Running tittle: a novel sugar beet aquaporin

BvCOLD1 a novel aquaporin from sugar beet (*Beta vulgaris* L.) involved in boron homeostasis and abiotic stress

Rosa Porcel[*1,2], Antonio Bustamante[*1], Roc Ros[3], Ramón Serrano[1], José Miguel Mulet[**1]

[1]*Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Ingeniero Fausto Elio s/n, 46022 Valencia, Spain*

[2]*Present Address: COMAV - Universitat Politècnica de València, CPI, Edificio 8E, Camino de Vera, s/n, 46022 Valencia, Spain*

[3]*Departament de Biologia Vegetal, Facultat de Farmácia, and Estructura de Recerca Interdisciplinar en Biotecnologia i Biomedicina (ERI BIOTECMED). Universitat de València. Dr Moliner 50, 46100 Burjassot, Spain.*

[*]These authors contributed equally to this work

[**]*Corresponding author: Dr. José Miguel Mulet Salort*

E-mail address: jmmulet@ibmcp.upv.es

Tel: +34 963 877 775

Fax: +34 963 877 859

ABSTRACT

Beta vulgaris (sugar beet) is one of the most important industrial crops. Screening of a cDNA library for sugar beet genes able to confer cold tolerance upon overexpression in yeast identified a novel aquaporin, which we named *BvCOLD1*. The amino acid sequence of BvCOLD1 indicated that is an acidic protein (pI 5,18) similar to TIP aquaporins. RNA expression analysis indicated that *BvCOLD1* is expressed in all sugar beet organs. Confocal microscopy of a GFP tagged version localized BvCOLD1 in the endoplasmic reticulum in yeast and in plant cells. Experiments in yeast showed that BvCOLD1 has an important role in transporting several molecules, among them boron, one of the most limiting micronutrients for sugar beet cultivation. Transgenic *Arabidopsis thaliana* plants overexpressing *BvCOLD1* showed enhanced tolerance to cold, to different abiotic stresses and to boron deficiency at different developmental stages. Searches in data bases only retrieved *BvCOLD1* orthologues in genomes from the *Chenopodioideae*, a subfamily of the *Amaranthaceae* family which includes the closely related crop *Spinacea oleracea* and halotolerant plants such as *Salicornia herbacea* or *Suaeda glauca*. Orthologues share a conserved sequence in the carboxy terminal, not present in other aquaporins, which is required for the functionality of the protein.

Keywords: Beta vulgaris, sugar beet, aquaporin, abiotic stress, cold, boric acid, tonoplast intrinsic protein

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is one of the world's most important sources of sugar, one of the major nutrients. Production worldwide is stabilized about 172 million tons per year. Sugarcane, cultivated in tropical climates, produces 80% of the world's sugar supply, while the remaining 20% comes from sugar beet which is cultivated under temperate climates in Europe, North America and some Asian regions, being France, the Russian Federation, Germany and the United States the main producers in 2014 (Food and Agriculture Organization of the United Nations. Statistics Division). Sugar beet cultivation is not only focused in the sugar industry, as is a major crop for cattle feeding (Evans & Messerschmidt, 2017), for bioethanol production (Zabed et al. 2014) and can also be cultivated to use its leafs as vegetables, specifically the cicla variety.

Environmental stress is the principal responsible of loss of sugar beet productivity. Sugar beet is grown in temperate areas which are prone to frost or low temperatures. The optimal temperature for plant germination is 25 ºC, although it can germinate in temperatures ranging from 3 ºC to 25 ºC. The required thermal time is around 130 ºCd (Villarías Moradillo, 1999). The crop is sown in early spring to improve root production and escape summer drought at maturity. In some areas of Southern Europe, sugar beet is sown in autumn (autumn sowing) to anticipate root harvest and escape drought and pathogens like *Cercospora*. Low-temperature stress is an important cause of decrease in crop quality and yield, as decreases sugar accumulation and plant growth (Moliterni et al. 2015). This susceptibility to low temperatures is especially damaging for the plant if temperatures fall below 0 ºC at early developmental stages. A temperature around -2 ºC can kill the plant at the cotyledon stage, while a three to four true-leaf plant can stand -10 ºC (Biancardi, 2005).

For several crops it has been shown that cold or chilling can alter boron nutrition. This essential element is needed throughout the plant life since plays a key role in the plant

cell wall structure. Boron deficiency causes a wide range of symptoms including the cessation of root elongation, reduced leaf expansion and the loss of fertility, depending on the plant species and developmental stage (Dell & Huang, 1997, Goldbach et al. 2001). Sugar beet has one of the largest requirements of boron for an industrial crop. Its deficiency is the main cause of the “heart rot” and “dry rot” diseases (Brandenburg, 1931). A deficiency in this element not only decreases yield but damages tap roots, decreasing their value and quality, as root growth is severely inhibited due to a reduction in the available surface for water and nutrient uptake (Brown et al. 2002, Shorrocks, 1997). Boron limitation can also alter plant water relations (reviewed in Wimmer & Eichert, 2013). In soil boron exists mainly as boric acid, which is leached by excessive rainfall constituting a general problem of insufficient boron in humid areas soils, most of them suitable for sugar beet production (Camacho-Cristobal et al. 2008). Therefore sugar beet cultivation often requires the addition of boron in the form of borate or boric acid, as foliar spray or as a part of a soil fertilizer. Sugar beet is generally tolerant of high boron soils, which can be found in arid or semiarid regions (Tanaka & Fujiwara, 2008), because of its ability accumulate the element in cell walls (Rozema et al. 1992).

At the molecular level there are several genes identified which are able to confer cold tolerance upon overexpression in transgenic plants, such as the transcription factor *GhDOF1* in cotton (Su et al. 2017) or the Arabidopsis ribosomal P3 protein *P3B* in sweet potato (Ji et al. 2017). There are also descriptions of genes which can increase growth under boron deficient conditions such as *BnaC4.BOR1;1c* from *Brassica napus* in Arabidopsis (Zhang et al. 2017) and the Arabidopsis gene *AtBOR1* in tomato plants (Uraguchi et al. 2014). In most organisms early response to environmental stress depends on transcription factors. Sugar beet genome has less transcription factors that any other flowering plant known genome (Dohm et al. 2014). In the particular case of *Beta vulgaris* there is a complete description of the trascriptomic changes induced by

cold. For instance, there is a homologue of the Arabidopsis CBF3 transcription factor which has been identified as upregulated upon cold stress (Moliterni et al. 2015), but no gene of sugar beet has been proved to confer cold tolerance by overexpression in any organism. The only GMO sugar beet commercially available has been transformed with a bacterial gene to induce tolerance to the herbicide glyphosate (Dewar et al. 2003). The identification of sugar beet genes whose function becomes limiting under cold condition can improve our understanding of this basic mechanisms, and at the same time, provide useful genes to develop biotechnological strategies to generate new and more efficient and resistant sugar beet varieties.

With this aim, we have screened a cDNA library from stress induced sugar beet leaves to identify genes able to confer tolerance to cold stress upon overexpression in the bakers' yeast *Saccharomyces cerevisiae*. We have previously used a similar strategy to identify sugar beet genes able to confer tolerance to salt (Kanhonou et al. 2001) or drought (Mulet et al. 2004) stress. In the present work using the yeast overexpression approach (Montesinos et al. 2003) we have identified a gene from a novel aquaporin (*BvCOLD1*), similar to TIP1 aquaporins, but which is only present in the genomes of the sugar beet related species *Spinacea oleracea* (Spinach), some halotolerant members of the *Chenopodioideae* subfamily of the *Amaranthaceae* family and *Mesembryanthemum crystallinum*. Overexpression of *BvCOLD1* is able to confer cold and abiotic stress tolerance to transgenic Arabidopsis plants and enhance growth under limiting boron conditions, therefore constituting an interesting target gene for future biotechnological improvement of this major crop.

MATERIAL AND METHODS

cDNA library construction, yeast strains and yeast culture conditions

A detailed description of the construction of the cDNA library from sugar beet leaves can be found in (Kanhonou et al. 2001). This library was constructed in the pYPGE15 plasmid (2µ derived multicopy plasmid with the *URA* marker gene) and was used to transform the yeast W303 strain (Wallis et al. 1989) by the LiCl method (Gietz et al. 1992). Diploid cells were used in the screening in order to prevent the isolation of recessive chromosomal mutations. The screening was performed as described in (Vicent et al. 2015) but using the above mentioned cDNA library from *Beta vulgaris*. In brief, transformants selected for uracil prototrophy on SD plates were pooled and replated on fresh SD plates at a density of 2×10^5 cells/plate (14 mm diameter). Colonies able to grow after 8 days at 10 ºC were chosen for further studies. The putative positive clones were drop-test assayed to reconfirm the cold tolerance phenotype. The plasmidic DNA was retransformed to confirm that the phenotype was dependent on the isolated plasmid and not a chromosomal mutation. After this last confirmation the positives were directly sequenced by the dye–primer cycle sequencing method using a DNA sequencer (Model ABI377, PE Biosystems). The pYPGE-*BvCOLD1* plasmid was named JM188.

Yeast were grown in either minimal synthetic glucose medium (SD), or rich medium (YPD) (as indicated in the corresponding figure). SD medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco) and 50 mM succinic acid, adjusted to pH 5 with Tris (Tris-(hydroxymethyl)-aminomethane), plus the required amino acids (100 µg/ml leucine, 30 µg/ml adenine, 30 µg/ml histidine and 80 µg/ml tryptophan). YPD medium contained 1% yeast extract (Difco), 2% Bacto peptone (Difco) and 2% glucose.

Measurement of intracellular boron concentration

Cells were grown in SD to an absorbance at 660 nm of 0.6 to 0.7, centrifuged for 5 min at 1.900 g and resuspended at the same concentration of cells in SD containing 90 mM of boric acid. Aliquots were taken at the indicated times, centrifuged in plastic tubes for 5 min at 1.900 g at 4 ºC, resuspended in 1 ml of ice cold water and transferred to an 1,5 mL microtube and washed twice with 1 ml of ice cold water. The cell pellets were resuspended in 0.5 ml of water. Ions were extracted by heating the cells for 15 min at 95 ºC. After centrifugation, aliquots of the supernatant were analyzed in a plasma emission spectrophotometer (Shimadzu).

Plasmids for *BvCOLD1* overexpression and GFP fusion protein

We constructed plasmid JM735 by cloning the superfolder green fluorescent protein (sGFP) (Cava et al. 2008) and the NOS terminator in the *Bam*HI and *Eco*RI sites of pBS-SK$^-$ (Stratagene, USA). An *Eco*RI-*Pvu*II fragment of JM735 containing the sGFP gene, the NOS terminator sequence and the polilinker of pBS-SK$^-$ was cloned in the *Sma*I site of plasmid pXCS-HA-STREPII (Witte et al. 2004) to create the plasmid JM743 which contains the 35S promoter, a 10 restriction site poli-linker and the sGFP sequence for constructing GFP fusion proteins. The *BvCOLD1* sequence was amplified from the JM188 plasmid using as a forward primer 5'-GTACTCGAGATGCCGATCAGCAGAATT-3' and as a reverse primer 5'-TATGGATTCAGCAGAAAGTCTTTGGTA-3'. The PCR product was cloned into the *EcoRV* site of pBS-SK$^-$ (Stratagene, USA), to create the plasmid JM866. The sequence of *BvCOLD1* was confirmed by sequenciation to discard the presence of mutations. A *Xho*I-*Eco*RI fragment of JM866 including the cDNA from *BvCOLD1* was subcloned into the JM743 plasmid by replacing the sGFP coding region between the XhoI and EcoRI

sites. This results in plasmid JM869 a construct for overexpression of *BvCOLD1* under the control of the CaMV 35S promoter.

For the BvCOLD1-GFP fusion we used as reverse primer 5'-CCCGGATTCAGCAGAAAGTCTTTGGTA to amplify the *BvCOLD1* sequence without the stop codon and cloned into the *EcoRV* site of pBS-SK$^-$ to create the plasmid JM865. A *XhoI*-*Bam*HI fragment of this plasmid was cloned in JM743 to create a BvCOLD1-GFP fusion protein (plasmid JM868).

For constructing the plasmid containing the BvCOLD1-GFP fusion for localization in yeast we used the yeast homologous recombination strategy. We amplified the sGFP gene from the JM735 plasmid using as forward primer 5'-ACCAAGAGCCTGCATCAGACTACCAAAGACTTTCTGCTATGGTGAGCAAGGGCGAG and as reverse primer 5'-CCACCAAAGGCCATCTTGGAACCGGGCCCCCCCTCGAGCTTGTACAGCTCGTCCAT this amplifies the sGFP sequence and includes in both borders a sequence homologous to the *XhoI* site of the pYPGE-*BvCOLD1* plasmid (JM188). We transformed yeast with the described PCR product and the plasmid JM188 digested with *Xho*I. Colonies containing a reconstructed plasmid where able to grow in SD medium without uracil. Plasmid was recovered from the obtained colonies and sequenced to confirm the integrity of the sequence and the absence of mutations. Plasmid was named JM883.

Constructions of *Arabidopsis thaliana* transgenic plants and growth conditions

Arabidopsis thaliana plants (ecotype Columbia 0) were grown under greenhouse conditions (16 h light/8 h dark, at 23±2ºC and 70±5% relative humidity) in pots containing a 1:2 vermiculite:soil mixture. Plants were irrigated twice a week with nutrient solution during 3 weeks as described in (Bissoli et al. 2012). The plasmid

JM869 and the empty control plasmid pXCS-HA.STREPII was introduced into *Agrobacterium tumefaciens* strain C58C1 by electroporation. Plants were transformed by flower infiltration (Bechtold et al. 1993). Transgenic plants transformed with the 35S:*BvCOLD1* containing plasmid (JM869) or with the empty control plasmid were screened on pots by adding BASTA 0.76 mM and the expression of the transgene was further confirmed in the plants transformed with the *BvCOLD1* gene by (qRT)-PCR, as described below. We also confirmed that none of the lines transformed with the control plasmid was presenting a distinctive phenotype (except the BASTA tolerance) when compared to the non-transgenic parental line (Col. 0).

For biomass determination under stress conditions *Arabidopsis thaliana* selected transgenic lines and control lines were germinated and growth in solid MS media until the development of real leaves (about two weeks) and then transplanted to 5x5 cm (height X diameter) individual plant pots. Plants were grown in optimal conditions until development of rosette leaves, but prior to the elongation of the hypocotyl. At this point some plants continued with the optimal conditions (control), other group continued with standard irrigation but was transferred to a growth chamber at 10 ºC (cold), another group was grown at 24 ºC but irrigation was stopped (water stress) or grown at 24 ºC irrigated twice per week with a 0,15 M NaCl solution (salt stress). After 30 days (50 days in the case of cold stress) aerial part of the plants was collected and fresh weight was determined. Then plants were dried and dry weight was determined.

In vitro growth conditions of _Arabidopsis thaliana_

A detailed description can be found in (Bissoli et al. 2012). For germination and early development assays in vitro, seeds were surface-sterilized with commercial bleach and rinsed with sterile water. The MS solid medium contained 0.8% phytoagar, Murashige and Skoog (MS) basal salt mixture (0.4%; Sigma), sucrose (1%) and 10 mM MES (2-

(N-morpholino) ethanesulfonic acid) buffer taken to pH 5.5 using Tris base (tris(hydroxymethyl)aminomethane). Stratification was performed over 3 days at 4 °C, and then plates were grown under long-day chamber conditions (16 h light / 8 h dark) (23 °C, 130 µE m−2 sec−1, 70% relative humidity). When indicated, the medium was supplemented as indicated in each case. Germination and early development was scored after 4 or 5 days (in control conditions), 6-7 days in stress conditions or 12-15 days in cold stress conditions.

Plant material and growth condition for sugar beet

Seeds of *Beta vulgaris* L. cv *Vulgaris* were sterilized for 3 min in pure ethanol and washed three times with sterile water to remove any trace of chemicals that could interfere in seed germination. Then seeds were placed on sterile vermiculite at 25 ºC to germinate. Ten-day-old seedlings were transferred to plastic pots containing 1 L of sterilized peat moss/vermiculite (1:1, v/v). Plants were grown under glasshouse conditions with 70–80% RH, day/night temperatures of 25/15 °C, and a photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 800 µmol m-2 s-1 (Li-Cor, Lincoln, NE, USA; model LI-188B). Water was supplied daily to maintain soil at field capacity during the entire period of plant growth. Plants were maintained under such conditions for five weeks until harvest, except for cotyledons (one week).

RNA isolation and synthesis of first strand cDNA

Total RNA was isolated from the indicated parts of sugar beet or Arabidopsis by phenol/chloroform extraction followed by precipitation with LiCl (Kay et al. 1987) and stored at -80 ºC.

For *A. thaliana*, the RNA was subjected to DNase treatment and reverse-transcription using the QuantiTect Reverse Transcription Kit (Qiagen), following the instructions provided by manufacturer. To rule out the possibility of a genomic DNA contamination, all the cDNA sets were checked by running control PCR reactions with aliquots of the same RNA that have been subjected to the DNase treatment but not to the reverse transcription step.

Northern blot analysis

Northern blot with *BvCOLD1* probe was carried out as described in (Porcel et al. 2006). Total RNA (15 μg) from different organs from sugar beet was separated by electrophoresis on 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto Hybond-N+ nylon membranes (Amersham, Little Chalfont, UK) by capillarity (Sambrook & Russell, 2001). Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hydridization (Herrin & Schmidt, 1988). Blots were prehybridized for 2–3 h at 42 ºC in 5X Denhardt's solution, 5X SSC and 0.5% SDS, and hybridized with *BvCOLD1* specific probe obtained by radioactive PCR labelling of plasmid inserts. Unincorporated ^{32}P was removed using Mini Quick Spin columns (Boehringer Manheim, Indianapolis, IN). A total of 10^7 cpm probe was heat-denatured and used to hybridize the blots overnight at 65 ºC under standard conditions (Sambrook & Russell, 2001). After washing twice for 5 min at room temperature in 2X SSC and 0.1% SDS, and twice for 15 min at 65 ºC with 0.53 SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at -70 ºC. Signals on autoradiograms were analysed and quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK). Transcript accumulation levels for each gene probe were normalized according to the amount of rRNA in the corresponding membrane, which had been also quantified with Quantity One software. Each quantification of signals on

autoradiograms and of rRNA was repeated three times and the average value was used for normalization. Northern blot analyses were repeated twice with different set of plants.

Quantitative real-time RT-PCR

Gene expression analyses in *Arabidopsis thaliana* transgenic plants were carried out by quantitative reverse transcription (qRT)-PCR using an iCycler iQ apparatus (BioRad, Hercules, CA, U.S.A.). The sequence of the primers used for PCR amplifications were as follows: for *BvCOLD1* forward primer 5'-GCCACCGCCATCGACCCAAA-3' and reverse primer 5'-GTCGAAGGCACCACCGGCAA-3' and for actin 8 (At1g49240) 5'-AGTGGTCGTACAACCGGTATTGT-3' and reverse primer 5'-GAGGATAGCATGTGGAAGTGAGAA-3' Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 µM each), 10.5 µl of 2x iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM dNTPs, 50 U/µl iTaq DNA polymerase, 6 mM MgCl2, 20 nM SYBR Green I, 20 nM fluorescein) plus 1 µl of a 1:10 dilution of each corresponding cDNA in a final volume of 21 µl.

The PCR program consisted in a 3 min incubation at 95 ºC to activate the hot-start recombinant Taq DNA polymerase, followed by 32 cycles of 30 s at 95º C and 30 s at 56 ºC, and 30 s at 72°C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 60 ºC to 100 ºC) after the final cycle of the PCR. Standardization was carried out based on the expression of the *A. thaliana* actin 8 gene in each sample. The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Experiments were repeated three times, with the threshold cycle (Ct) determined in triplicate, using cDNAs that originated from three RNAs extracted

from three different biological samples. Negative controls without cDNA were used in all PCR reactions.

Agrobacterium-mediated transient gene expression in *N. benthamiana*

Agrobacterium-based transient transformation was made as described in (Wieland et al. 2006) with some modifications. Briefly, *A. tumefaciens* culture transformed with plasmid JM868 was grown until saturation in LB medium (10 ml) at 28 ºC for 16 h. The culture was centrifuged and resuspended in the same volume of 10mM MgCl2, 10mM MES pH 5.6 and 200 mM acetosyringone. The culture was kept at room temperature for 3 h, without shaking. A syringe (without needle) was used to inject the *Agrobacterium* culture into the leaf. Plants were grown for 3–4 days in greenhouse before GFP localization was performed.

Confocal microscopy

Fluorescence of GFP was observed by a Leica TCS-SL confocal microscope and laser scanning confocal imaging system. A 488 nm excitation wavelength and a 510 nm emission wavelength were used, as described in (Peiro et al. 2014).

Statistical analysis

Statistical analysis was performed using the SPSS 16.0 statistical software package, (SPSS Inc., Chicago, IL, USA). The means were considered to be significantly different at $P < 0.05$ after the Duncan's multiple range test (Duncan, 1955).

RESULTS

Screening of a cDNA library from sugar beet leaves and isolation of *BvCOLD1.*

Heterologous gene expression of plant genes in yeast is a useful tool to identify rate limiting steps in stress response (Serrano et al. 2003, Mulet et al. 2004; Yu et al. 2017; Patankat et al. 2018). We used a cDNA library from sugar beet leaves (Kanhonou et al. 2001) to screen for plant cDNAs able to improve yeast growth at 10 ºC. After transforming about 254,000 cells with plasmid pYPGE15 containing the sugar beet cDNA inserts, colonies were pooled and selected for their ability to grow at 10 ºC as described in materials and methods. One of the isolated plasmids contained an 889 nucleotide cDNA that we named *BvCOLD1*. Overexpression of *BvCOLD1* in yeast conferred improved growth at 10 ºC (Fig. 1A). We confirmed that the *BvCOLD1* gene was being efficiently translated by yeast by fusing the Green Fluorescent Protein (GFP) to the carboxyl terminus of BvCOLD1. The localization was irregular, with a distinctive circle pattern. This could indicate localization in the endoplasmic reticulum (ER). To confirm that the signal that we were observing was the ER we stained the nuclei with 4',6-diamidino-2-phenylindole (DAPI) and confirmed that the structure we were observing was surrounding the nuclei so it can be attributed to the ER and the nuclear envelope (Fig. 1A).

Sequence analysis of BvCOLD1

The isolated cDNA contained an open reading frame of 762 bp. A BLAST analysis against the sugar beet genome database (Dohm et al. 2014) confirmed that *BvCOLD1* was indeed a sugar beet gene. Specifically *BvCOLD1* was 100% identical to the sequence annotated as Bv7_179210_kzkq.t1, which is located in the chromosome 7, between positions 532266 and 534970, in the minus strand. The genome structure showed that the *BvCOLD1* gene has two exons and a single 1.6kb intron that goes

from position 532850 to position 534517 (Fig. 1B). Additional analysis indicated that *BvCOLD1* has no paralogous genes in the sugar beet genome. A BLASTp analysis against the spinach (*Spinacea oleracea*) database identified a *BvCOLD1* orthologue annotated as Sp_212060_wajn.t1 which shares 83% of identity and 91% of positive homologies and an e-value of $1e^{-142}$. Comparison with a plant database indicated that the highest homology was obtained with tonoplast intrinsic proteins (TIP), although in yeast we detected the localization in the ER, but not in the tonoplast.

The calculated molecular weight of BvCOLD1 is 260,61 kD and is an acidic protein (pI 5,18) which contains 6 transmembrane domains. We identified the two highly conserved NPA pore forming motifs in position 85 and 198. The aromatic/arginine selectivity filter described for TIP proteins in BvCOLD1 is formed by histidine (position 65) in the helix2, Isoleucine (position 154) in helix 5, alanine (position 207) in loop E1 and valine (position 208) in loop E2 (Fig 1C), similar to what is described for TIP1 proteins (Soto et al. 2008). The WOLF pSORT localization algorithm predicted 14 nearest neighbours: 9 for plasma membrane, 4 for vacuolar membrane and 1 for endoplasmic reticulum. We further investigated the sequence of BvCOLD1 by modelling the sequence with the RAS MOL platform (Sayle & Milner-White, 1995) using the published structure of SoPIP2 in the open form (Törnroth-Horsefield et al. 2006) as a template. The software depicted a model in which we could determine a maximum pore size of 9,31 Angstroms. We could also observe the gating structure (Maurel et al. 2008) (Fig. 1D).

Phylogenetic analysis of *BvCOLD1*

BvCOLD1 has no orthologues in Arabidopsis. A BLASTp analysis against the TAIR database for expressed proteins in *Arabidopsis thaliana* showed that BvCOLD1 protein aligns with aquaporins, being the highest score for class 1 tonoplast intrinsic proteins

(TIPs), specifically BvCOLD1 shared 74% identity and an e-value $6e^{-129}$ with aquaporin TIP1-3 (gamma-TIP3; At4g01470) and 74% identity and an e-value of $3e^{-127}$ with aquaporin TIP1-1 (gamma-TIP; At2g36830), both from *Arabidopsis thaliana*. The intron structure, the NPA pore gating forming and the Aromatic/Arginine selectivity filter are conserved to TIP1 proteins, but a BLASTp analysis of TIP1-3 against the sugar beet genome data base indicated that the closest sugar beet gene is not *BvCOLD1*, but the gene annotated as Bv7_174560_ynzf.t1.

A phylogenetic analysis with the predicted amino acid sequence of *BvCOLD1* revealed that the closest sequences to BvCOLD1 are found in the halotolerant plants *Suaeda glauca*, *Salicornia herbacea*, *Mesembryanthemum crystallinum* and in the crop *Spinacea olereacea* (Fig. 2A). We confirmed this similarity by several phylogenetic trees by using either maximum parsimony or maximum similarity (data not shown). A detailed analysis confirmed that the three closest relatives to BvCOLD1 share an eleven amino acids motif in the carboxy terminal, with the sequence PASADYQRLSA. This motif is conserved in *Mesembryanthemum crystallinum* and with a single change in *Suaeda glauca*, and two changes in *Spinacea oleracea* (Fig. 2B). We repeated the phylogenetic tree without this motif and the aquaporin from *Mesembryanthemum crystallinum* was not located close to BvCOLD1, indicating that the presence of this conserved motif was increasing the score. The phylogeny of BvCOLD1 showed that this protein is only present in *Chenopodioideae*, a subfamily of the family of *Amaranthaceae*, which includes genus such as *Beta*, *Salicornia*, and *Suaeda* (Kadereit et al. 2010).

***BvCOLD1* expression and localization in sugar beet**

We investigated the expression pattern of *BvCOLD1*. Northern analysis from RNA extracted from sugar beet samples under normal growth conditions indicated that *BvCOLD1* is expressed in all investigated organs at a similar level (Fig. 3A). We

investigated whether *BvCOLD1* expression was induced by cold stress, but we did not find any significant change (data not shown). This is in agreement with the report of (Moliterni et al., 2015) in which the sequence of BvCOLD1 was not identified as up-regulated or down-regulated by cold stress. This result suggests that *BvCOLD1* is not regulated at the level of gene expression.

We further investigated the subcellular localization of BvCOLD1. For this purpose we constructed a version of *BvCOLD1* under the expression of the 35S-promoter and the GFP protein fused to the carboxy terminal. *Agrobacterium* cells transformed with this plasmid were used to infect *Nicotiana benthamiana* leaves. Four days after infection we could observe a GFP signal compatible with a reticular structure and an increase in the signal surrounding the nucleus, which could be identified as ER, thus confirming the localization observed in yeast (Fig. 3B). We further confirmed this pattern in protoplasts derived from *Nicotiana benthamiana* leaves (Fig. 3C).

Functional characterization of BVCOLD1 in yeast

We had shown that overexpression of *BvCOLD1* in yeast confers cold tolerance and that a GFP-tagged version was efficiently translated as could be observed by confocal microscopy (Fig. 1A). We then investigated whether we could identify the solutes transported by BvCOLD1 by functional expression in yeast. First we investigated the transport of glycerol by expressing it in a yeast *gpd1* mutant (Larsson et al. 1993). Glycerol is used by yeast cells as an osmoprotectant against osmotic stress. The *gpd1* mutant produces less glycerol than a wild type yeast. If the expressed aquaporin transports glycerol concentration diminishes in the cytoplasm and the cell is more sensitive to osmotic or oxidative stress. We compared the sensitivity against this stress in cells transformed with the empty plasmid (used as a control) and cells transformed with *BvCOLD1*, and confirmed that yeast were more sensitive in these conditions, thus

suggesting that BvCOLD1 was transporting glycerol (Fig. 4A). We also investigated if BvCOLD1 was able to transport other molecules such as ammonium (Jahn et al. 2004) but results were negative (data not shown).

Having confirmed that yeast is a good model system to characterize the ability of our aquaporin to transport diverse solutes we tried to determine whether BvCOLD1 is able to transport boron. Boron has no role in animal biology, but is an essential oligoelement in plant biology. Boron is required for the pollen tube growth and also for the formation of the cell wall due to the condensation of boric acid with pectic polisaccharides in the plant cell wall (Silva & Williams, 2001). Boron is a major limiting factor for sugar beet cultivation and its deficiency leads to the heart rot disease. It has been described that boron is transported mainly by aquaporins, so we wanted to confirm whether BvCOLD1 could be part of boron homeostasis. Boron extrusion in yeast is driven mainly by boron efflux transporter BOR1 (Nozawa et al. 2006). This transporter is conserved in plants as *AtBOR1* (Noguchi et al. 1997). The fact that BOR1 is extruding boron from the cytoplasm in a wild type yeast cell could be masking an hypothetical effect of BvCOLD1 in boron transport. We overexpressed *BvCOLD1* in a *bor1* yeast mutant which is defective in boron extrusion, and found that the overexpression of *BvCOLD1* induces yeast sensitivity to boron (Fig. 4B).

To further confirm that the expression of *BvCOLD1* in yeast alters boron homeostasis we compared the uptake kinetics of boron in a yeast strain transformed with an empty plasmid and with a yeast strain transformed with *BvCOLD1*. The strains expressing the plant aquaporin uptake boron faster that strains with the control plasmid, although at longer times both strains accumulate the same amount of boron (Fig. 4C). This is in agreement with the fact that BvCOLD1 is localized in the ER, so the heterologous expression of the plant protein could be affecting the internal distribution of boron.

BvCOLD1* overexpression improves cold and abiotic stress tolerance in *Arabidopsis thaliana

We have previously shown that BvCOLD1 protein is stable in yeast and in plants such as *Nicotiana benthamiana*. We have also shown that overexpression confers cold tolerance in yeast and is able to transport boron and probably also glycerol. We wanted to investigate whether ectopic overexpression of *BvCOLD1* could confer also stress tolerance in the model plant *Arabidopsis thaliana*. For this purpose we constructed a binary plasmid for stable expression in plants in which *BvCOLD1* expression was driven by the 35S promoter, and the NOS terminator, the plasmid we were using contained a Bar gene for selection based on resistance to the herbicide Basta (see materials and methods). We generated different transgenic lines by transforming *Arabidopsis thaliana* ecotype Columbia 0 plants with this construction and selected the three that presented higher expression of the transgene (Supp. Fig. 1). As a control we selected a representative transgenic line obtained by transformation of the same Arabidopsis genetic background with an empty plasmid.

BvCOLD1 was identified for its ability to improve growth under cold conditions upon overexpression in yeast. We wanted to determine whether this was also the case in plants during germination and early development. We sterilized and stratified the seeds and determined the ones germinating and developing green cotyledons after 10 days. The three lines exhibit enhanced germination and early development when compared to the line transformed with the empty plasmid (Fig. 5A). If plants were left for longer times at 10 ºC (28 days) most of the transgenic lines were surviving, and continued the development, while most of the plants transformed with the empty plasmid were dying.

There are several descriptions in the literature that the overexpression of aquaporins could confer pleiotropic resistance to abiotic stress (Sreedharan et al. 2015, Su et al. 2017). We tested whether the generated transgenic lines exhibited tolerance to other type of stress. We found that the overexpression of *BvCOLD1* enhanced germination

and early development under sodium chloride, lithium chloride and sorbic acid (Fig. 5C). We did not find any phenotype under osmotic stress induced by mannitol or toxic cations such as spermidine or norspermidine. We also did not observe any improvement in germination or early development when hydrogen peroxide was added to the medium (data not shown).

We have shown previously that in sugar beet *BvCOLD1* is expressed in all investigated organs. This finding suggests that its function may be required not only in germination, but in later stages of development. To investigate whether ectopic overexpression of *BvCOLD1* could increase stress tolerance during vegetative development, plants were germinated under normal conditions in solid media and then transplanted to individual plant pots with standard soil for Arabidopsis. Stress treatment was applied in the rosette stage, prior to the development of the hypocotyl (see materials and methods). After 30 days shoots were cut and fresh weight and dry weight was determined. We could observe that expression of *BvCOLD1* increases biomass under water and salt stress conditions and that slightly decreases biomass accumulation under cold stress (Fig. 6).

Plants overexpressing *BvCOLD1* are tolerant to low boron.

We have previously demonstrated that *BvCOLD1* is able to alter boron transport kinetics when is expressed in yeast. We wanted to confirm whether the expression in a plant system such as Arabidopsis could affect growth under low boron conditions. Normal boron concentration in standard medium as Murashige and Skoog (MS) is about 100 µM. We germinated plants in medium containing 0, 10, and 100 µM boron in order to determinate if the expression of *BvCOLD1* was increasing the ability of Arabidopsis transgenic lines to germinate and start development under limiting boron concentrations. Transgenic plants where germinating and expanded green cotyledons better than control plants in medium with low boron concentration (10 µM) or even

when no boric acid was added at all, indicating that could germinate with the traces present in the media (Fig. 7A). We further investigated whether overexpression of *BvCOLD1* was able to enhance growth under low boron conditions at later stages of development. It has been described that one of the main targets of boron deficiency is root elongation (Dell & Huang, 1997). We germinated control and transgenic plants in normal MS solid medium, transplanted them to plates with the indicated amount of boron and placed them in vertical position. After 17 days root length was recorded. Roots of transgenic plants where elongating the root better than control plants under low boron conditions (10 µM). We could also detect a minor elongation with no added boron, probably due to the traces present in the medium (Fig. 7B).

The carboxy terminal domain of BvCOLD1 is essential for its function.

The bioinformatics analysis of BvCOLD1 sequence rendered that the eleven amino acids in the carboxy terminal are conserved in in plants from the *Chenopodioideae* family such as *Spinacea oleracea* and in the non phylogenetically related plant *Mesembryanthemum crystallinum*. To determine if these amino acids are required for BvCOLD1 functionality we constructed transgenic plants overexpressing a truncated version of *BvCOLD1* in which the codon codifying the proline in position 244 was substituted by a stop codon. With three different transgenic lines overexpressing this truncated version we could not reproduce any of the phenotypes depicted in figures 5, 6 or 7, so we concluded that this domain is essential for BvCOLD1 functionality (data not shown).

DISCUSSION

In a context of climate change it is important to investigate the plant response to extreme environmental cues and identify genes able to confer stress resistance by overexpression. These genes could constitute targets for developing improved crops by genetic engineering or by new breeding techniques, and therefore to provide new tools to maintain food production under adverse environmental conditions. Sugar beet is a good source for stress resistance genes as is an important crop by itself, but is also a halotolerant plant, so its physiology and molecular biology is adapted to more limiting conditions than other crops. In this report we have identified a novel aquaporin from sugar beet using the yeast overexpression approach (Montesinos et al. 2003) under cold stress. This approach has been used before to identify yeast genes under cold conditions (Vicent et al. 2015), and plant genes under drought or salt stress but to our knowledge is the first description in which is used to identify plant genes able to confer cold resistance.

BvCOLD1 is expressed in all tissues investigated, pointing to a pivotal role in sugar beet physiology. It is localized in the ER, although its sequence its similar to TIP proteins (Yuan et al. 2017). We have confirmed this ER localization both in yeast and in plants. Another striking feature of *BvCOLD1* is that is not conserved in the model plant *Arabidopsis thaliana*. Sequence analysis identified that is only present in the *Chenopodioideae* subfamily of the *Amaranthaceae* family and presents a conserved amino acid sequence in the C terminal. This sequence can be found in others plants of the family adapted to hypersaline environments such as *Suaeda glauca*, *Salicornia herbacea* or in the unrelated plant *Mesembryanthemum cristallinum*, so BvCOLD1 could be a key protein for this adaptation. We have confirmed that the conserved carboxy terminal sequence is essential for BvCOLD1 function, as *Arabidopsis thaliana*

plants overexpressing a truncated version of this protein lost all the phenotypes observed in transgenic plants overexpressing the full length version of *BvCOLD1*.

There are reports in the literature of genes which can lead to cold tolerance upon overexpression (Ji et al. 2017, Liu et al. 2017), among them there are also several aquaporins (Ahamed et al. 2012, Huang et al. 2014, Li et al. 2009). It is also known that aquaporins can transport metalloids such as boric acid, silicic acid, and arsenite (Mukhopadhyay et al. 2014). We have shown that *BvCOLD1* can alter boron homeostasis in yeast and confer the ability to grow in low boron medium to transgenic Arabidopsis plants. This supports the idea of a key role of *BvCOLD1* in sugar beet physiology given that boron is one of the main limiting factors in sugar beet cultivation, and its deficiency is responsible of the heart rot disease, one of the major problems for its productivity. There are several proteins described to transport boron in plants. AtBOR1 is a member of the family of the bicarbonate transporters which is concentrated in the plasma membrane of different tissues upon boron deficiency (Takano et al. 2002). A Blast analysis indicated that AtBOR1 is present with two copies in the sugar beet genome (Bv5_120650_owkd.t1 and Bv5_115680_jrak.t1). The Arabidopsis aquaporins NIP5;1 and NIP6;1 are also boron transporters (Miwa et al. 2010). A BLASTp against the sugar beet database showed that the hypothetical protein Bv6_135670_oani.t1 shares a 75,78% of identity with NIP5;1 and that NIP6;1 shares 71,15% homology to Bv9_229530_hmzo.t1. NIP5;1 is responsible of boron uptake from the soil to the cytoplasm through the epidermis and cortex cells, while BOR1 is responsible of boron extrusion from endodermis and pericycle cells to the xylem (Miwa et al. 2010). NIP6;1 is important for preferential transport of boron to the growing shoot tissues (Tanaka et al. 2008). All three proteins have a high isoelectric point and are located in the plasma membrane, and preferentially present in roots and shoots. BvCOLD1 is not located in the plasma membrane and is an acidic protein, expressed in all investigated organs, so it's a novel participant in boron homeostasis whose

function must be related to the intracellular compartimentalization and distribution of boron. When we performed a boron uptake kinetics the final concentration of boron inside yeast is the same in cells transformed with the control plasmid or in cells transformed with *BvCOLD1* (Fig. 4), but yeast cells transformed with *BVCOLD1* are less tolerant to high boron concentrations. This indicated that BvCOLD1 is altering the intracellular distribution of boron. Our data indicate that *BvCOLD1* may be an important determinant of boron regulation in *Chenopodioideae*. It has been described that boron homeostasis may mediate changes in plant water relations, especially during boron deficiency (Wimmer & Eichert, 2013), this may explain the observed phenotypes of tolerance to drought, cold and salt stress observed in Arabidopsis transgenic lines and the fact that the overexpression of *BvCOLD1* induces cold tolerance during germination and early development, but induces sensitivity at later stages. An explanation for this experimental result may be that the translocation of boron due to the presence of BvCOLD1 may help plant development in young plantlets when there is a high production of new roots and leaves and therefore a high boron requirement, but at later stages without such requirement, the ectopic expression of *BvCOLD1* may interfere with the Arabidopsis response to cold stress.

In this report we have characterized a novel determinant for abiotic stress tolerance and boron homeostasis. This enables novel biotechnological strategies to develop abiotic stress resistant crops by genetic engineering of *BvCOLD1*. In addition the results present in this report open the possibility of using classical or new breeding techniques such as CRISPR/Cas9 or molecular marker assisted breeding in order to generate or identify varieties with increased expression of *BvCOLD1.* This may be a strategy to develop new sugar beet or spinach varieties with improved tolerance to abiotic stress or with less boron requirement, which can help to increase the yield of sugar, biomass, animal feed or food production under adverse environmental conditions.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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Figure legends.

Fig. 1. BvCOLD1 is a novel ER located aquaporin which confers cold tolerance upon overexpression in yeast. A. *BvCOLD1* confers cold tolerance in yeast upon overexpression. pYPGE: control yeast cells transformed with the empty plasmid. pYPGE*BvCOLD1* yeast cells transformed with the pYPGE plasmid containing the BvCOLD1 cDNA (upper panel). BvCOLD1-GFP is efficiently translated and located in the ER (lower panel). **B**. Genomic structure of *BvCOLD1*. The gene is located in chromosome 7 and has a single intron. **C**. *BvCOLD1* translated sequence analysis. Green: predicted transmembrane segments. Red: amino acids forming the Aromatic/ argininine selectivity filter. Blue: Amino acids forming the NPA sequence. **D**. BvCOLD1 model using the Rasmol software. Left: Zenital view with the open porus. Right: Lateral view.

Fig. 2. *BvCOLD1* is conserved in the Chenopodioideae family. A. Phylogenetic tree and multiple alignment and of amino acid sequences of BvCOLD1. The tree was built using the Minimum Evolution (ME) method, which uses distance measurements that correct multiple hits in the same sites, and a topology is chosen that shows the minimum value of the sum of all the branches (S) using the program .NCBI Tree Viewer (TV) and a sequence homology of more than 75%. **B.** Multiple alignments of the amino acids of the carboxyl terminal of BvCOLD1 from the sequences with the closest homology.

Fig. 3. *BvCOLD1* is expressed in all sugar beet organs and localized in the ER. A. Northern blot analysis of total RNA (15 μg) from petiole (P), young leaf (YL), old leaf (OL), hypocotyle (H), root (R), cotyledon (C) and epycotyle (E) from sugar beet. The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative expression (after

normalization to rRNA) as a percentage of the value for the treatment with the lowest one. **B**. Confocal laser scanning microscopy images of epidermal cells of *Nicotiana benthamiana* transiently expressing *BvCOLD1-GFP*. Image of a single slice (Slice) and composition of 30 different images along the Z axis (composition). **C**. Confocal laser scanning microscopy of a single protoplast derived from *Nicotiana benthamiana* leaves transiently expressing *BvCOLD1-GFP* with fluorescence (GFP) or with bright field (bright field).

Fig. 4. Overexpression of *BvCOLD1* alters glycerol and boron homeostasis in yeast. A. Cultures of the *gpd1* mutant strain transformed with the empty plasmid (upper lane), or with the plasmid containing *BvCOLD1* (BvCOLD1), were grown in selective SD medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of sorbitol (Sorb), hydrogen peroxide (H_2O_2) or sodium chloride (NaCl). Growth was recorded after 4 days. **B.** Cultures of the *bor1* mutant strain transformed with the empty plasmid (upper lane), or with the plasmid containing *BvCOLD1* (BvCOLD1), were grown in selective SD medium until saturation. Serial dilutions of each strain (1/10, 1/100 and 1/1000) were spotted onto YPD medium containing the indicated concentration of boric acid (H_3BO_3). Growth was recorded after 4 days. **C.** (a) *BvCOLD1* alters boron uptake kinetics of a yeast *bor1* mutant. Cultures of the *bor1* strain transformed with an empty plasmid (squares) and with the plasmid containing *BvCOLD1* (diamonds), were grown in YPD. At time 0 90 mM of H_3BO_3 was added to the medium from a concentrated stock. Aliquots were extracted at the indicated times and boron content was determined.

Fig. 5. Arabidopsis transgenic lines overexpressing *BvCOLD1* present better germination and early development under cold or abiotic stress conditions. A.

Overexpression of *BvCOLD1* increased germination and early development rate at 10 ºC. Germination and early development rates expressed as the mean percentages of seedlings with green expanded cotyledons for control line (filled bars) and transgenic lines 4 (dark grey bars), 6 (light grey bars), and 7 (empty bars) overexpressing *BvCOLD1* after 5 days (24 ºC) or after 15 days (10 ºC). Bars represent means ± standard error. Data with different letters differ significantly (P<0.05), as determined by the Duncan's multiple range test (n=300). **B.** Lines overexpressing *BvCOLD1* are able to develop under cold conditions. After recording germination and early development, plates were left to grow at 24 ºC (left) for 10 days or at 10 ºC (right) for 30 days. A representative plate of each treatment is shown. **C.** Overexpression of *BvCOLD1* improves germination under different abiotic stresses. Germination rates expressed as the mean percentages of seedlings with green expanded cotyledons for control line (filled bars) and transgenic lines 4 (dark grey bars), 6 (light grey bars) and 7 (empty bars) overexpressing *BvCOLD1* after 5 days in solid MS media (control) or in solid MS media containing 27mM LiCl (LiCl), 100mM NaCl (NaCl) or 0,5 mM Sorbic acid (Sorbic acid). Bars represent means ± standard error. Data with different letters differ significantly (P<0.05), as determined by the Duncan's multiple range test (n=300)

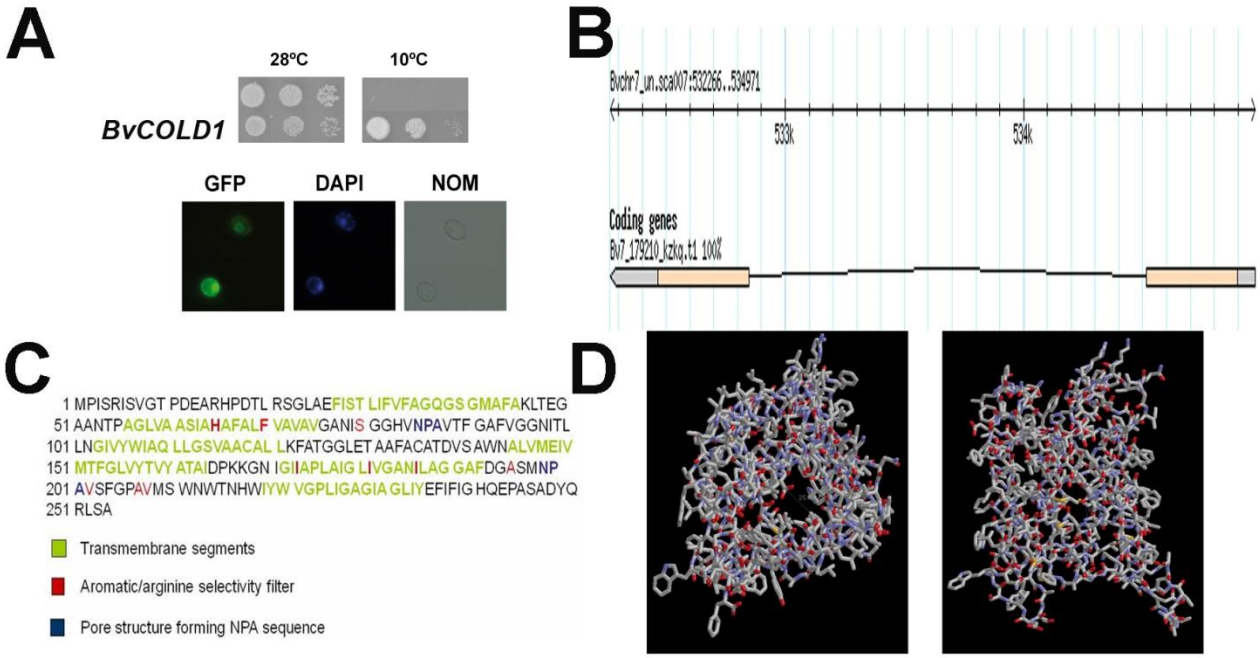
Fig 6. Overexpression of *BvCOLD1* increases biomass under abiotic stress conditions. Plants were germinated on solid MS medium, and then transferred to soil. Stress was applied as indicated in materials and methods. Fresh weight (**A**) and dry weight (**B**) of aerial part of control line (filled bars) and transgenic lines 4 (dark grey bars), 6 (light grey bars) and 7 (empty bars) overexpressing *BvCOLD1* was determined for plants cultivated at 24 ºC with standard irrigation (Control) in plants with standard irrigation growth at 10 ºC (Cold stress) in plants grown at 24 ºC subjected to water stress (Water stress) or in plants grown at 24 ºC irrigated with a NaCl solution (Salt stress). Upper lane in A and B represent a change in the scale to represent the data of

the control conditions (no stress). Means followed by the same letter are not significantly different (P<0.05) as determined by Duncan's multiple-range test (n=20).

Fig. 7. Overexpression of *BvCOLD1* increased germination, early development rate and root elongation under low boron conditions. **A**. Analysis of germination and early development of transgenic lines overexpressing *BvCOLD1* under boron limitation. Germination and early development rates expressed as the mean percentages of seedlings with green expanded cotyledons for control line (filled bars) and transgenic lines 4 (dark grey bars), 6 (light grey bars), and 7 (empty bars) overexpressing *BvCOLD1* after 5 days. Error bars represent means ± standard error. Data with different letters differ significantly (P<0.05), as determined by the Duncan's multiple range test (n=300 in six independent experiments) (upper panel). A representative plaque with each boron concentration is shown (lower panel). **B** Analysis of root elongation under low boron conditions. Plants of control line (filled bars) and transgenic lines 4 (dark grey bars), 6 (light grey bars), and 7 (empty bars) overexpressing *BvCOLD1* were germinated in MS medium and then transplanted to plates with the indicated concentration of boron and placed in vertical position. Root length was recorded after 17 days. Error bars represent means ± standard error. Data with different letters differ significantly (P<0.05), as determined by the Duncan's multiple range test (n=30 in six independent experiments) (upper panel). A representative plaque with each boron concentration is shown (lower panel).

Supplemental figure 1. Relative *BvCOLD1* expression in different transgenic lines generated in this work.

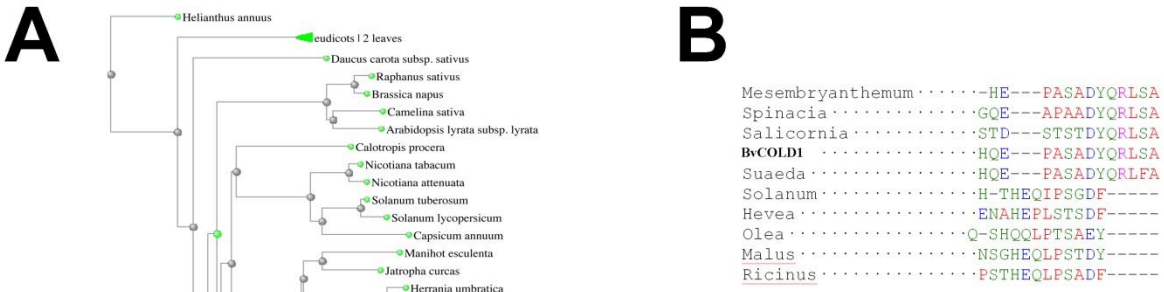
1 Fig. 1


A
28°C
10°C
BvCOLD1
GFP
DAPI
NOM
B
Bvchr7_un.scaf07:532266..534971
533k
534k
Coding genes
Bv7_179210_kzkq.t1 100%
C
1 MPISRISVGT PDEARHPDTL RSGLAEFIST LIFVFAGQGS GMAFAKLTEG
51 AANTPAGLVA ASIAHAFALF VAVAVGANIS GGHVNPAVTF GAFVGGNITL
101 LNGIVYWIAQ LLGSVAACAL LKFATGGLET AAFACATDVS AWNALVMEIV
151 MTFGLVYTVY ATAIDPKKGN IGIIAPLAIG LIVGANILAG GAFDGASMNP
201 AVSFGPAVMS WNWTNHWIYW VGPLIGAGIA GLIYEFIFIG HQEPASADYQ
251 RLSA
Transmembrane segments
Aromatic/arginine selectivity filter
Pore structure forming NPA sequence
D

2

3

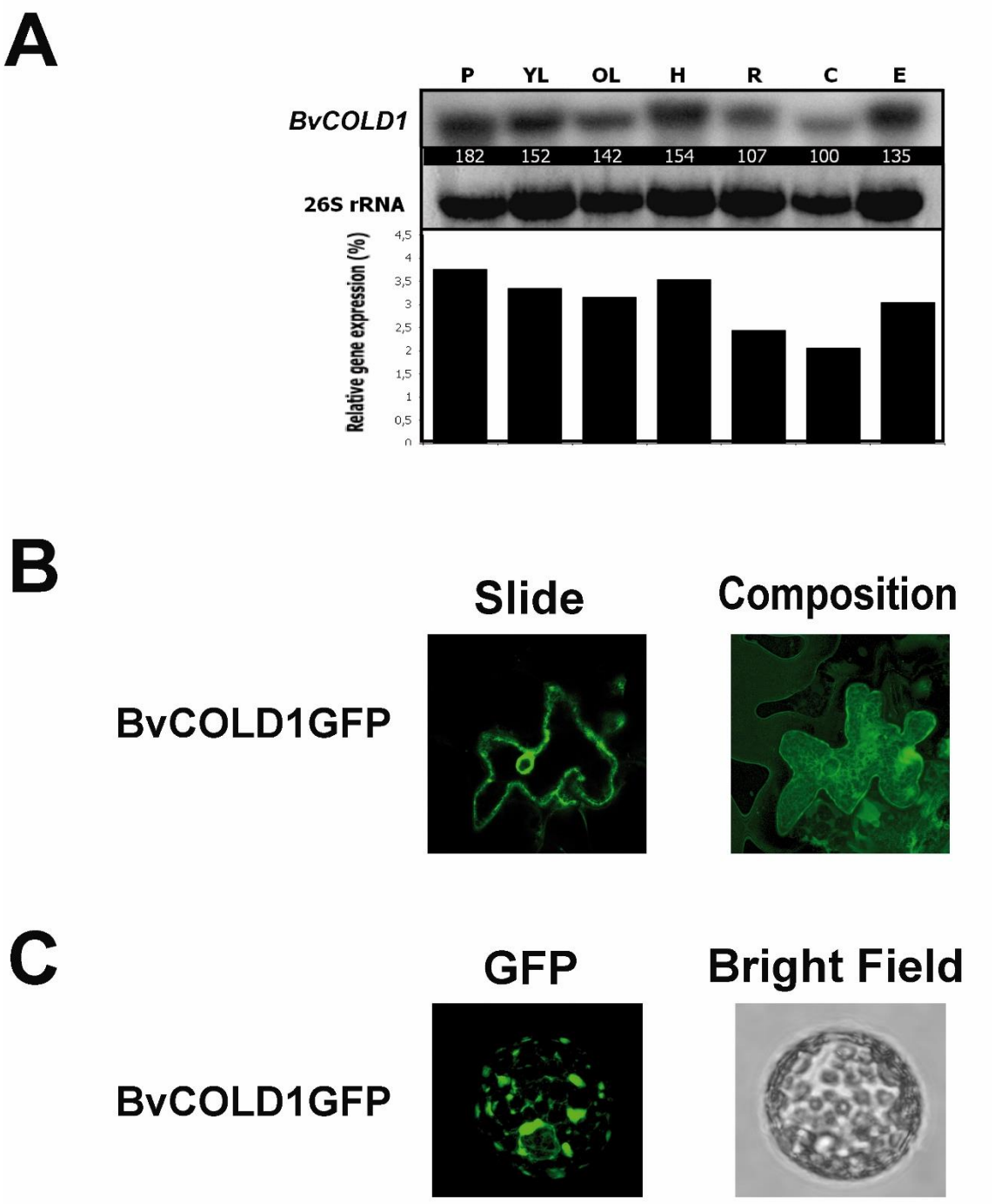
1 Fig 2.


A
Helianthus annuus
eudicots | 2 leaves
Daucus carota subsp. sativus
Raphanus sativus
Brassica napus
Camelina sativa
Arabidopsis lyrata subsp. lyrata
Calotropis procera
Nicotiana tabacum
Nicotiana attenuata
Solanum tuberosum
Solanum lycopersicum
Capsicum annuum
Manihot esculenta
Jatropha curcas
Herrania umbratica
Theobroma cacao
Fragaria vesca subsp. vesca
Mimosa pudica
Arachis ipaensis
Ziziphus jujuba
Ziziphus jujuba
Salix purpurea
Populus trichocarpa
Populus euphratica
Salix purpurea
Hevea brasiliensis
Manihot esculenta
Manihot esculenta
Mesembryanthemum crystallinum
Chenopodium quinoa
Spinacia oleracea
(BvCOLD1)
Suaeda glauca
Salicornia herbacea
0.1
B
Mesembryanthemum ·····-HE---PASADYQRLSA
Spinacia ·····GQE---APAADYQRLSA
Salicornia ·····STD---STSTDYQRLSA
BvCOLD1 ·····HQE---PASADYQRLSA
Suaeda ·····HQE---PASADYQRLFA
Solanum ·····H-THEQIPSGDF-----
Hevea ·····ENAHEPLSTSDF-----
Olea ·····Q-SHQQLPTSAEY-----
Malus ·····NSGHEQLPSTDY-----
Ricinus ·····PSTHEQLPSADF-----

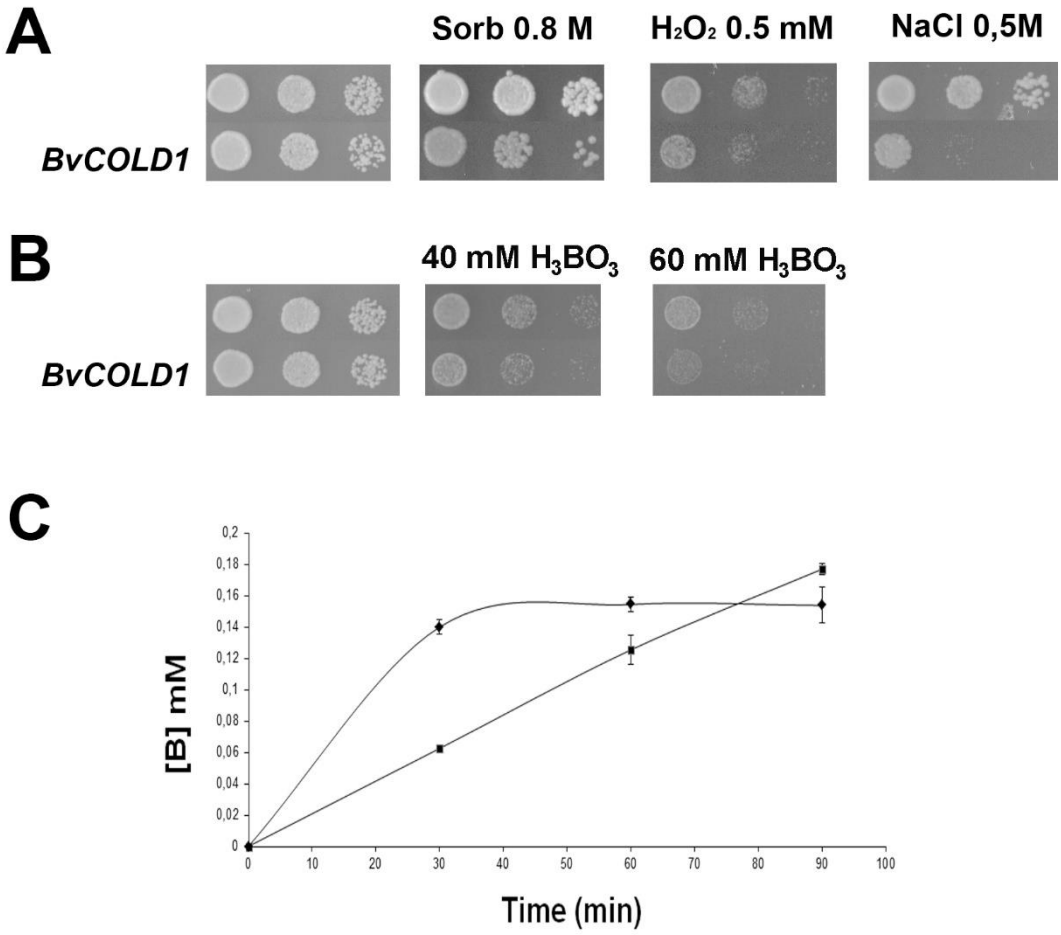
2

3

Fig 3:


A
P
YL
OL
H
R
C
E
BvCOLD1
182
152
142
154
107
100
135
26S rRNA
Relative gene expression (%)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
B
Slide
Composition
BvCOLD1GFP
C
GFP
Bright Field
BvCOLD1GFP

1 Fig. 4

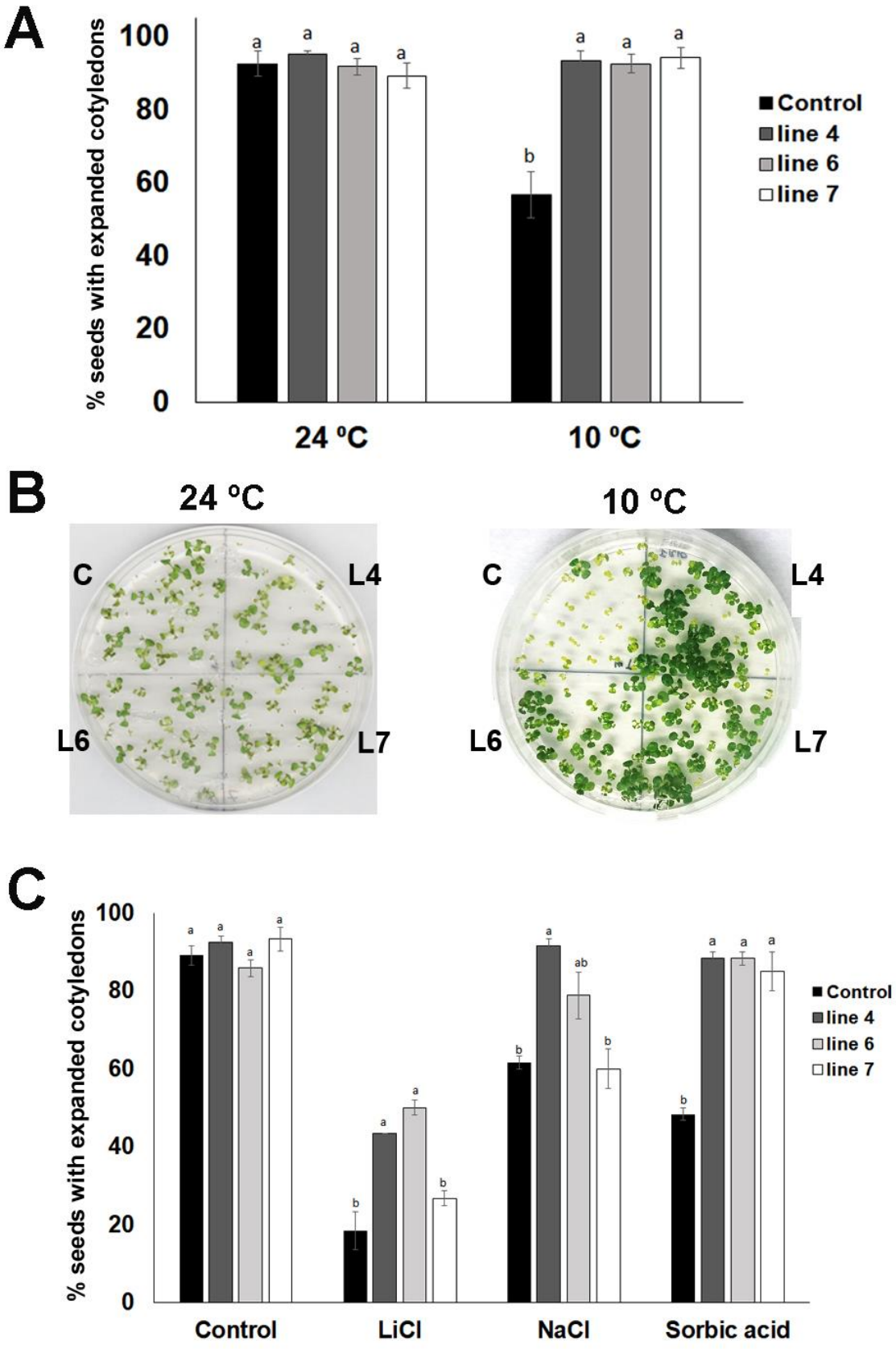

A
Sorb 0.8 M
H2O2 0.5 mM
NaCl 0,5M
BvCOLD1
B
40 mM H3BO3
60 mM H3BO3
BvCOLD1
C
[B] mM
0,2
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
0
10
20
30
40
50
60
70
80
90
100
Time (min)

2

3

4

1 Fig. 5


A
% seeds with expanded cotyledons
100
80
60
40
20
0
a
a
a
a
b
a
a
a
24 ºC
10 ºC
Control
line 4
line 6
line 7
B
24 ºC
10 ºC
C
L4
L6
L7
C
L4
L6
L7
C
% seeds with expanded cotyledons
100
80
60
40
20
0
Control
LiCl
NaCl
Sorbic acid
Control
line 4
line 6
line 7

2

3

Fig. 6

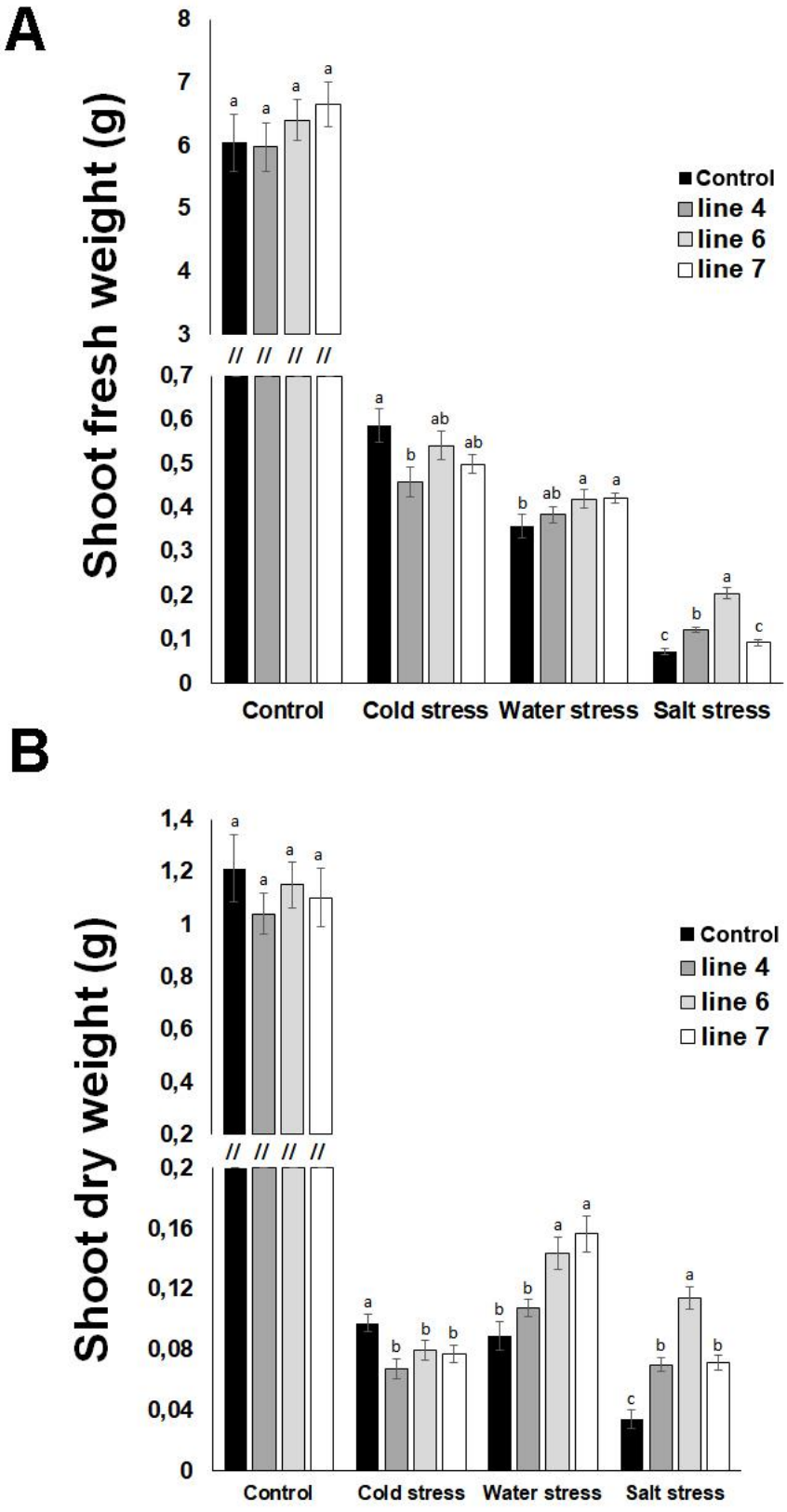
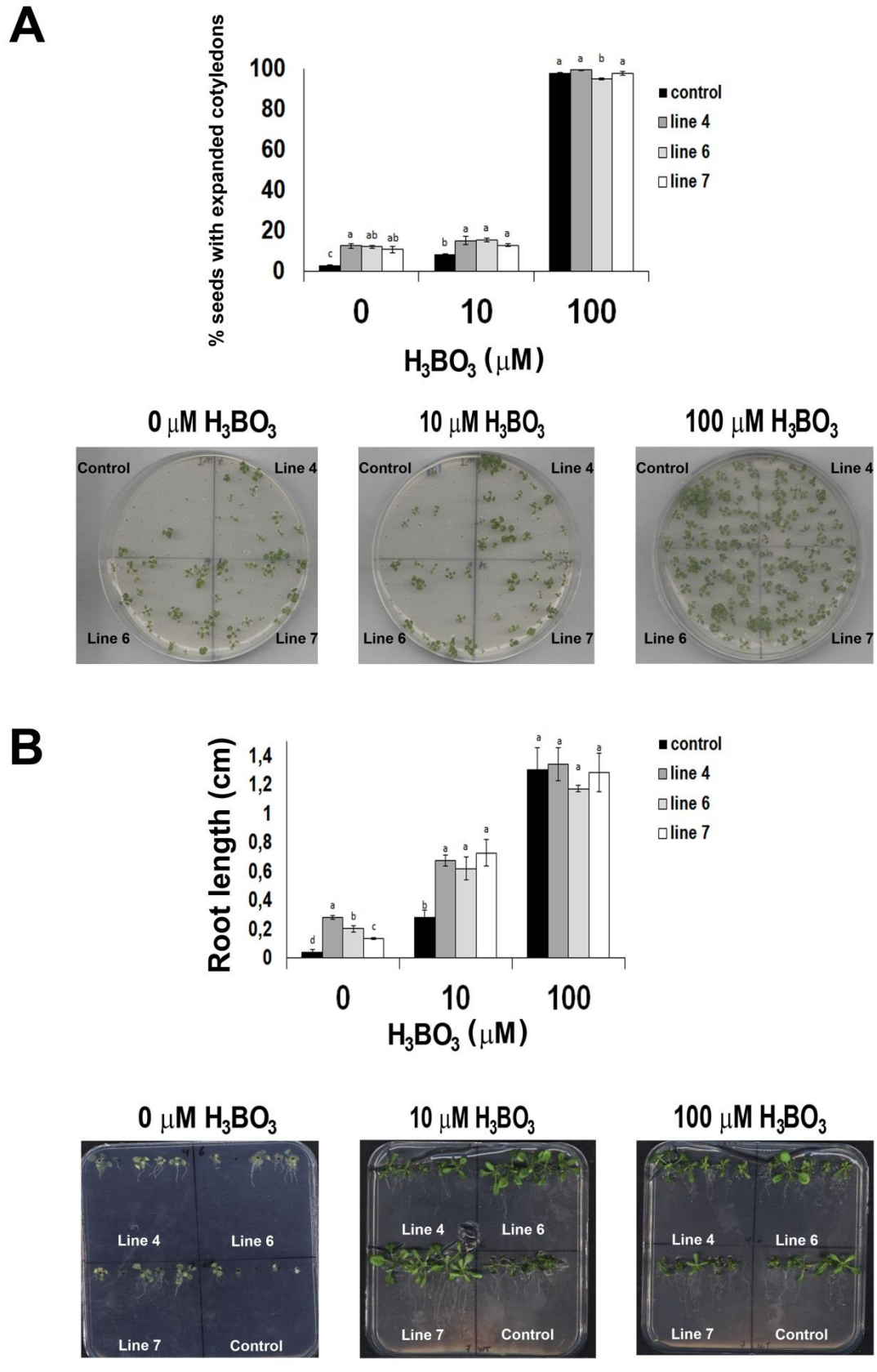

A
Shoot fresh weight (g)
Control
line 4
line 6
line 7
Control
Cold stress
Water stress
Salt stress
B
Shoot dry weight (g)
Control
line 4
line 6
line 7
Control
Cold stress
Water stress
Salt stress

Fig. 7


A
% seeds with expanded cotyledons
100
80
60
40
20
0
a
ab
ab
b
a
a
a
a
b
a
a
a
control
line 4
line 6
line 7
0
10
100
H3BO3 (μM)
0 μM H3BO3
10 μM H3BO3
100 μM H3BO3
Control
Line 4
Line 6
Line 7
B
Root length (cm)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
d
a
b
c
b
a
a
a
a
a
a
a
control
line 4
line 6
line 7
0
10
100
H3BO3 (μM)
0 μM H3BO3
10 μM H3BO3
100 μM H3BO3
Line 4
Line 6
Line 7
Control

Supplemental 1