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# Two Lateral Organ Boundary Domain Transcription Factors HvCRL1 and HvCRL1L1 Regulate Shoot-Borne Root Formation in Barley (*Hordeum vulgare* L.)

**Thu Dieu Nguyen[1] · Mathieu Gonin[2] · Michal Motyka[1] · Antony Champion[2] · Goetz Hensel[1,3] · Pascal Gantet[1,2] · Véronique Bergougnoux[1]**

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## Abstract

Plant-specific LOB-domain (LBD) transcription factors are crucial in post-embryonic root initiation. In cereals, the fibrous root system comprises primary and seminal roots that develop during embryogenesis and lateral- and crown roots that develop post-embryonically from root or stem, respectively. In rice, the CROWNROOTLESS1 (CRL1) LBD transcription factor is the core regulator of crown root initiation and a direct target of the auxin response factor (ARF)-mediated auxin signaling pathway. Orthologs of *CRL1* have been identified and characterized in several species, where their role in crown root initiation has been validated. In barley, we identified two genes phylogenetically closely related to the rice *CRL1* genes that we named *HvCRL1* and *HvCRL1L1*. Using a crown root inducible system (CRIS), we identified that both *HvCRL1* and *HvCRL1L1* are expressed in response to auxin during the early steps of crown root differentiation in stem base, with *HvCRL1* transcripts being accumulated quickly during the first hour of treatment. Transient activation assays in rice protoplast showed that HvCRL1 could bind the *LBD-box*, a consensus DNA sequence recognized by LBD transcription factors, whereas HvCRL1L1 did not. Both genes can partially complement the *crl1* rice mutant. Loss-of-function mutation in each gene drastically impairs crown root formation in barley. These data show that HvCRL1 and HvCRL1L1 are both involved in the regulation of crown root formation in barley but that these two transcription factors likely act through distinct and complementary pathways in this developmental process.

**Keywords** Crown root formation · LBD transcription factor · Cereal · Barley · Rice

## Introduction

LATERAL ORGAN BOUNDARIES (LOB) Domain (LBD) proteins define a family of plant-specific transcription factors (TFs) that play crucial roles in plant growth and development, especially in post-embryonic root formation both in monocot and dicot species (Kidwai et al. 2023). In Arabidopsis, AtLBD16, AtLBD18, and AtLBD29 transcription factors control early lateral root formation (Okushima et al. 2007; Lee et al. 2009). In addition, AtLBD16 and AtLBD29 also control adventitious root formation from leaf explant (Liu et al. 2014). In tomato, *SHOOTBORNE ROOTLESS* (*SBRL/SlLBD17*) controls adventitious root development, whereas *BROTHER OF SHOOTBORNE ROOTLESS BSBRL/SlLBD16a* controls lateral root initiation (Omary et al. 2022). In rice, the crown root initiation is controlled by the LBD transcription factor CROWN ROOTLESS1 (CRL1)

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✉ Pascal Gantet
pascal.gantet@umontpellier.fr

✉ Véronique Bergougnoux
veronique.bergougnoux@upol.cz

[1] Czech Advanced Technology and Research Institute, Palacký University Olomouc, Olomouc, Czech Republic

[2] UMR DIADE, Université de Montpellier, IRD, CIRAD, Montpellier, France

[3] Centre for Plant Genome Engineering Institute of Plant Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

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(Inukai et al. 2005; Liu et al. 2005). CRL1 is involved to a lesser extent in the control of lateral root formation. *CRL1* is an auxin-responsive gene regulated by ARF1 (Inukai et al. 2005). In maize, *ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS* (*RTCS*) and *RTCS-like* (*RTCL*) are *CRL1* orthologs and are also auxin-responsive genes involved in the initiation of shoot-borne root primordium formation (Taramino et al. 2007; Xu et al. 2015; Hochholdinger et al. 2018). In wheat, the LBD transcription factor MORE ROOT IN WHEAT (TaMOR) also controls the formation of crown roots (Li et al. 2022). Thus, these *LBD* genes remarkably perform similar biological functions in different plant species and constitute useful regulators of crown root system architecture.

In cereals, the crown root system dominates and ensures resource acquisition during vegetative growth as well as during reproductive and grain-filling phases (Hoppe et al. 1986). Crown root number predominantly influences root biomass and the soil portion that the plant will explore and exploit. Different species of grasses adapt to water deficit by inhibiting the growth of newly formed crown roots and promoting the growth of already anchored soil roots to go deeper (Sebastian et al. 2016). In contrast, numerous crown roots favor plant soil anchorage and produce a multiaxial and redundant system, enabling a larger volume of soil exploration, rapid capture of water, and security to minimize damaging effects due to biotic stresses (Hochholdinger et al. 2004).

In barley (*Hordeum vulgare* L.), like in other cereals, the root system is mainly composed of post-embryonic shoot-borne roots, named crown roots (Orman-Ligeza et al. 2013; Gonin et al. 2019; Jia et al. 2019; Wahbi and Gregory 1995). Despite the availability of the genome-wide analysis of the *LBD* gene family in barley (Guo et al. 2016), their particular function in different developmental programs is unknown, especially during crown root differentiation. In the current research article, we specifically investigated the function of two barley *LBD* genes, *HvCRL1* and *HvCRL1L1*, that are phylogenetically closely related to the rice *CRL1* gene and the maize *RTCS* and *RTCL* genes whose function in crown root initiation and elongation has been proven in those species (Liu et al. 2005; Xu et al. 2015). Using real-time PCR analysis, we showed that both genes are accumulated in the stem base of barley seedling in response to auxin-mediated root initiation, with *HvCRL1* transcripts being accumulated earlier than *HvCRL1L1* transcripts. The *in-silico* analysis of the *HvCRL1L1* promoter indicated that this gene could be a downstream target of HvCRL1. In addition, the complementation of the rice *crown root-less1* (*crl1*) mutant, as well as the knockout barley lines in each of the two genes, demonstrated that both genes are involved in crown root development in barley.

## Methods

### Plant Material

The two-rowed spring barley (*Hordeum vulgare* L.) cultivar Golden Promise (cv. GP) was used for the study. Plants were sown and grown in 2-L pots containing a 3:1:2 mixture of garden mold/sand/white and black peat (Klasmann Substrate 2). At the tillering stage (BBCH code 29/30), plants were fertilized with 15 g Osmocote (N, P, K: 19%, 6%, 12%; Scotts, The Netherlands). Cultivation was performed in a glasshouse in Olomouc (Czech Republic) maintained at 18 °C-day/16 °C-night, under natural light variation. When necessary, the 14 h-photoperiod was maintained by artificial lighting ensured by sodium-vapor lamps coupled with mercury-vapor lamps (500 µmol $m^{-2}$ $s^{-1}$ at the top of the plant).

For the rice (*Oryza sativa* L.), the two cultivars cv. Kitaake and cv. Taichung 65 (TC65), as well as the *crl1* mutant in the genetic background TC65 were used. TC65 and *crl1* seeds were previously obtained from Prof. Y. Inukai, Japan (Inukai et al. 2005). For the purpose of grain multiplication and selection of transgenic plants, plants were grown in glasshouses. Grains were sown in 2-L pots containing ProfiSubstrate (Gramoflor) and placed in pot plates filled with water to maintain wetland growth conditions. Plants were fertilized twice a month with 0.3 g of AGRO NPK 11/7/7 fertilizer during vegetative development and with Kristalon Plod a květ fertilizer (NPK: 15/5/30; AGRO CS a.s., Czechia) from flowering to grain maturity. The temperature was maintained at 25 °C. During the winter, the 14 h-photoperiod was ensured by sodium-vapor lamps coupled with mercury-vapor lamps (500 µmol $m^{-2}$ $s^{-1}$ at the top of the plant); in the summer, no additional lighting was provided.

### Identification of LBD Proteins in Barley and Phylogenetic Analysis

Sixteen barley LOB-domain (LBD) protein sequences were retrieved from the Plant Transcription Factor Database (PlantTFDB) (Jin et al. 2017). Table S1 provides the correspondence of IDs between the 2012 annotation (Mascher et al. 2013) and the last available annotation of the barley Morex reference genome V3 (Mascher et al. 2021). One sequence (MLOC_72525) had no correspondence in the HORVU.MOREX.r3 annotation and was, therefore, not considered for the study. Using MEGA 11 (Tamura et al. 2021), the 15 sequences were aligned by MUSCLE and a maximum likelihood tree was built using the Jones-Taylor-Thornton (JTT) model with uniform rates among sites

(all sites used) and 1000 bootstraps. Sequences, as well as exon/intron structures, were extracted from Ensembl Plants (https://plants.ensembl.org/index.html); the zinc finger-like motif, the GAS block, and the leucine-zipper-like coiled-coil motif were determined manually (Zhang et al. 2020). The visualization of gene features was done with GSDS 2.0 (http://gsds.gao-lab.org/) (Hu et al. 2015) and modified with Method Draw Vector Editor (https://editor.method.ac/#move_down; visited Jan. 2024).

To predict the barley close orthologs of the LBD protein described as being involved in crown root initiation in rice and maize, barley sequences were aligned to the sequences from rice (OsCRL1/Os03t0149100-01 and OsCRL1-like/Os03t0149000-01), and maize (RTCS/GRMZM2G092542_P01 and RTCL/AC149818.2FG009) using the same conditions as described above. The characterization of the barley LBD protein family (Guo et al. 2016) and the recent barley annotation (Mascher et al. 2021) evidenced that the barley LBD proteins family contains 31 genes.Therefore, a phylogenetic analysis has been run with learnMSA (Becker and Stenke 2022) and default parameters; the sequences of rice (Os), maize (Zm) and Arabidopsis (At) *LBD* genes. The phylogenetic tree was analyzed with IQtree 1.6.2. (Nguyen et al. 2015). The best protein model was identified using -m MFP option, and the branch supports were tested by UFBoot2 (Hoang et al. 2018) with 10000 replicates.

## Crown Root Inducible System

The study of crown root initiation is challenging because it occurs in an unpredictable manner only in a few cells inside the stem base. To circumvent this problem, the lateral root inducible system (LRIS) described (Crombez et al. 2016) was adapted for crown root. For this purpose, grains of barley cv. GP were sterilized for 3 min in 70% ethanol (v/v), 5 min in 3% sodium hypochlorite with regular shaking, and intensively rinsed in sterile $H_2O$. Grains were sown in Petri dishes on filter paper wet with water containing *N*-1-naphthylphthalamic acid (NPA), an inhibitor of the polar auxin transport, to a final concentration of 50 µM (stock solution 100 mM in DMSO). Petri dishes were sealed and placed in the dark at 4 °C for 3 days to stimulate homogeneous dormancy breakage. Dishes (after re-saturating filter paper with 50 µM NPA) were then placed in a phytotron in controlled conditions (photoperiod: 12 h/12 h; 13 °C-night/16 °C-day; light intensity: 170 µmol photons $m^{-2}$ $s^{-1}$; 60% relative humidity) to induce germination. Three-day-old, germinated seedlings were transferred into a mini-hydroponic system filled with ½ strength Hoagland solution containing 50 µM NPA. After 5 days, the seedlings were transferred in a ½ strength Hoagland solution containing 50 µM 1-naphthaleneacetic acid (1-NAA). After 24 h of growth in the presence of 1-NAA, the seedlings were transferred to a ½ strength Hoagland. Stem bases were collected before induction with 1-NAA (0 h) and at 3, 6, 12, and 24 h after induction (Fig. S1). In parallel, samples were obtained from seedlings kept in ½ strength Hoagland solution containing 50 µM NPA for the whole duration of the experiment; those samples were used as a control to decipher between induction by 1-NAA and NAA-independent endogenous developmental programs. For each time-point, the sample comprised five stem bases that were flash frozen in liquid nitrogen. Each sample was prepared as six independent biological replicates.

## Gene Expression Analysis by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Samples prepared as described above were ground to a fine powder in liquid nitrogen using mortar and pestle. Total RNA was extracted using Quick-RNA™ Plant Miniprep kit (ZYMO RESEARCH, USA) according to the manufacturer's instructions and treated by TURBO™ DNase I (Thermo Fisher Scientific) to prevent contamination by genomic DNA. The concentration of total RNA was determined with a NanoDrop™ One/OneC Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific). The RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) was used to synthesize cDNA from 2 µg of the total RNA with oligo$(dT)_{18}$.

For real-time PCR, cDNA samples were diluted 5 times and used in a reaction containing 1X gbSG PCR master mix (Generi biotech), 250 nM of each primer, and 500 nM of ROX as a passive dye. Primers for qPCR were designed with Primer3Plus (https://www.primer3plus.com/) for the 5 barley *LBD* genes representing putative orthologs of the rice *CRL1* and maize *RTCS* genes based on the phylogenetic study. A set of putative genes selected based on the literature and in silico prediction using Genevestigator® (Nebion AG; data not shown) was analyzed for their stability across our samples with RefFinder (Xie et al. 2023). Finally, three reference genes (Hv*ACT*: AK248432.1; Hv*EF2α*: AK361008.1; Hv*5439*: AK360511.1) were selected. The specificity of the primers was checked by BLAST restricted to the barley genome and sequencing of the amplified product (Seqme, Czechia). Primers and amplification conditions are described in Table S2. Amplification and detection were carried out on a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) in an optical 96-well plate, as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The dissociation curve analysis was performed after 40 cycles. The cycle threshold value for the gene of interest was normalized to the three reference genes and the geometric mean of expression was calculated. The relative expression was determined using the

ΔΔCt mathematical model corrected for the PCR efficiency (Pfaffl 2001). The relative quantification was compared to the T0 sample corresponding to stem bases of seedlings harvested before treatment with 1-NAA. Each sample was analyzed in three technical replicates and six independent biological replicates. Each independent biological replicate represented a pool of five explants.

Nonparametric Kruskal–Wallis ANOVA supported statistical significance followed by a post hoc multiple comparison of mean rank using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

### Trans-Activation Assays in Rice Protoplasts

For transient protoplast transformation, the coding sequence of Hv*CRL1* (MLOC_10784/HORVU.MOREX.r3.4HG0408280) and Hv*CRL1L1* (MLOC_61947/HORVU.MOREX.r3.6HG0630410) was amplified from cDNA obtained from 2 µg of RNA extracted from the stem base of seedlings grown in the presence of 1-NAA. High-fidelity PCR amplification was performed using a Phusion® High-Fidelity DNA Polymerase (New England Biolabs) using primers designed to introduce the *Bam*HI and *Nco*I restriction sites (Table S2). The amplified PCR products were cloned inside the *Bam*HI/N*co*I restriction sites of the pRT104 vector (Töpfer et al. 1993) to generate the pRT104::35S-HvCRL1 and pRT104::35S-HvCR1L1 effector vectors. The vectors were verified by a commercial sequencing service (Seqme, Czechia).

The normalizer vector, p2rL7::35SrLUC, containing the *Renilla luciferase* (LUC) gene, and the pGusSH-47 reporter plasmids carrying the *GUS* reporter gene placed under the control of a minimal *CaMV 35S-47* promoter and an LBD-box tetramers are described in (Gonin et al. 2022).

Protoplast isolation and transfection were conducted as previously described (Gonin et al. 2022; Zarei et al. 2011). Briefly sterilized hulled seeds of the rice cv. Kitaake were sown in cultivation boxes containing ½ strength Murashige & Skoog medium supplemented with Gamborg B5 vitamin (Duchefa Biochemie B.V., Haarlem, The Netherlands). Etiolated seedlings were grown for 9 days in the dark at 19 °C. Leaves and shoots of 9-day-old rice seedlings were sliced with a sharp razor blade into small pieces that were quickly transferred into 30 ml of enzymatic solution consisting of 1.5% [w/v] cellulase R10 (Duchefa Biochemie B.V., Haarlem, the Netherlands), 0.4% [w/v] macerozyme R10 (Duchefa Biochemie B.V., Haarlem, the Netherlands), 0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$, BSA 0.1% [w/v], and pH 5.7. The vacuum was applied for 10 min, and the tissues were incubated in the dark for 4 h at 28 °C. Following filtering and washing, rice protoplasts were co-transformed by polyethylene glycol (PEG) (Yoo et al. 2007) with (1) pGusSH-47 reporter plasmid, (2) the p2rL7 normalization plasmid, and (3) pRT104 effector plasmid empty or carrying either Hv*CRL1* or Hv*CRL1L1* gene under the control of the CaMV35S promoter, in a ratio 2:2:6, respectively. The protoplasts were collected 18 h after transformation and cultivation at 28 ± 2 °C in the dark, frozen in liquid nitrogen, and stored at − 80 °C until further analysis. GUS and LUC activities were measured as described previously (Zarei et al. 2011) using a Varioscan LUX (Thermo Fisher Scientific) installed with SkanIt™ Software for Microplate Readers to measure the fluorescence and luminescence. GUS activities were related to LUC activities in the same samples considering transformation and protein extraction efficiencies. For both genes, 4–6 independent biological replicates were analyzed. Values were expressed as relative to the highest value observed with Hv*CRL1*. Statistical analysis was performed with GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

### Knock-Out Barley *crl1* Mutant Generated by CRISPR-Cas9

Small guide RNAs (sgRNA) were designed using the DESKGEN KNOCKIN tool (Hough et al. 2016) to target Hv*CRL1* or Hv*CRL1L1*. The sgRNA was selected upstream of the conserved active LOB domain of the LBD protein. The preparation of the CRISPR-Cas9 vectors was performed as previously described (Holubova et al. 2018). Briefly, the gene-specific oligos containing a *Bsa*I restriction site (Table S2) were annealed and integrated into the *Bsa*I-digested pSH91 vector. The sgRNA-Cas9 expression cassette was sequenced by Sanger sequencing (Seqme, Czechia) and subcloned into the binary p6i-d35S-TE9 vector (DNA-Cloning-Service, Hamburg, Germany) using *Sfi*I restriction enzyme giving rise to the vectors p271-35S::*10,784*-gRNA1, p271-35S::*10,784*-gRNA2 and p271-35S::*61,947*-gRNA1, p271-35S::*61,947*-gRNA2 and p271-35S::*61,947*-gRNA3. The Sanger-sequenced vectors (Seqme, Czechia) were electro-transformed into the supervirulent *Agrobacterium tumefaciens* strain AGL1 and used for the stable transformation of barley immature embryos.

Plants of the spring barley cv. GP were grown in a phytotron under control conditions (15 ºC/16 h/light and 12 ºC/8 h/dark). The light source was a combination of mercury tungsten lamps and sodium lamps, providing an intensity of 500 µmol.$m^{-2}$.$s^{-1}$ at the level of plant tops. Plants were cultivated in a 2:1 mixture of soil and perlite (Perlit Ltd., Czechia) and were fertilized every 14 days with YaraMila Complex (AgroCS, Czechia). Loss-of-function *hvlbd* mutants were generated as previously described (Hensel et al. 2008; Marthe et al. 2015). Their diploidy was validated by flow cytometry as previously described

(Doležel et al. 1989). All T0 transformants were selected on 50 mg.$L^{-1}$ hygromycin and by PCR amplification using T-DNA-specific primers. Three pairs of primers were used to amplify different regions of the T-DNA: *hpt* gene, *Cas9* gene, and the region containing the gRNA fused to the RNA scaffold. All primers and PCR conditions are described in Tables S2 and S3, respectively. To determine the type of mutation, a PCR reaction using primers flanking the targeted DNA region (Tables S2 and S3) was performed; the PCR product was Sanger-sequenced by commercial service (Seqme, Czechia), and the mutation was predicted using DECODR (https://decodr.org; Bloh et al. 2021). Barley plants that show a potential mutation were retained for homozygous selection and phenotyping.

The selection of homozygous lines was done according to the Cleaved Amplified Polymorphic Sequences (CAPS) assays, which are based on the fact that SNP polymorphisms identified between different mutant and non-mutant lines, as well as wild type, are converted into CAPS markers (Hodgens et al. 2017). The webtool indCAPS (http://indcaps.kieber.cloudapps.unc.edu/) was used to design the PCR primers and the conditions for CAPS assay.

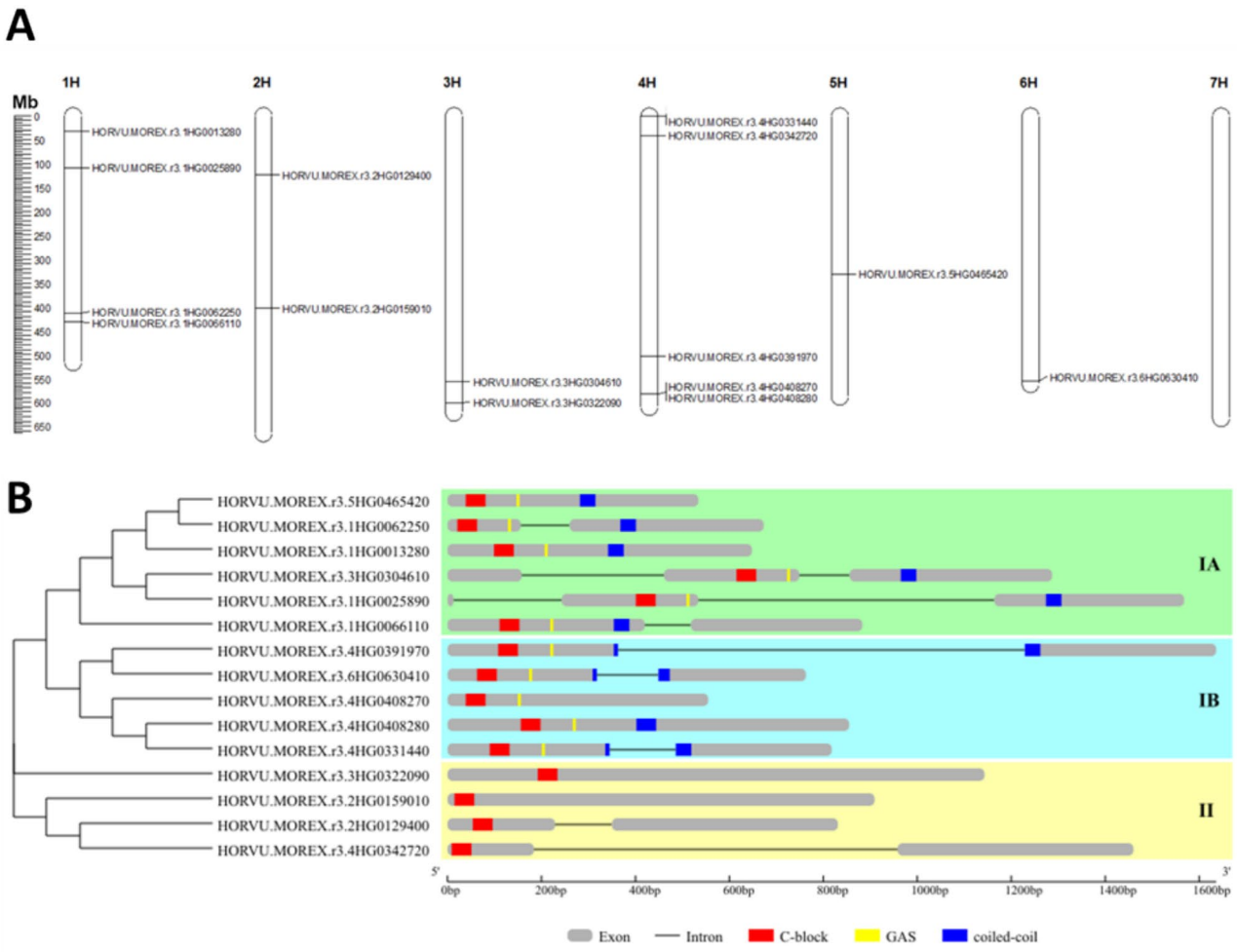
To phenotype the root system of mutant, the WT cv. GP as well as a line that integrated the empty vector were considered as negative control. The grains were sterilized in 70% ethanol for 30 s, rinsed once in sterile deionized water before immersion in 3% hypochlorite for 3 min and finally extensively rinsed with sterile deionized water. The sterilized grains were then gently dried on sterile filter papers, placed in Petri dishes on three layers of water-wet filter papers, and finally stored at 4 °C for 3 days to induce homogeneous germination. Germination was initiated by placing the Petri dishes under controlled conditions in a phytotron with a photoperiod of 15 °C/16 h/day and 12 °C/8 h/night. Two days after germination (DAG), young seedlings with approximately 1–2 cm of shoot length were transferred into pots (Ø:10 cm × h:10 cm) containing cleaned beach sand over a 1 cm layer of soil in the bottom to avoid sand leaching during watering. The plants were grown for 8 weeks in the same conditions and regularly watered with a nutritive solution (Kristalon™ start, Agro, Czechia). Eight-week-old barley plants were removed from the pots. Roots were carefully washed under tap water to remove the excess sand and soil. The number of crown and embryonic roots was manually determined, the length of the longest root (deepness of the root system) was measured with a ruler to the nearest mm, and the total fresh root weight was determined with a scale. Finally, each plant's root system was scanned using an EPSON scanner at 600 dpi equipped with the ImageScanner III LabScan 6.0 software; pictures were saved as tiff-formatted photos.

## Complementation of the Rice *crl1* Mutant

The two barley genes (Hv*CRL1* and Hv*CRL1L1*) were amplified from cDNA obtained from RNA extracted from stem bases of cv. GP seedlings, grown in the crown root inducible system for 6 h after induction by 1-NAA. The primers and amplification conditions are described in Tables S2 and S3, respectively. PCR amplified products were introduced into the Gateway™ binary vector pCAMBIA5300.OE (Khong et al. 2015) using BP clonase (Invitrogen). The resulting plasmids were validated by a commercial sequencing service (Seqme, Czechia) and introduced into the *Agrobacterium tumefaciens* AGL1 strain for the stable transformation of the rice *crl1* mutant (Coudert et al. 2015). To validate that the insertion of the T-DNA itself did not affect the overall fitness of the plants (negative control), an empty pCAMBIA5300.OE vector was prepared by the removal of the *ccdB* cassette. Three constructs were established for the complementation assay: (1) pC5300.OE::HvCRL1, (2) pC5300.OE::HvCRL1L1, and (3) pC5300.OE-empty as a negative control.

The A*grobacterium*-mediated transformation of the rice *crl1* mutant in the cv. TC65 background (Coudert et al. 2015) was performed as previously described (Toki et al. 2006; Sallaud et al. 2003). The presence of the T-DNA in obtained plants was confirmed by PCR amplification of either one of the Hv*LBD*s. All primers and PCR amplification conditions are provided in Tables S2 and S3, respectively. A line containing an empty vector was considered as a negative control. Homozygous lines for the T-DNA and single insertion were selected until the T2 generation.

To determine the effect of the overexpression of *HvCRL1* and *HvCRL1L1* on the root system of rice seedlings, seeds of the wild-type TC65, *crl1* mutant, *crl1* mutant that integrated the empty T-DNA and the T2 generation of *crl1* mutant complemented with either *HvCRL1* (OE-*HvCRL1*) or *HvCRL1L1* (OE-*HvCRL1L1)* were first disinfected for 30 s in 70% ethanol, fast rinsed in sterile deionized water, sterilized for 3 min in 3% hypochlorite with regular shaking, and finally extensively rinsed in sterile deionized water. The seeds were sown in the middle line of a square petri dish (24 × 24 cm) containing 250 ml of ½ Murashige and Skoog medium (MS; pH 5.8). Dishes were sealed with 2 layers of parafilm, and the lower half was covered by aluminum foil to limit the influence of light on the root growth. Plates were placed vertically in a phytotron with a photoperiod of 25 ± 2 °C/12 h light and 22 ± 2 °C/12 h darkness. Twelve days after germination, the plates were scanned with ImageScanner III LabScan 6.0 with a 600-dpi resolution and saved as a tiff-formatted photo. The total number of roots per plant was determined.


A
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Mb
HORVU.MOREX.r3.1HG0013280
HORVU.MOREX.r3.1HG0025890
HORVU.MOREX.r3.1HG0062250
HORVU.MOREX.r3.1HG0066110
HORVU.MOREX.r3.2HG0129400
HORVU.MOREX.r3.2HG0159010
HORVU.MOREX.r3.3HG0304610
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HORVU.MOREX.r3.4HG0342720
HORVU.MOREX.r3.4HG0391970
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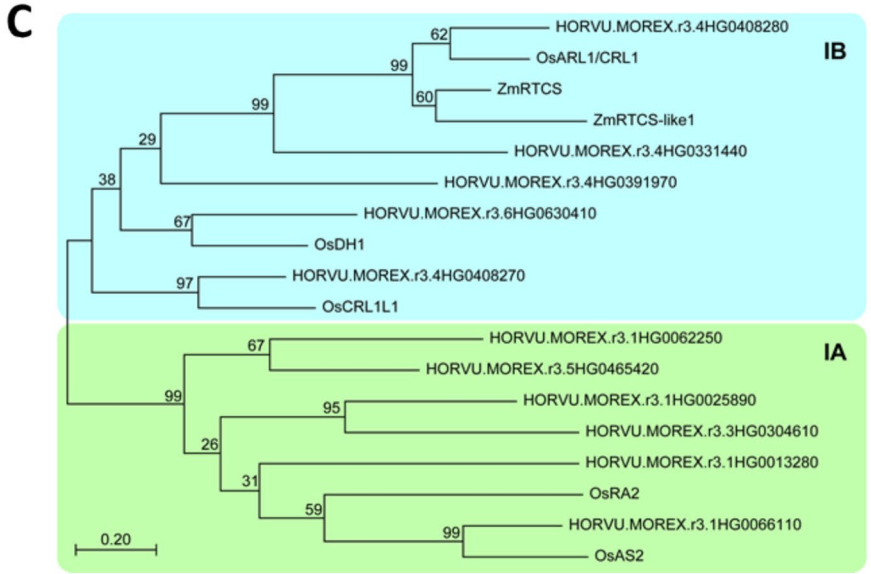
C
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IA
HORVU.MOREX.r3.4HG0408280
OsARL1/CRL1
ZmRTCS
ZmRTCS-like1
HORVU.MOREX.r3.4HG0331440
HORVU.MOREX.r3.4HG0391970
HORVU.MOREX.r3.6HG0630410
OsDH1
HORVU.MOREX.r3.4HG0408270
OsCRL1L1
HORVU.MOREX.r3.1HG0062250
HORVU.MOREX.r3.5HG0465420
HORVU.MOREX.r3.1HG0025890
HORVU.MOREX.r3.3HG0304610
HORVU.MOREX.r3.1HG0013280
OsRA2
HORVU.MOREX.r3.1HG0066110
OsAS2
62
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60
99
29
38
67
97
67
99
95
26
31
59
99
0.20

◂**Fig. 1** In silico characterization of barley genes encoding LBD proteins. **a** Localization of the putative *HvLBD* genes on the 7 chromosomes of barley. The chart has been built with MapChart. **b** Gene structure and conserved domains of the 15 barley *HvLBD* genes. The chart has been drawn with GSDS 2.0. **c** The phylogenetic tree of the LBD proteins including known rice (Os) and maize (Zm) sequences was built by MEGA11 (Tamura et al. 2021). Accession numbers: ZmRTCS/ZmLBD2-GRMZM2G092542; ZmRTCL/ZmLBD43: AC149818.2_FG009; OsARL1/CRL1: Os03g0149100; OsDH1/LBD16: Os02g0820500; OsCRL1L1: Os03g0149000; OsRA2: Os01g0169400; OsAS2: Os01g0889400

## Results and Discussion

### *LBD* Gene Family Analysis in Barley and Identification of the Putative Orthologues of Rice *CRL1* and Maize *RTCS* Genes

Initially, only fifteen sequences identified in the PlantTFDB as putative LBD proteins in barley were known and, therefore, considered in this study. For ease of comparison with the recent barley genome annotation (Mascher et al. 2021), the correspondence between former and recent annotations is provided in Table S1. Except for chromosome 7, barley *LBD* genes were localized on all chromosomes with an overrepresentation on chromosomes 4 and 1 (Fig. 1a). The analysis of the gene structure identified that genes comprised 1–3 exons (Fig. 1b). The barley *LBD* genes clustered into three distinct groups. Four sequences (2HG0129400, 2HG0159010, 3HG0322090, and 4HG0342720) missed two of the conserved domains (GAS block and leucine zipper-like coiled-coil motif) characteristic of the LBD proteins. They clustered in one group corresponding to the class II LBD proteins, lacking an intact leucine-zipper-like domain. Other barley LBD genes clustered into the described LBD groups IA and IB (Fig. 1b) based on their homology with known rice LBD proteins (Fig. 1c) (Coudert et al. 2013; Zhang et al. 2020). With the characterization of the barley LBD protein family (Guo et al. 2016) and the recent barley annotation (Mascher et al. 2021), the number of genes encoding LBD proteins in barley rose to 31. However, integrating these sequences in the phylogenetic analysis did not affect the overall structure of the original tree obtained with the 15 sequences (Fig. S1). While combining the function reports and evolutionary relationship between LBD proteins, molecular functions can be attributed to different groups. In this regard, the barley HvLBD proteins clustering in class II might regulate anthocyanin synthesis and nitrogen responses. HvLBD proteins belonging to class IA might play primary roles in the lateral root formation, whereas HvLBD of class IB may have main functions in leaf adaxial–abaxial polarity, plant reproduction, and adventitious rooting (Zhang et al. 2020). The barley class IB contains 5 HvLBD proteins, one of them (HORVU.Morex.r3.4HG0408280) clustered with the well-characterized rice OsARL1/CRL1and maize ZmRTCS proteins (Liu et al. 2005; Xu et al. 2015), suggesting that it could be their ortholog and play a role in the crown root initiation in barley. Consequently, we named it HvCRL1.

### Expression Profiles of the *HvLBD* Genes During Crown Root Initiation

The study of lateral- or crown root initiation is tedious because it occurs only in a few cells inside the root or the stem base, respectively. Moreover, it occurs unpredictably. To circumvent this problem, a Lateral Root Inducible System (LRIS) has been developed in Arabidopsis and maize to study lateral root initiation (Crombez et al. 2016). By treating seedlings consecutively with an auxin transport inhibitor and a synthetic auxin, one can control the synchronous initiation of lateral roots, consequently allowing abundant sampling of a desired developmental stage. Here, we used the same system to synchronously initiate crown roots (Fig. S2) and named it Crown Root Inducible System (CRIS). The expression of the five Hv*LBD* genes belonging to Class IB was analyzed by qRT-PCR during crown root initiation. Two genes (*HORVU.Morex.r3.4HG0331440* and *HORVU.Morex.v3.4HG0408270*) were not detected in our conditions, suggesting that they are not expressed in the stem base of barley. Whereas *HORVU.Morex.r3.4HG0391970* was detected in our conditions, its expression was very low and not affected by auxin treatment, indicating that it most probably has no function during crown root initiation. The transcripts of the *HvCRL1 (HORVU.Morex.r3.4HG0408280)* gene accumulated to reach a maximum within the first 3 h of auxin treatment in the stem base of young seedlings and stayed at high level even after 24 h. Finally, *HORVU.Morex.r3.6HG0630410* gene was found to accumulate significantly in the stem base of young seedlings 3 h after treatment, to reach a maximum after 12 h of auxin induction and to stay high even after 24 h (Fig. 2).

Using our transcriptomic study designed to identify genes differently regulated during the first 3 h of auxin-induced crown root initiation (GSE171320), we observed that *HvCRL1* transcripts are significantly accumulated during the first hour following auxin treatment (Fig. 3), indicating that *HvCRL1* is a prime target of the auxin signaling pathway. In contrast, *HORVU.Morex.r3.6HG0630410* was not detected earlier during the kinetic of induction, suggesting that despite a possible role in regulating crown root initiation in barley, it might not be a direct target of auxin. Moreover, *HORVU.Morex.r3.6HG0630410* is phylogenetically the closest ortholog of the rice *DEGENERATED HULL1/LBD16* (*DH1/LBD16/Os02g0820500)* gene (Fig. 1c; 73.50% of identity as determined by ClustalOmega), which, besides its function in glume formation (Li

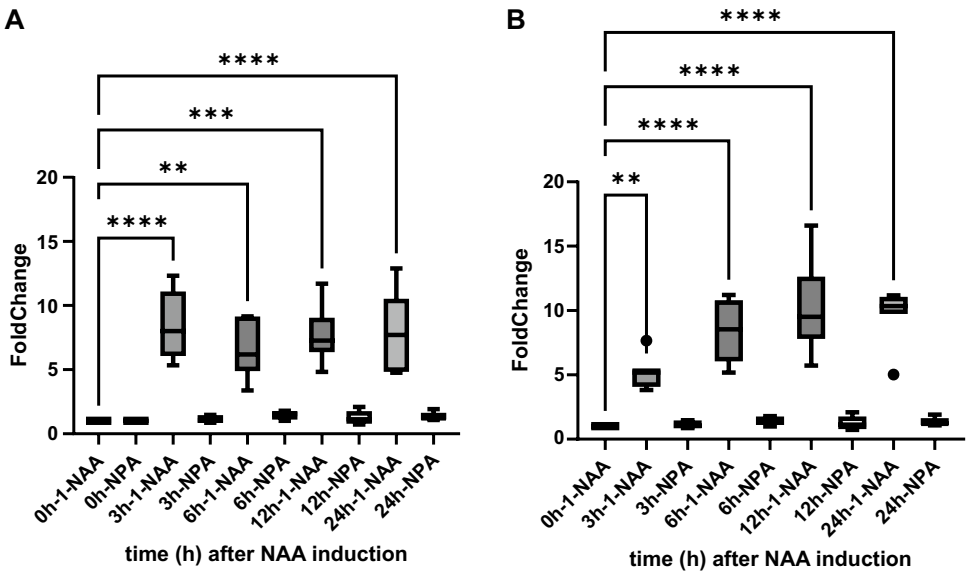


**Fig. 2** Gene expression analysis by qRT-PCR of two *LBD* genes of class IB expressed in the stem base of young barley seedlings after auxin induction at 3, 6, 12, and 24 h. **a** *HvCRL1* (HORVU.MOREX.r3.4HG0408280). **b** *HvCRL1L1* (HORVU.MOREX.r3.6HG0630410). Normalization was done using three reference genes: *Actin*, *Hv5439* and *EIF152*. The graphs show the Tukey box plot representation of the data ($n=6$). The statistical significance was assessed by a non-parametric Kruskal–Wallis followed by a multiple comparisons test (GraphPad Prism 10). **Statistically significantly different from the control "0 h-NPA" ($p$ value $<0.01$)

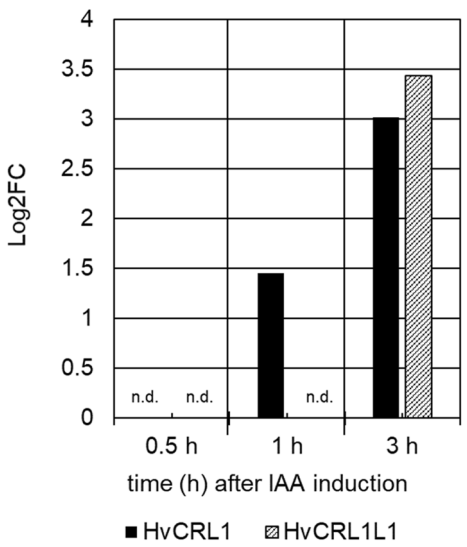


**Fig. 3** Changes in expression of *HvCRL1* (HORVU.MOREX.r3.4HG0408280) and *HvCRL1L1* (HORVU.MOREX.r3.6HG0630410) genes as determined by transcriptomic data (GEO accession: GSE171320). Barley seedlings were grown using the crown root inducible system (CRIS) and samples were collected at 0.5, 1, and 3 h after induction

et al. 2008), has been demonstrated to promote emergence and growth of crown roots by affecting cell division (Geng et al. 2024). Therefore, *HORVU.Morex.r3.6HG0630410* is referred to as *HvCRL1-Like1* (*HvCRL1L1*).

For both genes, a very low basal expression was observed and did not change when the seedlings were kept in the presence of the inhibitor of the polar auxin transport (NPA; Fig. 2), confirming that changes in their respective expression were related to auxin treatment only.

We analyzed the 5000 bp promoter region of both *HvCRL1* and *HvCRL1L1* genes (Fig. S3). Using the consensus Auxin Response Factor (ARF) binding sequence, Auxin Response Element (*AuxRE:TGTCNN*), we highlighted only *AuxRE* that form tandems with a maximum of 24 bp spacing (Cance et al. 2022). In the promoter of *HvCRL1*, we found 10 *AuxRE* tandems. Except for two tandems that form everted repeats (ER: two *AuxREs* orientated back-to-back in different strands of DNA), all others form direct repeats (DR: two *AuxREs* following each other in the same DNA strand) (Freire-Rios et al. 2020). In the promoter of *HvCRL1L1*, out of the five identified *AuxRE* tandems, four presented a DR conformation, whereas the fifth one was made of inverted repeats (IRs; double sites in which two *AuxREs* are oriented toward each other in different strands of DNA). Further analysis is required to determine which *AuxRE cis*-regulating elements are responsible for the auxin-mediated regulation of both genes.

With the assumption that *HvCRL1L1* might be a downstream target of HvCRL1, we also analyzed the promoter sequence of *HvCRL1L1* for the presence of (1) the palindromic *LBD-box* (GCGGCG) that is the consensus sequence recognized by LBD transcription factors (Husbands et al. 2007), and (2) the *CRL1-box* (CAC[A/C]C) that

is recognized with high affinity by the rice CRL1 (Gonin et al. 2022). Interestingly, we observed that the promoter of *HvCRL1L1* contains both *LBD*- and *CRL1-boxes* (Fig. S3), forming notably a cluster of 6 boxes in the region – 1953 to – 1695 bp. The DNA binding activity of LBD transcription factors requires homo- or heterodimerization of the transcription factors (Lee et al. 2017). The recent crystallographic structure of the wheat LBD Ramosa2 (TtRa2LD) indicated that the dimerization of the LOB domain determines the exact spatial arrangement of the conserved $CX_2CX_6CX_3C$ motifs (zinc fingers), defining the distance between the DNA binding sites (Chen et al. 2019). This mechanism is identical to the molecular caliper described for ARF transcription factors (Korasick et al. 2015).

In the promoter of *HvCRL1*, we did not identify *LBD-boxes* but several *CRL1-boxes*. However, the regulation of *HvCRL1* by LBD proteins is most probably unlikely because the *CRL1-boxes* are too far apart from each other to allow binding of the LBD dimer at the DNA binding sites.

## HvCRL1, but not HvCRL1L1, Binds the LBD-Box In Vivo

To validate that HvCRL1 and HvCRL1L1 are transcription factors that recognize and bind to the *LBD-box*, initially identified as the consensual DNA binding site of the ASYMMETRIC LEAVES2 (AS2) (Husbands et al. 2007) LBD protein, we performed a *trans*-activation assay in rice protoplasts. For this purpose, rice protoplasts were co-transformed with a tripartite vector system that comprises (1) a reporter plasmid triggering the expression of the β-D-glucuronidase (GUS)-encoding *uidA* gene under the control of a minimal promoter and a tetramer of either the *LBD-box* or its mutated version, (2) an effector plasmid allowing the expression of either *HvLBD* genes under the control a minimal *CaMV35S* promoter, and (3) a reference plasmid carrying the *Renilla luciferase* gene. While the empty vector had minimal or no effect on GUS activity, HvCRL1 significantly *trans*-activated the *LBD-box* promoter in rice protoplasts (Fig. 4). This activation was strongly reduced when the *LBD-box* was mutated.

In contrast, the increase in the GUS reporter activity of the *LBD-box* by HvCRL1L1 was not found to be significant.

It has been demonstrated that the *LBD-box* can be bound by different LBD transcription factors such as RTCS in maize, HvRAMOSA2 in barley or AS2, and AtLBD18 in Arabidopsis. LBD transcription factors form dimers to bind to the DNA, recognizing pairs of *LBD-boxes* with different affinities depending on the number of bases between two consecutive LBD-boxes (Chen et al. 2019). Recent studies have highlighted that LBD transcription factors have a relaxed DNA binding specificity, explaining how they could regulate a plethora of developmental programs (Gonin et al.

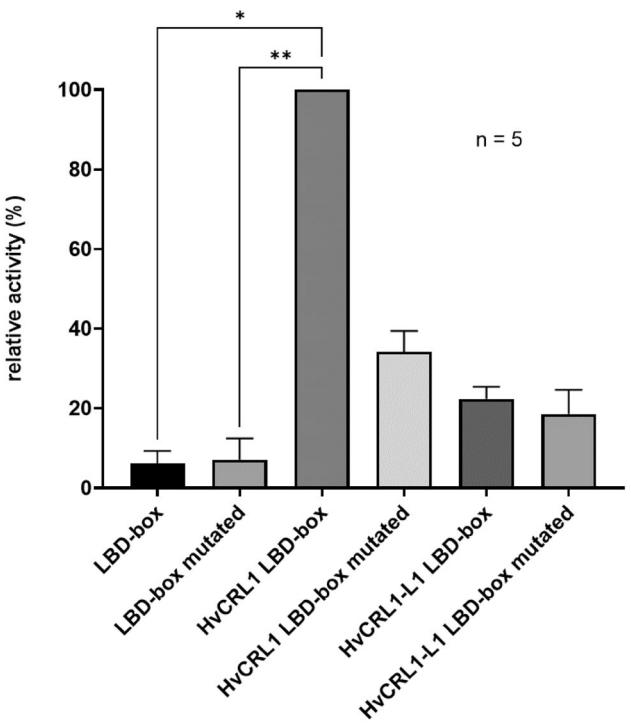


**Fig. 4** Transactivation assay in rice protoplast with *HvCRL1* and *HvCRL1L1*. Rice protoplasts were co-transformed with an effector plasmid carrying either *HvCRL1* or *HvCRL1L1* gene under the control of the *35S* promoter and reporter plasmids carrying LBD-box motif (*LBD-box*) or its mutated version (*LBD-box* mutated) fused to GUS. A reference plasmid carrying the *Renilla luciferase* gene under the control of the *35S* promoter was co-transformed to correct for transformation and protein extraction efficiencies. The control represents protoplasts that were transfected with an empty effector plasmid. The data were expressed as a percentage of the highest activity observed for *HvCRL1*. The graph represents the average ± SEM of 5 independent experiments. The statistics were assessed with GraphPad Prism 10 (One-way ANOVA non-parametric Kruskal–Wallis followed by Dunn's multiple comparisons test)

2022). Thus, the fact that HvCRL1L1 did not significantly induce changes in GUS reporter activity might reflect that this specific LBD transcription factor might recognize a different DNA binding site. In addition, LBD transcription factors can form heterodimers or interact with other regulatory proteins. For example, in maize, RTCS and RTCL can form heterodimers (Majer et al. 2012). AS2 LOB can interact with bHLH048, decreasing the affinity of AS2 for the *LBD-box*, and consequently suggesting that such a heterodimerization process could be a post-translational mechanism that regulates LBD transcription factor activity (Husbands et al. 2007). More recently, the rice CRL1 was shown to interact with *WUSCHEL*-related Homeobox11 (WOX11), a homeodomain transcription factor also involved in crown root differentiation in rice (Geng et al. 2023). The interaction of WOX11 with CRL1 promotes the binding of WOX11 on the promoter of *CYTOKININ OXYDASE/DEHYDROGENASE4*

(Os*CKX4*), and the expression of this latter, which encodes a cytokinin oxidase that favors crown root initiation by reducing locally cytokinin levels. In addition, WOX11 can interact with another rice LBD transcription factor, LBD16, which facilitates the initiation and elongation of rice crown roots by modulating cell division (Geng et al. 2024). WOX11 binds the *LBD16* promoter, interacting with a demethylase JMJ706 that epigenetically unlocks *LBD16* expression. In turn, when LBD16 is expressed enough, it can compete with JMJ706 to bind with WOX11 on the *LBD16* promoter, which results in re-methylating the locus, leading to the repression of LBD16 expression. This stresses that LBD transcription factors can interact with a wide range of proteins and are also involved in post-translational regulatory mechanisms, particularly for regulating crown root formation.

## Knock-Out *hvcrl1* and *hvcrl1l1* Barley Plants are Affected in Crown Root Formation

To assess the role of the *HvCRL1* and *HvCRL1L1* genes in the initiation of the crown root, we generated different independent lines of barley knocked-out either in *HvCRL1* or *HvCRL1L1* genes using the CRISPR-Cas9 methodology and *Agrobacterium*-mediated transformation of the immature embryo of barley. For the *HvCRL1* gene, all independent mutants contained a 1-base deletion. For the *HvCRL1L1* gene, different types of deletion-based (− 1, − 10, − 13, and − 19 bp) and insertion-based mutation (+ 1 bp) were obtained (Fig. S4). In all cases, the mutation resulted in a frameshift and, consequently, a predicted non-functional LBD protein. Except of an apparent reduction in the number of tillers in the knockout lines, no significant changes in the vegetative part of the plants could be observed in comparison with WT plants or plants transformed with an empty vector (Fig. S5). For both genes and all lines analyzed, a reduction in the number of crown roots was observed (Fig. 5a), correlated with a reduction in the fresh weight of the total root system (Fig. 5b).

## *HvCRL1* and *HvCRL1L1* Overexpression in the Rice *crl1* Mutant Reverts Crown Rootless Phenotype

Concurrently, we overexpressed the two barley genes in the rice *crl1* mutant (Inukai et al. 2005), which exhibits a crown rootless phenotype due to a defect in crown root initiation. In 12-day-old seedlings, the rice *crl1* mutant is characterized by a unique root developing long lateral roots (Fig. 6a). The expression of both *HvCRL1* and *HvCRL1L1* genes resulted in a significantly higher number of roots in the rice seedlings, indicating that both genes can partially complement the *crl1* rice mutant by restoring crown root initiation (Fig. 6b). Therefore, it is hypothesized that there is some functional duplication between rice *CRL1* and barley *HvCRL1* and *HvCRL1L1* genes. However, the partial

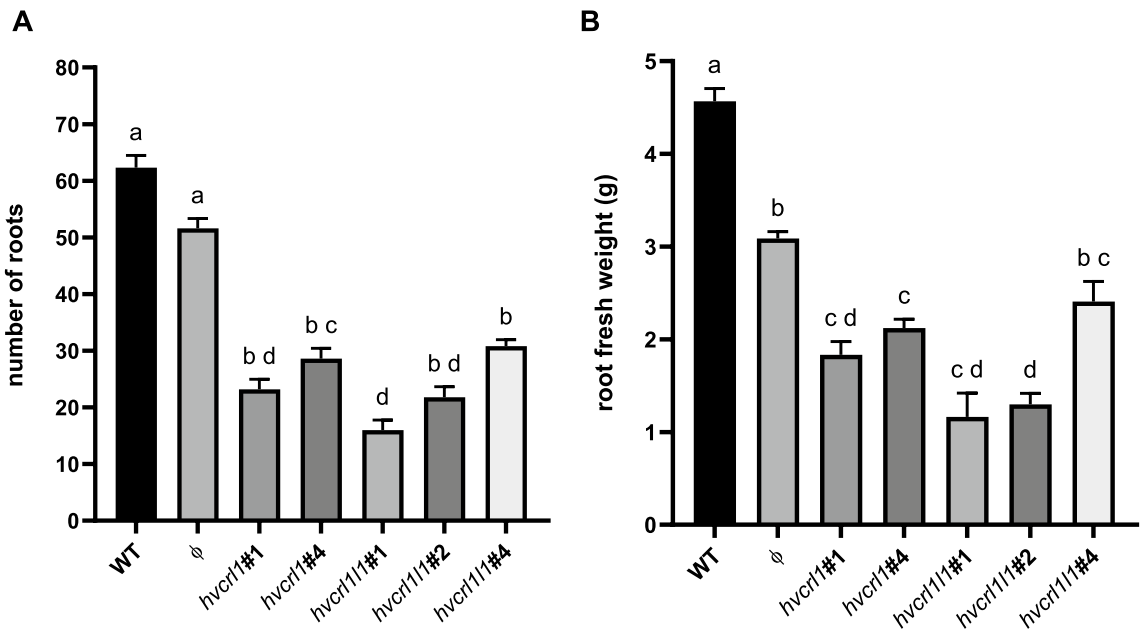


**Fig. 5** Effect of CRISPR-Cas$^9$-mediated knockout of *HvCRL1* and *HvCRL1L1* genes on the number of crown roots (**a**) and fresh weight of the total root system (**b**) of 8-week-old plants grown in sand. T2 generations, homozygous for the mutation without the T-DNA, were considered. The graphs represent average ± SEM ($n = 5$). Statistical significance was assessed by a Brown-Forsythe and Welch ANOVA test followed by Dunn's multiple comparison (GraphPad Prism 10.2.2). Bars with identical letter are not significantly different ($p < 0.05$)

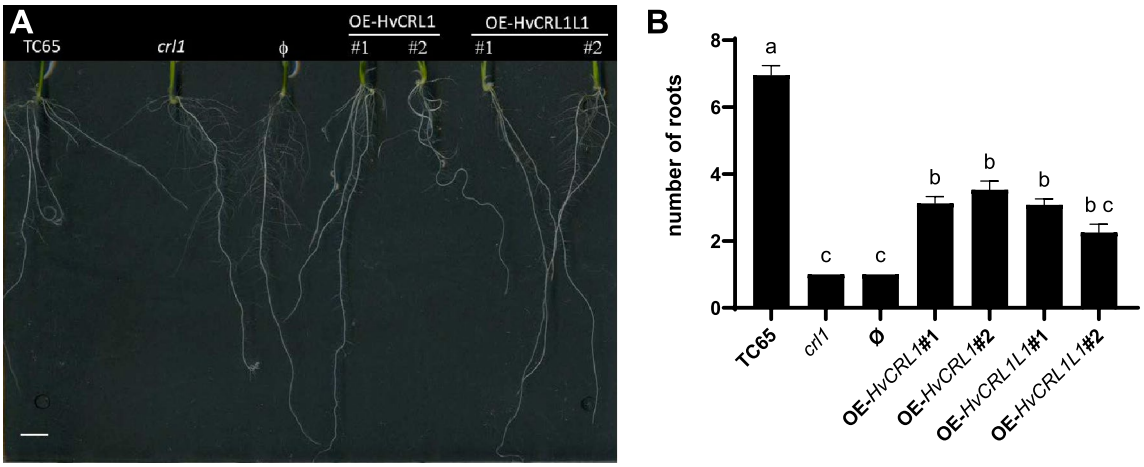


**Fig. 6** Complementation of the crown rootless phenotype of the rice *crl1* mutant by overexpression of *HvCRL1* or *HvCRL1L1* genes. **a** Representative photographs of the root system of 12-day-old seedlings. Bar represents 1 cm. **b** Total number of roots. *HvCRL1* or *HvCRL1L1* genes were overexpressed in the rice *crl1* mutant in the TC65 genetic background. An empty vector was used as a control ($\phi$). T2 generations, homozygous for the T-DNA, were considered for the analysis. The graphs represent average ± SEM; $n = 15$. Statistical significance was assessed by a Brown-Forsythe and Welch ANOVA test followed by Dunn's multiple comparison (GraphPad Prism 10.2.2). Bars with identical letter are not significantly different ($p < 0.05$)

reversion observed in the rice *crl1* mutant suggests the potential requirement of the two barley genes to fully reverse the crown rootless phenotype.

Altogether, our data confirmed that both *HvCRL1* and *HvCRL1L1* play a role in barley's crown root formation and development.

## Conclusion

The current study identified two barley LBD transcription factors, HvCRL1 and HvCRL1L1, that are closely phylogenetically related to the rice CRL1 transcription factor and are involved in the crown root formation in barley. Indeed, not only both genes partially complement the rootless phenotype of the rice *crl1* mutant, but the loss-of-function of *HvCRL1* and *HvCRL1L1* results in the reduction in the number of crown roots, showing that they are both involved in this developmental process. We showed that the expression profile of these two genes during crown root formation presents a slight time delay and that HvCRL1 can bind the LBD-box, whereas HvCRL1L1 cannot. This suggests that both proteins likely cooperate through different molecular pathways in regulating crown root formation in barley. This is reminiscent of what was described in maize for RTCS and RTCL (Xu et al. 2015) or, more recently, in rice for CRL1 and LBD16 (Geng et al. 2024, 2023). Further investigations are required to better understand the mechanisms of actions of these two LBD transcription factors during crown root initiation and development.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00344-025-11667-x.

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**Data Availability** The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Declarations

**Conflict of interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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