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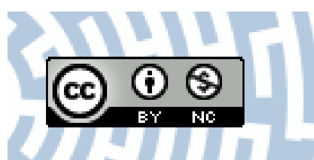

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PLANT GENETICS · ORIGINAL PAPER

# New allele of *HvBRI1* gene encoding brassinosteroid receptor in barley

**Damian Gruszka · Iwona Szarejko · Miroslaw Maluszynski**

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**Abstract** The aim of these studies was to characterize nucleotide substitutions leading to the phenotype of brassinosteroid-insensitive, semi-dwarf barley mutant *093AR*. Two substitutions in the sequence of barley *HvBRI1* gene, encoding leucine-rich repeats receptor kinase (LRR-RK), which participates in brassinosteroid (BR) signalling, were identified in this chemically-induced barley mutant of the cv. Aramir. The LRR-RK is a transmembrane protein phosphorylating downstream components. The identified substitutions CC>AA at positions 1760 and 1761 in the *HvBRI1* gene of this mutant led to a missense mutation, causing the Thr-573 to Lys-573 replacement in the protein sequence. The threonine residue is situated in the distal part of a 70-amino acids island responsible for binding of BR molecules. As this residue is conserved among BRI1 protein homologs in *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Oryza sativa* and *Hordeum vulgare*, it was postulated that this residue is crucial for the protein function. The genetic analyses indicated that the mutant *093AR* was allelic to the spontaneous, semi-dwarf mutant *uzu* which carries A>G substitution at position 2612 of the *HvBRI1* gene (GenBank acc. no. AB088206). A comparison of the genomic sequence of *HvBRI1* in the mutants *uzu*, *093AR* and in the cv. 'Aramir' confirmed the presence of the single-nucleotide A>G substitution at position 2612 in the sequence encoding kinase domain of HvBRI1 polypeptide in *uzu*, but not in *093AR* mutant, indicating that a new allele of the *HvBRI1* gene was identified.

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## Introduction

### The importance of semi-dwarf mutants

Dwarfism or semi-dwarfism of crop plants has been recognized as an indispensable attribute of intensive agriculture. Numerous spontaneous mutations have been observed in crop plants, leading to dwarf or semi-dwarf phenotypes (for review see Maluszynski and Szarejko 2005). Some of these mutants display defects in gibberellin metabolism, however, several of them, including the barley spontaneous mutant *uzu* ('swirl' in Japanese) show a malfunctioning of the brassinosteroid (BR) signal transduction pathway. Mutation techniques have been employed as a very important source of genetic variation for crop improvement many decades ago. In rice various mutation breeding experiments yielded more than 534 varieties which have so far been released (FAO/IAEA Mutant Varieties Database; www-infocris.iaea.org/MVD/default.htm). For this crop, mutation experiments led to the release of two semi-dwarf mutant cultivars: Reimei (from cv. Fujiminori) which was released in Japan in 1966, and Calrose76 (from cv. Calrose), released in 1976 in USA. Both these mutant cultivars were obtained after gamma rays treatment. Semi-dwarf cultivars gained some preponderance over ancestral cultivars due to the fact that they exhibited reduced plant height conferring lodging resistance. At the same time, Calrose76 produced 14% more grain yield (and 13% less straw mass) as the second desired characteristic. These rice varieties had significant economic impact and were used as sources of semi-dwarfism in numerous

breeding programmes (for review see Rutger 1992). Both Reimei and Calrose76 carry a mutation in the *sd1* (*semi-dwarf1*) gene, the same as *DGWG* (Dee-Geo-Woo-Gen) gene present in the semi-dwarf rice cv. IR8, developed at the International Rice Research Institute (IRRI) which has been a forerunner of the Green Revolution. Molecular analyses led to the suggestion that the product of the *sd1* gene participates in gibberellin biosynthesis (Monna et al. 2002; for review see Maluszynski and Szarejko 2005). Reimei and Calrose76 have been widely cited as examples of how useful induced mutations in plant breeding are.

There are at least thirteen mutated genes described for semi-dwarfism, induced by chemical or physical mutagens in wheat. Some of them have high breeding value and significant economic impact. The mutant line Krasnodarskij Karlik, previously widely used in USSR/Russia and Eastern Europe, has recently become a major source of semi-dwarfism in many countries around the world. The wheat *Rht* (Reduced height) genes, whose spontaneous mutations lead to the semi-dwarf phenotype, and contributed to the Green Revolution, were identified as homologous to *GAI* gene in *Arabidopsis* and *D8* in maize, encoding transcription factors belonging to GRAS family and acting as negative regulators of gibberellin signalling (Ikeda et al. 2001). Induced mutations have contributed significantly to the development of high yielding cultivars in many crops so far (for review see Maluszynski and Szarejko 2005). Despite the fact that several mutants impaired in BR synthesis or signaling were identified in monocotyledonous crops, the only mutant which gained significant agronomical importance is barley spontaneous, semi-dwarf mutant *uzu*.This allele was introduced into many varieties cultivated in East Asia (Chono et al. 2003).

## Brassinosteroids are steroid hormones perceived by transmembrane BRI1 receptor kinase

Brassinosteroids (BRs) are hormones displaying high activity in stimulation of plant growth and development (for review see Bajguz and Tretyn 2003; Fujioka and Yokota 2003). They are present at low concentrations in pollen grains, anthers, seeds and vegetative tissues undergoing early developmental stages in a broad range of species representing various evolutionary groups (for review see Clouse and Sasse 1998). Biochemical and genetic analyses allowed for determination of BRs signal transduction model in *Arabidopsis thaliana* which is commenced by the perception of these molecules on the cell membrane leading to changes in gene expression (for review see Gendron and Wang 2007). BRs are perceived by the transmembrane polypeptide BRI1 (Brassinosteroid-Insensitive1), which belongs to the family of serine-threonine protein kinases (Li et al. 2001) containing leucine-rich repeat (LRR) extracellular domain (Li and Chory 1997; He et al. 2000; Wang et al. 2001). Isolation of mutants defective in BR perception leads to determination of the structure of *BRI1* gene and its protein product (Noguchi et al. 1999). Molecular genetic and biochemical analyses of BRI1 protein have revealed a functional receptor kinase with leucine-rich repeats, an extracellular domain involved in binding of BR molecules and an active cytoplasmic kinase domain that autophosphorylates multiple serine and threonine residues (Friedrichsen et al. 2000; Wang et al. 2001). The N-terminal portion of BRI1 protein contains a signal peptide followed by a leucine zipper motif and a pair of cystein. The last two elements are responsible for homo- and heterodimerization (Nam and Li 2002). This part of the protein is followed by 25 tandem LRRs, each of them spanning 24 amino acids, forming secondary structures of α-helises and β-sheets (Bishop and Koncz 2002). Between the 21$^{st}$ and 22$^{nd}$ LRRs there is a 70-amino acid island (Noguchi et al. 1999), which is responsible, together with the 22$^{nd}$ LRR, for the binding of BR molecules (Kinoshita et al. 2005). The portion of the protein comprising the LRRs mediates interactions with other polypeptides (Li 2003; for review see Li and Jin 2006). The extracellular and cytoplasmic parts of the BRI1 protein are separated by a transmembrane fragment followed by a highly conserved serine-threonine kinase domain, responsible for the initiation of signal transduction by phosphorylating of the downstream components (Li and Chory 1997; Noguchi et al. 1999; Friedrichsen and Chory 2001).

Barley *BRI1* homolog was identified using the PCR with degenerate primers designed from amino acid sequences of *Arabidopsis* BRI1 and rice BRI1 homolog (Chono et al. 2003). A complete, full-length coding sequence of intronless barley *HvBRI1* gene, 3558 bp in length, was determined and published in GenBank database (accession no. AB088206). So far, one missense substitution in barley *HvBRI1* gene has been identified in mutant *uzu*. Genetic analysis indicated that the semi-dwarf phenotype of *uzu* mutant is caused by a single recessive gene, located on chromosome 3H. The single A>G missense mutation causes the His-857-Arg substitution, which evokes the semi-dwarf phenotype without any reduction in plant fertility (Chono et al. 2003). Taking into account these favourable features of plant architecture that allow for lodging resistance after the application of elevated levels of fertilizers (Khush 1999), the *uzu* allele was introduced into all hull-less barley varieties cultivated currently in Japan, the South Korean peninsula and East China. It was demonstrated that all *uzu* landraces cultivated in these regions carry the same SNP within the *HvBRI1* gene sequence encoding kinase domain of the BR receptor (Chono et al. 2003; Saisho et al. 2004).

The Department of Genetics, University of Silesia has developed a wealthy collection of dwarf and semi-dwarf barley mutants. Genetic analysis indicated that the chemically-induced, semi-dwarf barley mutant *093AR,* which was the material of the present research, was allelic to the spontaneous mutant *uzu*, which has a substitution in *HvBRI1* gene (Gruszka et al. 2006). The objective of the presented studies was to characterize the mutation causing the semi-dwarf phenotype of the mutant *093AR*, since up to now this has been the only chemically-induced, semi-dwarf and fully-fertile mutant related to BR signalling, carrying mutation in the fragment of *HvBRI1* gene encoding extracellular domain of the BR receptor, derived from the European spring barley. So far, the only known barley mutant *uzu* has been used as a source of semi-dwarfism for cultivars grown exclusively in East Asia.

## Materials and methods

### Plant material

Plant material used in the study included the spontaneous, semi-dwarf mutant *uzu* obtained from the Svalöf AB (Sweden) collection, and the chemically-induced, semi-dwarf mutant *093AR* from the collection of the Dept. of Genetics, Univ. of Silesia and its parental cv. Aramir. The mutant *093AR* was selected after a mutagenic treatment of seeds of the cv. Aramir with N-methyl-N-nitrosourea (MNU). The seeds were pre-soaked in distilled water for 8h and mutagenically treated with MNU (0.7 mM) for 3h. After the first treatment the seeds were rinsed several times in tap water and germinated on trays with filter paper for 6h before a second 3h treatment with the same mutagen concentration. The mutant *093AR* was selected as a semi-dwarf form with the height of about 70% of the parental cultivar. Plants of the *uzu* mutant are of similar height at maturity as those of mutant *093AR* (Fig. 1). The height of both semi-dwarf mutants was measured in a two-year field experiment, in which 30 plants per genotype/year were analysed. A detailed characteristic of these genotypes is given in Table 1. The origin of *093AR* from the cv. Aramir was confirmed by AFLP marker technique analysis (data not shown). As a result of crossing with the parental cv. Aramir, it was found that the phenotype of the mutant *093AR* is conferred by a recessive mutation of a single gene. A genetic analysis of both these mutants, *093AR* and *uzu*, indicated that they are allelic (Table 1).

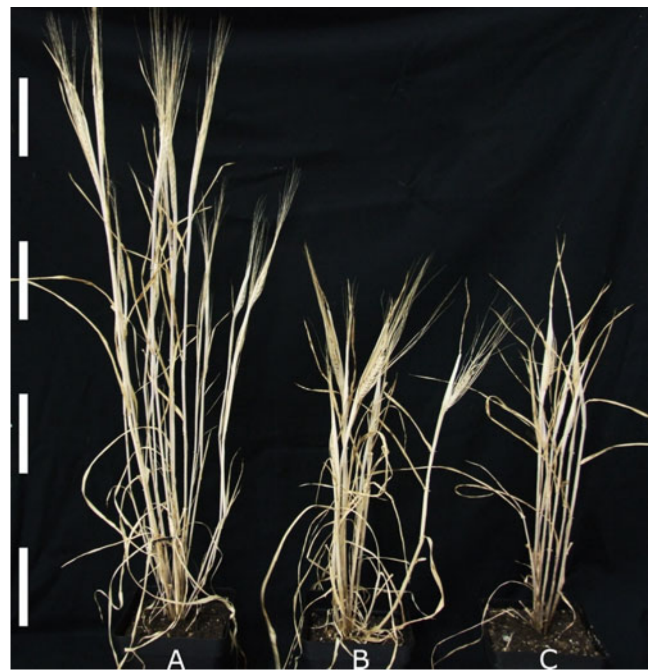


**Fig. 1** Phenotypes of the semi-dwarf barley mutant *093AR* (**b**), its parental cv. Aramir (**a**) and mutant *uzu* (**c**). Bar - 10 cm

### Physiological tests

Barley BR-mutants are selected on the basis of an etiolation test during which seedlings of each genotype are grown for seven days in two different conditions: in light and in darkness under black hoods covering the pots in order to eliminate the influence of light, which is known to reduce the BR synthesis. After the growing period, the length of roots and leaves was measured and compared between the

**Table 1** Characteristics of plants of the cv. 'Aramir', semi-dwarf mutants *093AR*, *uzu* and $F_1$ generation of a cross between both mutants performed in allelism test the seedling length was measured upon two-week growth period in both conditions

| Genotype | Seedling length [cm] | | Plant height at maturity [cm] | Length of internodes [cm] | | | | | Spike length [cm] | Awns length [cm] |
|---|---|---|---|---|---|---|---|---|---|---|
| | in light | in darkness | | 1st | 2nd | 3rd | 4th | 5th | | |
| Aramir | 13.1 | 15.5 | 67.8 | 18.2 | 16.6 | 11.7 | 12.2 | 9.1 | 6.95 | 12.5 |
| *093AR* | 8.3 | 8.4 | 51.3 | 13.8 | 11.4 | 9.5 | 9.5 | 7.1 | 5.1 | 9.9 |
| *uzu* | 8.25 | 8.35 | 52.3 | 13.7 | 11.5 | 9.4 | 9.7 | 8.9 | 5.2 | 9.5 |
| $F_1$ (*093AR* × *uzu*) | 8.3 | 8.4 | 52.4 | 13.6 | 11.1 | 10.0 | 9.2 | 8.5 | 5.0 | 9.8 |

seedlings of a given genotype grown in different conditions. The lack of an enhanced elongation of seedlings grown in darkness (when compared to those grown in light) indicates that the given genotype is the BR-mutant.

In order to determine whether the phenotype of the analysed mutants is caused by the malfunctioning of the BR synthesis or the signalling pathway, the leaf segment unrolling test was performed according to the protocol by Chono et al. (2003). Three concentrations of 24-epi-brassinolide (24-eBL; Sigma) were applied: $10^{-8}$, $10^{-6}$ and $10^{-5}$ M with distilled water as a reference. Leaf segments of the cv. Aramir were analysed as a control. Ten leaf segments per genotype were analysed after incubation in each solution. The width of leaf blade segments was measured after the incubation period.

DNA extraction, PCR, cloning and sequencing

Grains of the genotypes *uzu*, *093AR* and cv.Aramir were sown into a mixture of soil and vermiculite (1:1) and grown for four weeks in a growth chamber at $18^0$C under 16h photoperiod and 200 µ$Em^{-2}s^{-1}$ light intensity. After four weeks, leaf tissue samples were collected and dried in Silica Gel, Type III (Sigma) for two weeks. Samples were ground with an electric grinder (Retsch) and subjected to DNA extraction. DNA extraction was performed according to "micro C-TAB" protocol (Doyle and Doyle 1987). Six primer pairs: F1 5`cgcttctcgcatggtctca, R1 5`gcagttggtgaagtccgaga, F2 5`ctcgacttgtccagcaacaa, R2 5`gttgggatcttggcagagg, F3 5`cgacctcagctccaacaact, R3 5`tccttgtgaagttgcacagc, F4 5`cggtccgaggaccttagtc, R4 5`cagaatgtgaccggctatca, F5 5`ggaggcagaagaatgacgag, R5 5`cagcaacacaacaccgtagc, F6 5`ggtatgtgccaccggagta, R6 5`agcttgcgtgggaacctca, designed with the use of the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3) were used for the *HvBRI1* gene cloning (NCBI GenBank acc. no. AB088206). Three independent "touch-down" PCR reactions were performed for each genotype per primer pair. Composition of PCR mixture (20 µl) was: 1 µl DNA of given genotype (0.1µg/µl), 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 10 pM of primer F, 10 pM primer R, 0.7 unit of polymerase and distilled water to final volume. The following PCR amplification profile for primer pairs 2FR, 3FR, 5FR was used: (1) 94°C, 3 min, (2) 94°C, 45 sec, (3) 62°C, 45sec, (4) 72°C, 1 min 30 sec, steps 2 – 4 repeated 3 times, (5) 94°C, 45 sec, (6) 60°C, 45 sec, (7) 72°C, 1 min 30 sec, steps 5 – 7 repeated 3 times, (8) 94°C, 45 sec, (9) 57.5°C, 45 sec, (10) 72°C, 1 min 30 sec, steps 8 – 10 repeated 32 times. The final extension: 72°C, 5 min. For the primer pairs 1FR, 4FR, 6FR the following PCR profile was used: (1) 94°C, 3 min, (2) 94°C, 45 sec, (3) 59°C, 45 sec, (4) 72°C, 1 min 30 sec, steps 2 – 4 repeated 3 times, (5) 94°C, 45 sec, (6) 57°C, 45 sec, (7) 72°C, 1 min 30 sec, steps 5 – 7 repeated 3 times, (8) 94°C, 45 sec, (9) 55°C, 45 sec, (10) 72°C, 1 min 30 sec, steps 8 – 10 repeated 32 times. The final extension: 72°C, 5 min. Amplification products were extracted from 1.5% agarose gels using QIAEX II Gel Extraction Kit (QIAGEN) and cloned into a pGEM-T Easy vector (Promega). The transformed *Escherichia coli* JM109 Competent Cells were incubated at 37°C on an LB medium containing Blue-White Select Screening Reagent (Sigma) overnight. Three white colonies for each insert were chosen and transferred into a liquid LB medium for plasmid isolation as a sequencing control. Plasmid purification was carried out using QIAprep Spin Miniprep Kit (QIAGEN). The inserts were sequenced using the SequiTherm EXCEL II LC DNA Sequencing Kit (EPICENTRE) utilizing the LI-COR sequencer and e-Seq v2.0 software (LI-COR).

*In silico* analysis of sequences obtained and mutations identified

The analysis of the sequencing data was performed with the AlignIR software (LI-COR), which allowed the assembly and alignment of sequences derived from different genotypes in order to identify mutations (LI-COR). The sequences carrying mutations were transferred to Jellyfish program in which *in silico* translation was performed in order to determine the impact of the identified mutation on the sequence of the encoded polypeptide. Translation was carried out using six different reading frames, but for the final analysis one specific amino-acid sequence was used that shared the highest level of similarity with homologous polypeptide derived from other species. In order to find out to what extent the amino-acid residue that was substituted as a result of identified mutation is conserved, the amino-acid sequences of homologous polypeptides were aligned and compared using ClustalW program (www.ebi.ac.uk/Tools/clustalw2/index). A further characterization of the mutational changes involved the use of the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/), which performs a schematic superimposition of predicted secondary spatial structures on the amino-acid sequence of the analysed polypeptides (Jones 1999; McGuffin et al. 2000; Bryson et al. 2005). This method allows the localization of amino-acid residues within secondary spatial structures of alfa-helises and beta-sheets. Their localization within a secondary structural domain was also confirmed with the use of SOPMA program (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_sopma).

RNA extraction, reverse transcription and RT-PCR

RNA was extracted from combined leaves and roots of 6-days seedlings of the cv. Aramir and mutant *093AR* according to the TRIZOL Reagent protocol (Life Technologies). After isolation 2 µg of total RNA were treated with

RQ1 DNase. 1μl of RQ1 10 × DNase Buffer and 3μl of RQ1 DNase was added to each sample. DEPC-treated water was added to the final volume of 10 μl. The mixtures were incubated at 37°C for 30 min. 1μl of RQ1 DNase Stop Solution was added to each sample. The samples were incubated at 65°C for 10 min.

Reverse transcription was performed utilizing the RevertAid First Strand cDNA Synthesis Kit (Fermentas). DNase-treated RNA (2.5 μl) with 1 μl of Oligo(dT)$_{18}$ and 8.5 μl of DEPC-treated water per sample were incubated at 70°C for 5 min. and placed immediately on ice. Four μl of 5 × reaction buffer, 1 μl of RNase inhibitor and 2 μl of dNTPs (10 mM) were added to each sample. The solutions were incubated at 37°C for 5 min. One μl of RevertAid MuLV Reverse Transcriptase was added to each sample and the solutions were incubated at 42°C for 1h and at 70°C for 10 min. For each RT-PCR reaction, 1.5 μl of cDNA was used. The following PCR amplification profile was used for primers pair 4FR in order to determine the overall amount of product obtained after PCR reactions on cDNA from the mutant *093AR* and cv. Aramir: (1) 94°C, 3 min, (2) 94°C, 45 sec, (3) 59°C, 45 sec, (4) 72°C, 1 min 30 sec, steps 2 – 4 repeated 3 times; (5) 94°C, 45 sec, (6) 57°C, 45 sec, (7) 72°C, 1 min 30 sec, steps 5 – 7 repeated 3 times; (8) 94°C, 45 sec, (9) 55°C, 45 sec, (10) 72°C, 1 min 30 sec, steps 8 – 10 repeated 32 times. The final extension: 72°C, 5 min.

To determine the presence of the *HvBRI1* gene transcript, RT-PCR was carried out on the basis of cDNA derived from the mutant *093AR* with the cDNA from the cv. Aramir as a control. The reactions were performed with the use of primers pair 4FR and the corresponding PCR reaction profile. In order to determine the relative transcription level of *HvBRI1* gene in investigated forms, semi-quantitative RT-PCR reactions were performed on cDNA of these genotypes. The reactions were performed with the use of primers pair 4FR and by applying five different duration times of the PCR program: 23 cycles, 25 cycles, 27 cycles, 29 cycles and the original 32 cycles.

## Results

### Mutant *093AR* displays lack of enhanced elongation during its growth in darkness and is BR-insensitive

Physiological tests were carried out on two mutants – spontaneous, semi-dwarf *uzu* and allelic, chemically-induced semi-dwarf *093AR* as well as on the cv. Aramir. The etiolation test indicated that seedlings of cv. Aramir that were grown in darkness were significantly longer than those grown in light (Fig. 2a). In contrast to the cv. Aramir, both mutants showed a lack of an enhanced rate of elongation during growth in darkness. Seedlings of these genotypes grown under two different conditions were of similar length (Fig. 2b and c). This result indicated that 093AR is a BR-related mutant.

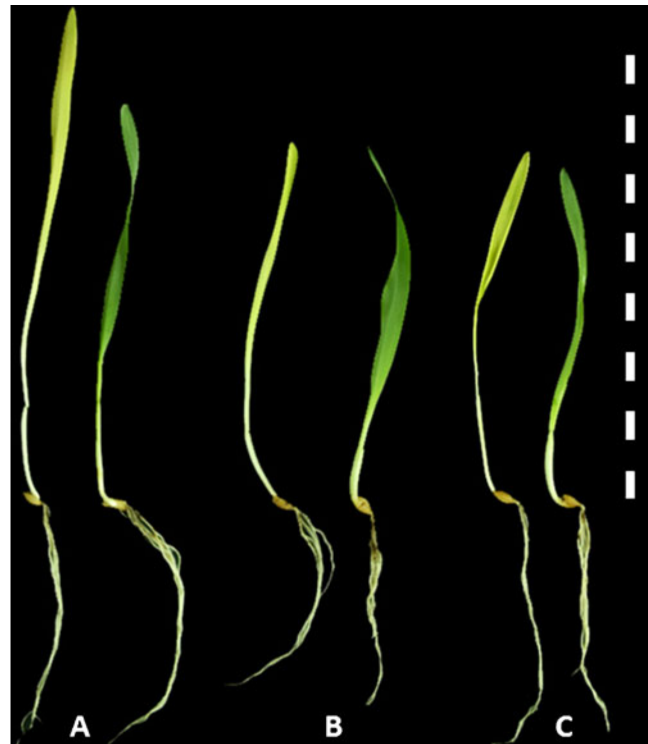


**Fig. 2** Result of the etiolation test for the cv. 'Aramir' with normal BR signal transduction pathway (**a**) and the mutants *093AR* (**b**) and *uzu* (**c**) displaying defects in signalling of this hormone and de-etiolation. In each pair of plants presented (A, B or C), the seedling on the left was grown in the dark. Bar – 1 cm

To determine whether this abnormality is connected with the malfunctioning of synthesis or signalling pathway of this hormone, leaf segment unrolling tests were performed. Leaf segments of the cv. Aramir displayed gradual unrolling corresponding to the increase in 24-eBL concentration within incubation solutions (Fig. 3a), whereas, both analysed mutants did not show any response to the applied concentrations of the hormone (Fig. 3b and c). These results indicate that both mutants show defects in BR signalling pathway. It confirms that the analysed mutants are allelic.

### Identification of a mutation within the *HvBRI1* gene in the mutant *093AR*

The comparison of genomic sequences of the *HvBRI1* gene in the mutants *uzu*, *093AR* and cv. Aramir confirmed the presence of a mutation previously identified by Chono et al. (2003) in the *uzu* genotype. The single-nucleotide A>G substitution has been identified at the position 2612 in the *uzu* mutant sequence, which proved to be specific for this genotype. This mutation leads to the change of histidine-

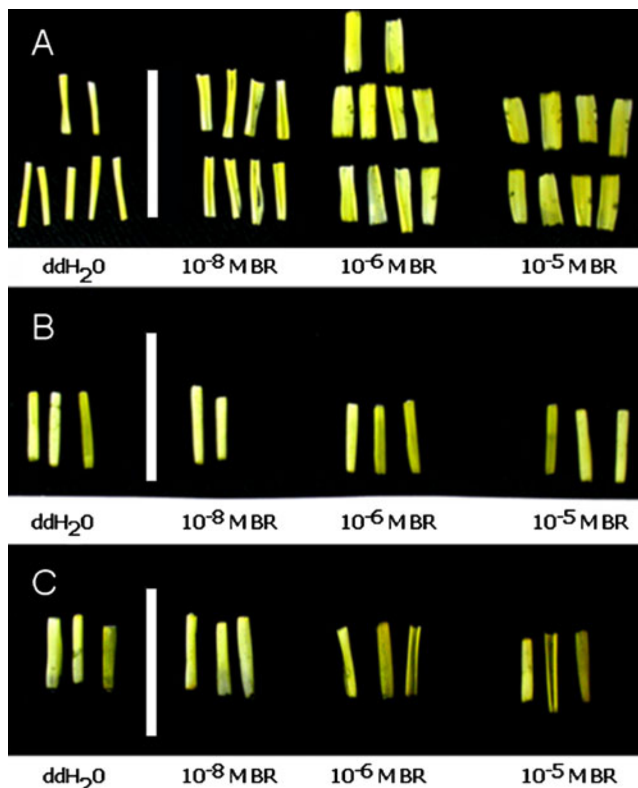


**Fig. 3** Leaf-blade segment unrolling in response to various concentrations of 24-eBL. Leaf-blade segments of the cv. 'Aramir' (**a**) showed a positive response to the applied concentrations of BR - gradual unrolling. Leaf-blade segments of the mutant *093AR* (**b**) and mutant *uzu* (**c**) displayed a lack of reaction, indicating that these genotypes have defects in the BR signalling pathway. White bars - width of 2 mm

857 into arginine (Fig. 4a). In the case of the mutant *093AR* and cv. Aramir, adenine was present at this position, indicating the lack of mutation in the gene fragment encoding a highly conserved kinase domain.

However, a comparison of other fragments of *HvBRI1* gene sequences between these genotypes leads to the identification of double-nucleotide substitutions CC>AA at the positions 1760 and 1761 in the sequence of the mutant *093AR*. These mutations are specific for this genotype (Fig. 4a). On the basis of a translation *in silico* with the use of Jellyfish program, it was demonstrated that the substitutions identified in mutant *093AR* cause the change of threonine-573 into lysine. Threonine-573 is the last amino-acid residue located within the 70-amino acid island separating the LRR domains, whereas histidine-857 which is substituted in mutant *uzu* is situated within the kinase domain (Fig. 4b).

Threonine-573 is a highly conserved amino-acid residue among the homologous BRI1 polypeptides from mono- (barley, rice) and dicotyledonous (*Arabidopsis*, tomato) species which was demonstrated by the ClustalW alignment. With the ClustalW tool, polypeptide sequences of BRI1 homologs were aligned and the positions of the mutations, which have been identified so far in BRI1 homologs from various species, were depicted and superimposed on the alignment. The mutations that cause substitutions of amino-acid residues located within the 70-amino acid island, in which the substituted threonine-573 in mutant *093AR* is situated, have so far also been identified in rice and *Arabidopsis* (Fig. 5).

In order to determine the location of the substituted threonine-573 residue within secondary spatial structures, an analysis was performed with the use of PSIPRED Protein Structure Prediction program. This analysis indicated that this residue is situated within the short alpha-helix domain encompassing residues 572-574 (Fig. 6). Moreover, it was demonstrated that this alpha-helical domain is present in the corresponding locations in the homologous proteins from mono- (barley, rice) and dicotyledonous species (*Arabidopsis*, tomato) (Fig. 6). The location of threonine-573 within the alpha-helical domain was also confirmed with the use of SOPMA program (data not shown). This conservation of a topological arrangement of spatial domains may indicate that the alpha-helical domain is of critical importance for protein conformation.

## Analysis of transcription level of the *HvBRI1* gene in the mutant *093AR*

In order to determine the presence of *HvBRI1* gene transcript in the mutant *093AR*, RT-PCR reactions were carried out on cDNA derived from this genotype and the cv. 'Aramir' as a reference. The aim of this survey was to check if there are any qualitative or semi-quantitative differences between the transcription levels of *HvBRI1* gene in these genotypes. Results indicated that there is no qualitative difference in transcription level of this gene between the analysed genotypes. Additionally, the semi-quantitative RT-PCR reactions were performed on the same cDNA derived from these genotypes in order to determine a quantitative difference between transcription levels of the *HvBRI1* gene. No significant differences between the amounts of RT-PCR products at each stage of the reaction were demonstrated between these genotypes (Fig. 7), indicating that there is no mutation within the regulatory region of *HvBRI1* gene in the mutant *093AR* affecting the transcription level of this gene. Hence, it may be concluded, that double substitutions CC>AA identified in the mutant *093AR* within the coding sequence of *HvBRI1* gene, which cause the change of highly conserved threonine-573, are responsible for the semi-dwarf, BR-insensitive phenotype of this mutant.

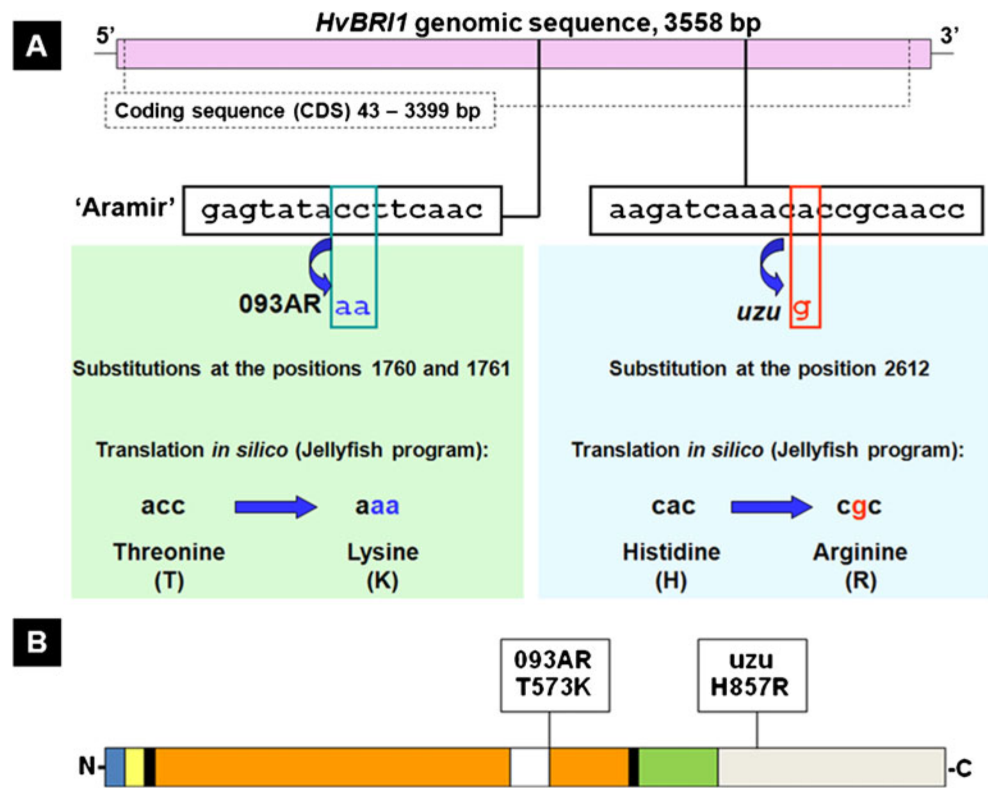


**Fig. 4** Type and location of substitutions identified in two allelic barley mutants: chemically-induced *093AR* from cv. 'Aramir' and spontaneous mutant *uzu* within the coding sequence of *HvBRI1* gene and their impact on the sequence of encoded polypeptide (**a**).Structure of co-receptor encoded by barley *HvBRI1* gene with denotation of domains: signal peptide (blue), leucine zipper motif (yellow), pairs of cysteines (black), LRR domains (orange), 70-amino acid domain (white), transmembrane fragment (green) and kinase domain (grey) (**b**). Location of amino-acid residues substitutions identified in mutants *093AR* and *uzu* are depicted in frames. 'T573K' indicates the substitution of threonine-573 by lysine in mutant *093AR*, H857R denotes the replacement of histidine-857 by arginine in *uzu* mutant

## Discussion

In the BR-insensitive mutant *093AR* of the cv. Aramir, two vicinal substitutions causing changes of cytosines to adenines at positions 1760 and 1761 were identified. These substitutions led to the change of the single amino-acid residue, threonine-573, to lysine. Analysis, performed with the use of ClustalW program, indicated that this residue is highly conserved among homologous BRI1 polypeptides derived from mono- and dicotyledonous plant species. The results indicate that threonine-573 plays an important role in the function of the BRI1 co-receptor. A high conservation level of amino-acid residue among homologous polypeptides is broadly accepted as an indication of the critical role of this residue in the function of the enzyme (Hong et al. 2002). Threonine-573 is a hydrophilic and a neutral amino acid carrying a hydroxyl group. This amino acid was substituted in mutant *093AR* by lysine, which is basic, contains an amino group, being positively charged when reduced. Both amino acid residues have different values of isoelectric point: 5.6 for threonine and 9.6 for lysine. The fact that these amino-acid residues have different structures and additionally display distinct physical and chemical properties, suggests that their substitution leads to conformational changes of the polypeptide and, as a consequence, an alteration in its catalytic efficiency.

Threonine-573 is the last amino-acid residue situated within the 70-amino acid island (70-aa domain) separating the LRR domains. The presence of this island is the unique feature of BR receptors, belonging to the vast family of LRR-RLK (Leucine-Rich Repeat Receptor-like Kinases) proteins. This domain of BRI1 polypeptides together with directly preceding LRR domain span about a hundred-amino-acid region responsible for the binding of BR molecules (Kinoshita et al. 2005; Belkhadir and Chory 2006; Nakamura et al. 2006;). The importance of the 70-amino acid island along with the flanking LRR domain in maintaining a proper conformation, crucial for the binding of BR molecules, was demonstrated by the alignment of BRI1 proteins' sequences (Szekeres 2003). The aim of this survey was to determine the functional domains displaying the highest level of conservation. The 70-amino acid island showed the second highest level of sequence conservation after the kinase domain. The result indicated that this fragment of BRI1 receptors plays an important role in maintaining protein conformation. In transgenic *A. thaliana* plants that displayed an overexpression of a mutated form of BRI1 receptor carrying substitutions of amino acids within the 70-amino acid island, a lack of BR binding was noted (Wang et al. 2001). It was suggested that this domain of the BRI1 receptor is responsible for the recognition of BR molecules, most probably through interaction with another BR-binding protein (Nomura et al. 2003).

Threonine-573, which was substituted in the mutant *093AR*, is situated three residues away from a highly

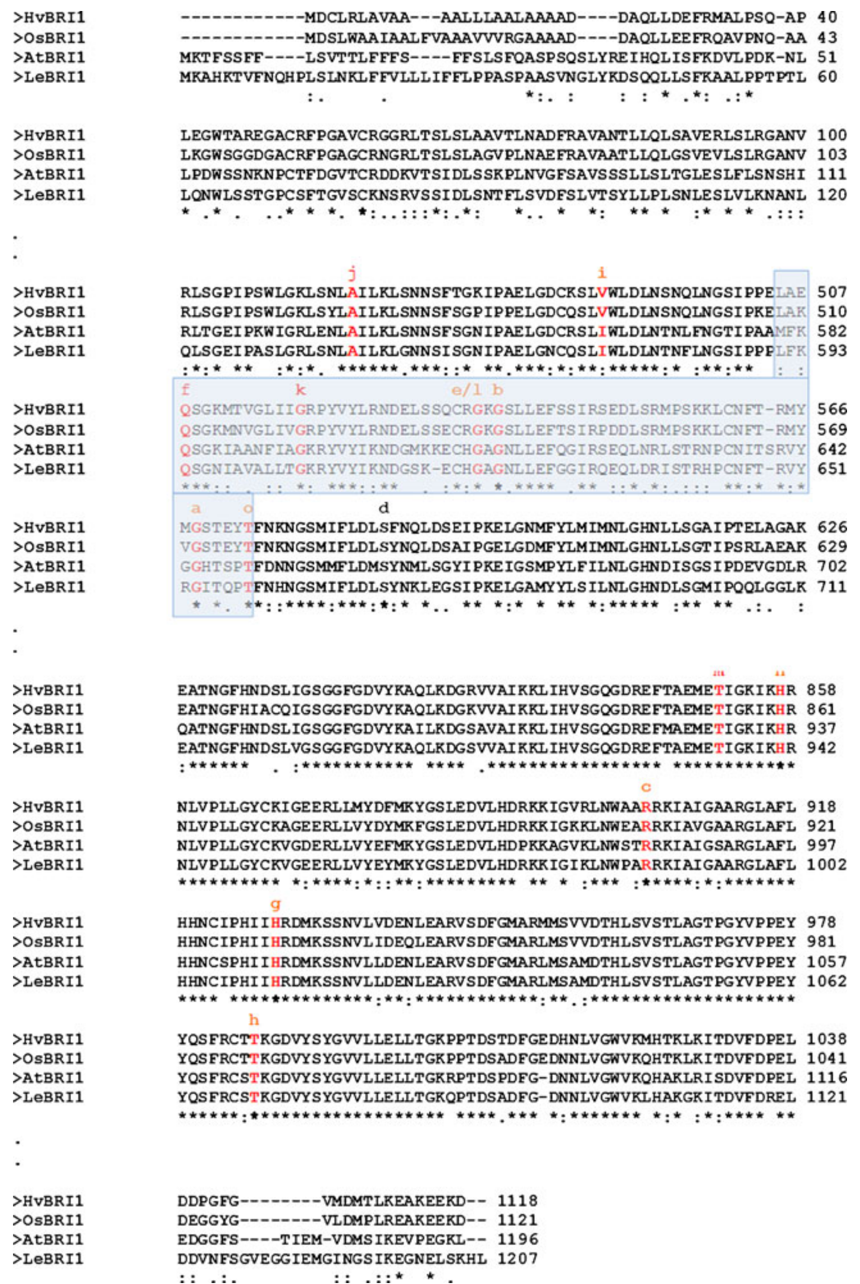


**Fig. 5** Comparison of protein sequences of BRI1 homologs from barley, rice, *Arabidopsis* and tomato with the use of ClustalW tool. Asterisks indicate a complete conservation of given amino-acid residue in all compared sequences: HvBRI1 (BAD01654), OsBRI1 (NP916669), AtBRI1 (AAC49810) and LeBRI1 (AAN85409); ':' indicates, that a given amino-acid residue is not fully conserved, however, corresponding residues in compared polypeptides share a significant similarity of chemical properties. '.' denotes that the amino-acid residue is not conserved and, additionally, corresponding residues in compared polypeptides do not share chemical similarity. The distribution of mutations identified in BRI1 homologs in these species: in *Arabidopsis*: a – *bri1-6,* b - *bri1-7*, c – *bri1-8,* d – *bri1-9*, e – *bri1-113,* f – *bri1-116* allele, respectively; in tomato: g – *abs* mutant; in rice: h – *d61-1*, i – *d61-2*, j – *d61-7*, k -*d61-8*, l - *d61-9*, m - *d61-10* allele, respectively; in barley: n – *uzu* mutant, o – *093AR* mutant. The 70-amino acid island was depicted in blue

conserved domain (Asn-Gly-Ser-Met), which is present in all protein homologs functioning as BR receptors and is responsible for interactions with BR molecules during perception (Kinoshita et al. 2005; Nakamura et al. 2006; Holton et al. 2007). Together with a high level of conservation among the homologous BRI1 polypeptides, this observation indicates that threonine-573 plays an important role in maintaining the proper conformation of the receptor that is necessary for BR perception. It was also demonstrated with the use of two different programs (PSIPRED and SOPMA) that this residue is located within the short alpha-helix present in corresponding fragments of all homologous polypeptides. This conservation of a topological arrangement of spatial domains within these fragments of the homologous BRI1 proteins may indicate that the alpha-helical domains are of critical importance for protein conformation. No significant differences between the amounts of PCR products at each stage of semi-quantitative RT-PCR reactions were demonstrated between the mutant *093AR* and cv. Aramir indicating that there is no mutation within the regulatory region of *HvBRI1* gene in mutant *093AR* that could influence transcription of this gene. This means that the identified vicinal nucleotide substitutions within the coding sequence of this gene are responsible for the semi-dwarf, BR-insensitive phenotype of the selected mutant.

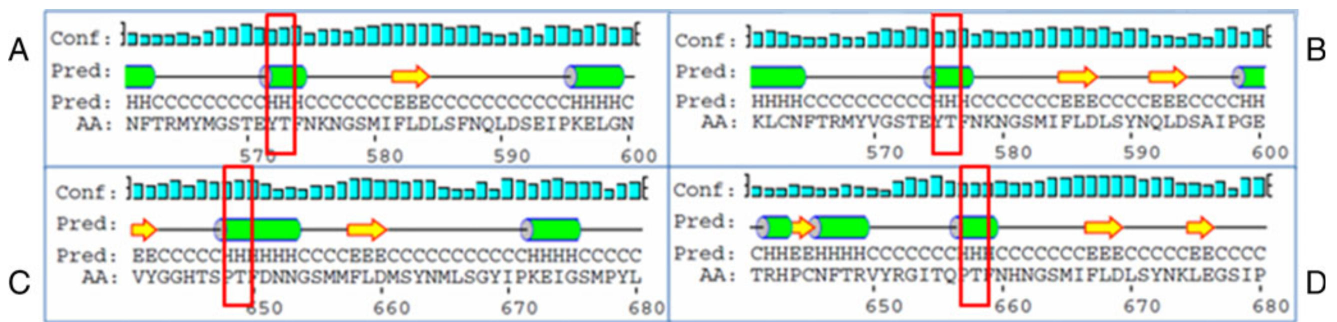


**Fig. 6** Fragments of diagrams generated by PSIPRED program as a result of analysis performed on the basis of polypeptide sequence of HvBRI1 (**a**), OsBRI1 (**b**), AtBRI1 (**c**) and LeBRI1 (**d**), respectively. Corresponding positions of threonine (T) which was substituted in the mutant *093AR* are shown in a red frame in these homologous polypeptides. Alpha-helices (**h**) are depicted as green cylinders, beta-sheet (**e**) as yellow arrow, and coils (**c**) as black, horizontal lines, respectively. 'Conf' denotes confidence of prediction, and the height of blue bars represents this confidence on the basis of sequence similarity between query and protein sequences deposited in the repository

Mutations causing changes of amino acids that are situated within the 70-amino acid island were also identified in other plant species. In rice, such substitutions causing alterations in the sequence of this domain were characterized in two mutants, *d61-8* and *d61-9*. In both cases, it was a G>A substitution, which caused the change of glycine-522 into glutamate in mutant *d61-8* and glycine-539 into aspartate in the second genotype. Both substituted residues are located within the proximal part of the 70-amino acid domain (Fig. 5). Mutants are semi-dwarf with a 20% reduction in plant height and fully fertile (Morinaka et al. 2006; Nakamura et al. 2006), similarly to the phenotype of the examined barley mutant *093AR* which carries an amino-acid substitution within the same receptor domain. Rice mutant *d61-2* that carries a G>A substitution causing a change of valine-491 into methionine displays a similar phenotype. The substituted amino acid is situated within the LRR preceding the 70-amino acid island (Yamamuro et al. 2000). A similar phenotype is displayed by another rice mutant, *d61-7*, carrying a C>T substitution causing a change of alanine-467 into valine in the same LRR domain (Fig. 5). This mutant shows a lower level of plant height reduction, an increased production of biomass and grains per spike when compared to the parental variety (Morinaka et al. 2006).

Substitutions that cause changes of amino acids located within the 70-amino acid island were also identified in *Arabidopsis* (Li and Chory 1997; Noguchi et al. 1999;

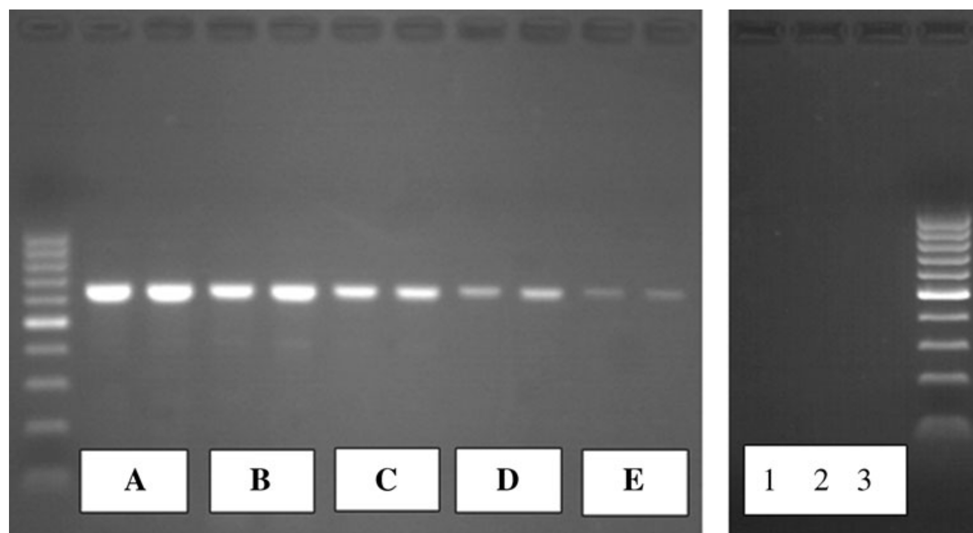


**Fig. 7** The results of semi-quantitative RT-PCR reactions on the basis of cDNA derived from mutant *093AR* and cv. 'Aramir'. The reactions were performed with the use of primers pair 4FR and by applying five different duration times of the PCR program: (**a**) original 32 cycles, (**b**) 29 cycles, (**c**) 27 cycles, (**d**) 25 cycles, and (**e**) 23 cycles. RT-PCR reactions performed on cDNA from the cv. 'Aramir' are the first in each A – E pair. Size marker: Gene Ruler$^{TM}$ DNA Ladder (Fermentas). PCR product size: 650 bp. Control reactions are included: 1 - PCR reaction devoid of cDNA template, 2 - PCR reaction devoid of PCR primers, 3 - PCR reaction devoid of either ingredient

Friedrichsen et al. 2000). In the *Arabidopsis* mutants *bri1-6*, *bri1-7*, *bri1-113* and *bri1-116*, such substitutions caused changes of glycine residues. The first of these mutant carries a G>A substitution leading to the change of glycine-644 into aspartate. The same substitution is also present in the mutant *bri1-7*, which leads to the change of glycine-613 into serine and in the mutant *bri1-113*, in which glycine-611 is substituted by glutamate. The mutant *bri1-116* was characterized as carrying a C>T transition that was a nonsense mutation leading to the formation of a truncated polypeptide. Taking into account that this mutation is located in the sequence encoding the proximal part of the 70-aa domain, this polypeptide was nonfunctional as it was devoid of the transmembrane region as well as the kinase domain. This mutant displays severe dwarfism and significant reduction of fertility (Friedrichsen and Chory 2001).

Substitutions in the mutants *bri1-7* and *bri1-113* are placed in close vicinity, in the central part of this domain, while nucleotide change identified in mutant *bri1-6* causes a change of amino acid localized in the distal part of the 70-aa domain (Fig. 5). Mutants *bri1-6* and *bri1-7* are classified as semi-dwarf and fertile. This is of particular significance, taking into account that glycine-644, which is substituted in the mutant *bri1-6,* is localized only four residues up-stream from threonine, which in the analogous position is changed into lysine in the barley mutant *093AR*. In the case of both mutants, polar amino-acid residues are substituted by charged ones. In both mutants this leads to the semi-dwarf phenotype with full fertility. The *Arabidopsis* mutant *bri1-113,* classified as dwarf, carries a change of glycine-611 to a negatively charged glutamate (Li and Chory 1997). The critical role of glycine-611 is based on its small size, which allows for conformational changes of the polypeptide after perception of the BR molecule and leads to an activation of the kinase domain. This mutation also influences the process of receptors dimerization (Friedrichsen et al. 2000). With the exception of the two *Arabidopsis* mutants, *bri1-113* and *bri1-116* (nonsense mutation), the rest of the mutants, which carry substitutions of amino-acid residues situated within BRI1 proteins' fragments responsible for the binding of BR molecules, exhibited semi-dwarf phenotypes without any reduction in fertility. This is the case for all the *d61-2*, *d61-7*, *d61-8*, *d61-9* mutants from rice, *bri1-6, bri1-7* from *Arabidopsis* and the examined barley mutant *093AR*, described in the paper. These mutants carry an amino-acid substitution in the corresponding fragment of the HvBRI1 protein and display a very similar phenotype. Taking this into account, it is concluded that in the case of both mono- and dicotyledonous species, mutants carrying missense mutations within the 70-amino acid domain of homologous BRI1 proteins show very similar phenotypes in terms of fertility and semi-dwarfism.

The barley mutants described in this paper (*uzu* and *093AR*) share very similar phenotypes in terms of plant height reduction (both of them are semi-dwarf; Fig. 1), they show a lack of enhanced seedling elongation during their growth in darkness (Fig. 2), a lack of response to various concentrations of exogenously applied 24-eBL (Fig. 3), and both are fully fertile. These two mutants exhibit their similar phenotypic features despite the fact that they carry amino-acid substitutions within different domains of the HvBRI1 receptor, namely within the kinase domain (*uzu*) and 70-amino acid island (*093AR*). As the result of allelism test, $F_1$ generation, which was obtained after crossing between mutants *uzu* and *093AR* showed very similar phenotype to both mutant lines. When interpreting this result it should be kept in mind, that alleles carried by these mutants are not loss-of-function given that they do not lead to serious abnormalities in phenotypes and fertility of plants. Besides, altered forms of HvBRI1 proteins encoded by these alleles do not interact with each other, but with another component of BR receptor – HvBAK1 (BRI1-Associated Kinase1). A similar situation, where amino-acid substitutions located within these two different domains of the BRI1 receptor cause very similar effect in terms of plant height reduction and retention of full fertility, was reported in rice. The rice mutants *d61-8* and *d61-9,* carrying amino-acid substitutions within the 70-aa island, and two other rice mutants, *d61-1* and *d61-10*, with amino-acid substitutions within the kinase domain of OsBRI1 receptor (Fig. 5) show a very similar, semi-dwarf phenotype and full fertility (Morinaka et al. 2006; Nakamura et al. 2006). Noteworthy, these barley and rice mutants with mutations in different domains of homologous BRI1 receptors display very similar phenotypes. This is also reflected by the fact that threonine-854 substituted by isoleucine in the rice mutant *d61-10* is located only six amino-acid residues away from the conserved histidine residue, which was substituted in analogous position in the barley mutant *uzu* (Fig. 5). However, even in the same species, missense mutations causing substitutions of amino acids, located in different domains of BRI1 receptor, may lead to various phenotypes. This is the case in rice mutants *d61-3*, *d61-2* and *d61-1*. Mutants *d61-3* and *d61-2* carry amino-acid substitutions in different fragments of the Leucine-Rich Repeat domain, in its central and distal parts, respectively, whereas mutant *d61-1* has a substitution of amino-acid residue located within the kinase domain. Mutant *d61-3* displays a severe reduction in plant height and sterility, mutant *d61-2* shows an intermediate reduction of plant height (ca. 40%) and full fertility, whereas mutant *d61-1* displays a slight height reduction (ca. 10%) and full fertility (Nakamura et al. 2006). Taking this into consideration, it may be concluded that identifying new genes related to the BR signalling and exploring new alleles of already identified genes, provides a

great potential for developing a range of new semi-dwarf forms of crops.

## Conclusions

In the study, a new allele of barley *HvBRI1* gene encoding BR receptor was identified. Genetic analysis indicated that the chemically-induced, semi-dwarf mutant *093AR* is insensitive to high concentrations of brassinolide and allelic to the spontaneous mutant *uzu*, which carries a mutation in the *HvBRI1* gene. These results were confirmed by a molecular analysis which led to the identification of missense nucleotide substitutions within the sequence of the *HvBRI1* gene. This mutation caused a change of the highly conserved amino-acid residue and is responsible for the phenotype of the mutant *093AR*. In this study, the mutation identified in an extracellular domain of the barley HvBRI1 receptor is described for the first time. Despite its great value for agriculture, the *uzu* allele was introduced to barley varieties cultivated solely in East Asia, whereas the examined mutant *093AR* has so far been the only semi-dwarf mutant related to BR signalling, derived from the European spring barley germplasm. Identified mutation, which caused the semi-dwarf phenotype of the mutant *093AR* did not entail any reduction in fertility and seed production. Since it is well known that semi-dwarf forms of cereals are more resistant to lodging under unfavourable conditions when supplied with high concentrations of fertilizers, the identified mutant may potentially be a source of semi-dwarfism for barley breeding. Apart from this, identification of the new allele with mutation in *HvBRI1* gene fragment encoding domain mediating BR binding provides information about an acitivity of the receptor. The results of our study demonstrate that chemical and physical mutagenesis significantly improves exploring new genes and/or alleles of known genes, leading to the development of new attractive semi-dwarf fully-fertile forms of crops.

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