 V  D  P  V  T  L  C  T  G  Q  T  Y  E  R  A  N  I  S  R  W   99

                                           TAG in ari-l.145 and ari-l.214
 541 CTGGCGTTGGGCCACAGGACCTGCCCGACCACGATGCAGGAGCTCTGGGACGACGCGCTC  600
 100 L  A  L  G  H  R  T  C  P  T  T  M  Q  E  L  W  D  D  A  L  119

 601 ACCCCCAACGCCACGCTCCGCCAGCTCATCGCCGCCTGGTTCTCCCGCCGGTACACCCGC  660
 120 T  P  N  A  T  L  R  Q  L  I  A  A  W  F  S  R  R  Y  T  R  139

 661 TTCAAGAAGCGCTCGGCAGACTACCACGGCCGCGCCGCGGACCTCGTCCACGGTCTCCGC  720
 140 F  K  K  R  S  A  D  Y  H  G  R  A  A  D  L  V  H  G  L  R  159

 721 GGCACGGCCGTCCCGAGGAGGCACCCCCTCAAGGGCCAGGCCCGCGTCGCCGCGCTCCGG  780
 160 G  T  A  V  P  R  R  H  P  L  K  G  Q  A  R  V  A  A  L  R  179

                   GCT in Kristina and ari-l.257 (Silent mutation)
 781 GAGTTGCGCTCCCTCGCCTCCGCCCACCAGTCGGTGACCAAGGCCATAGCTGAGGCCGGC  840
 180 E  L  R  S  L  A  S  A  H  Q  S  V  T  K  A  I  A  E  A  G  199

 841 GGCGTGTCGTTGCTGACCTCGCTTCTTGGCCCCTTCACGTCTCATTCCGTGGGGTCCGAG  900
 200 G  V  S  L  L  T  S  L  L  G  P  F  T  S  H  S  V  G  S  E  219

 901 GCGGTGGCCATTCTTGTCAGCGGCGTGCCGCTCGACGGCGACGCTAAGGCGGCGCTGATG  960
 220 A  V  A  I  L  V  S  G  V  P  L  D  G  D  A  K  A  A  L  M  239

 961 CAGCCGGCAAAGGTGTCCCTCGTGGTGGACATGCTCAACGAGGGCGCCGTCGACACCAAG 1020
 240 Q  P  A  K  V  S  L  V  V  D  M  L  N  E  G  A  V  D  T  K  259

1021 ATCAACTGCGTCCGCCTCATCCGCATCCTCATGGAGGAGAAAGGCTTCCGGCCGGAGACG 1080
 260 I  N  C  V  R  L  I  R  I  L  M  E  E  K  G  F  R  P  E  T  279

1081 GTGGCGAGCCTGAGCCTCTTGGTCGGCGCCATGCGCCTGGTCCGGGACAAGCGGCACCCG 1140
 280 V  A  S  L  S  L  L  V  G  A  M  R  L  V  R  D  K  R  H  P  299

1141 GACGGTGTGGCCGCCGGGCTAGAACTGCTCAATTCCATCTGCGCCGTGCACAGGCCGGCC 1200
 300 D  G  V  A  A  G  L  E  L  L  N  S  I  C  A  V  H  R  P  A  319

1201 AGGAGCATGATTGTCAGCATTGGTGCGGTGCAGCAGCTGGTGGAGCTGCTGCCGGAGCTG 1260
 320 R  S  M  I  V  S  I  G  A  V  Q  Q  L  V  E  L  L  P  E  L  339

1261 GCGACGGAGTGCGTGGAGCCAGCCCTGGACATCTTGGATGCGCTCGCCTCGGTCCCTGAA 1320
 340 A  T  E  C  V  E  P  A  L  D  I  L  D  A  L  A  S  V  P  E  359

1321 GGCAGGACGGCTCTGAAGGATTGCCCGAGGACGATACCCAATGCCGTCCGATTGCTGATG 1380
 360 G  R  T  A  L  K  D  C  P  R  T  I  P  N  A  V  R  L  L  M  379

            TTC in ari-l.135 (Ser-382 to Phe)
1381 AGGGTGTCCGAGGCCTGCACGCAGCGTGCCCTGTCGATGCTCTGGGTGGTGTGCAGGATG 1440
 380 R  V  S  E  A  C  T  Q  R  A  L  S  M  L  W  V  V  C  R  M  399

1441 GTGCCCGAGGAGTCTGCACCCGCTGCCCTGGAGGTTGGGCTGGCCGCCAAGCTTCTCCTG 1500
 400 V  P  E  E  S  A  P  A  A  L  E  V  G  L  A  A  K  L  L  L  419

1501 GTGATCCAAAGCGGGTGCGGGCCGGAGCTGAAGCAGCAAGCCTCGGAGTTGCTCAAGCTC 1560
 420 V  I  Q  S  G  C  G  P  E  L  K  Q  Q  A  S  E  L  L  K  L  439

1561 TGCACCGTGAATTGCACGTCCACCGTCTTCCTCGCGAAATGCAAGCTCACCAAGACAATT 1620
 440 C  T  V  N  C  T  S  T  V  F  L  A  K  C  K  L  T  K  T  I  459

1621 CAGTGAGAAGACTCCGGGACATTGTCCTTCTGGTTGTGATTTGAGTCGATGATGCCGTTC 1680
 460 Q  *

                                      GGGG in Kristina and ari-l.257
1681 CCCTCGGTCGGTCTCGCATAATCTGGACTGGGAATGGATGAGAGAAACAAATTGCTGCTG 1740

1741 GATTAAGACCCTGTGTGTAGATAAACTCAGCAATGCAGGAAGCTTGTCGTGTCATTTAAA 1800

1801 GACATGGGGCATGGGGGTGGATCAATGGATCACCCTGCCCATCGACCGGAAGCTAGGATC 1860

1861 GAGAAGCAATGCGGCAGCATAAGCACCACC                               1890
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encoding E3 ubiquitin ligases in plant genomes (Du et al. 2009; Smalle and Vierstra 2004). In contrast, there are only a few genes encoding E1 ubiquitin-activating enzymes and E2 ubiquitin-conjugating enzymes. The E3 ubiquitin ligases are divided into three classes depending on the presence of one of three protein domains; HECT, RING or U-box (Ardley and Robinson 2005; Deshaies and Joazeiro 2009; Rotin and Kumar 2009). The rice *TUD1* and the barley *Brh2* gene products belong to the U-box class of E3 ubiquitin ligases. There are 64 and 77 genes encoding U-box E3 ubiquitin ligases in Arabidopsis and rice, respectively (Yee and Goring 2009; Zeng et al. 2008). The barley Brh2 polypeptide shows 92% identical residues to that of rice TUD1 and the two genes are located in a syntenic region comparing barley chromosome 4H and rice chromosome 3. The barley and rice genomes show no other gene products that are more than 35% identical to Brh2 or TUD1. Thus, we strongly believe that *Brh2* and *TUD1* are orthologous genes.

In short-culm rice *TUD1* mutants as well as in short-culm barley *brh2* and *ari-l* mutants reported in this study, a functional E3 ubiquitin ligase is absent as a consequence of the mutations. Since the function of the ubiquitin machinery is to degrade proteins, the *TUD1* encoded E3 ubiquitin ligase probably initiate the removal of a factor that represses elongation in a wild type plant. Thus, absence of a functional E3 ubiquitin ligase in a *tud1, brh2* or *ari-l* mutant results in a semi-dwarf phenotype because the repressor of elongation is not degraded. Therefore, the *TUD1/Brh2/Ari-l* encoded E3 ubiquitin ligase should be regarded as an activator of plant elongation. The physical interaction between *TUD1* encoded E3 ubiquitin ligase and the Gα subunit of a heterotrimeric G protein in rice (Hu et al. 2013) suggests that the Gα subunit is the degradation target of the *TUD1* encoded E3 ubiquitin ligase. However, the Gα subunit functions as an activator of elongation rather than a repressor since mutations in the gene of Gα in rice and barley cause a short-culm phenotype (Braumann et al. 2017; Wang et al. 2006). Therefore, further analyses of the relationship between the *TUD1* encoded E3 ubiquitin ligase and the Gα subunit are required.

The rice *tud1-2* mutant showed normal sensitivity to gibberellic acid and cytokinin but less sensitivity to exogenously applied brassinosteroid (Hu et al. 2013). It was suggested that the *TUD1* encoded E3 ubiquitin ligase participates in a second brassinosteroid signalling pathway in rice, which is independent of the BRI1 brassinosteroid receptor (Hu et al. 2013). In the present study we found that the apparent brassinosteroid deficient phenotype of barley *brh2* and *ari-l* mutants (Fig. 1) is not caused by deficient brassinosteroid signalling; in leaf-unrolling and leaf lamina inclination bioassays we did detect a clear response to exogenously added 24-epi-brassinolide. In order to provide an explanation to the apparent brassinosteroid deficient phenotype of barley *brh2* and *ari-l* mutants, we suggest that *TUD1/Brh2/Ari-l* might be related to brassinosteroid synthesis or degradation rather than signalling, since mutants deficient in brassinosteroid biosynthesis or signalling are phenotypically very similar in barley, if not indistinguishable (Dockter et al. 2014). We also want to point out that our idea about a brassinosteroid deficient ideotype (Dockter et al. 2014) could be wrong in the sense that also mutations in non-brassinosteroid related genes might cause a phenotype that we interpret as brassinosteroid deficient. We suggest that further analyses are required in order to decipher the molecular mechanisms underlying the short-culm phenotype of *tud1, brh2* and *ari-l* deficient mutants and the relation of this gene to brassinosteroid signalling and metabolism, as well as heterotrimeric G proteins.

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Author contributions IB, CD and MH designed research. WU, AP and SZ performed experimental work. MH wrote the paper.

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