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Enhancement of root growth by a drought-induced Ran-GTPase CLRan1 from a xerophyte wild watermelon

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Title:

Enhancement of root growth by a drought-induced Ran-GTPase *CLRan1* from a xerophyte wild watermelon

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Root growth enhancement by drought-induced *CLRan1*

Abbreviations:

2DE, two-dimensional gel electrophoresis; BM, basal medium; CDS, coding sequence; GUS, β-glucuronidase;; MS medium, Murashige and Skoog medium; PEG, polyethylene glycol; RACE, rapid amplification of cDNA ends; TAIL-PCR, thermal asymmetric interlaced-PCR

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Abstract

Drought avoidance via enhanced water uptake is one of the survival strategies of plants under water deficit conditions; however, the molecular mechanisms underlying this adaptive response are not fully understood. Wild watermelon (*Citrullus lanatus* sp. no. 101117-1) is highly resistant to drought and enhances root growth under drought stress to build a highly developed root architecture. Previous proteome analysis has shown that diverse arrays of functional proteins were induced in the roots at earlier phases of drought, which included Ran GTPase, an essential regulator of cell division and proliferation. In this study, we characterized the molecular properties and physiological significance of Ran GTPase in wild watermelon. Two cDNAs were isolated from wild watermelon, *CLRan1* and *CLRan2*, which showed a high degree of structural similarity with those of other plant Ran GTPases. Quantitative RT-PCR and promoter-GUS assays suggested that *CLRan1* was expressed mainly in the root apex and lateral root primordia, whereas *CLRan2* was more broadly expressed in other part of the roots. Immunoblotting analysis confirmed that the abundance of CLRan proteins was elevated in the root apex region under drought stress. Transgenic Arabidopsis overexpressing CLRan1 showed enhanced primary root growth, and the growth was maintained under polyethylene glycol-induced osmotic stress. These results indicated that CLRan functions as a key regulator of drought avoidance response in the roots of wild watermelon.

Key words:

Drought avoidance, Ran-GTPase, root growth, wild watermelon

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Introduction

Plant growth is strongly influenced by environmental stresses, of which water deficit caused by drought is one of the major factors impeding crop plant development and yield worldwide (Passioura 2007; Cramer et al. 2011). Depending on the stress intensity and duration, developmental stage and the plant species, plants show significantly different physiological, biochemical and molecular responses to drought stress (Chaves et al. 2002; Verslues et al. 2006). It is recognized that adaptive responses of plants to drought can be categorized into three major strategies: drought escape by growth season adjustments and early flowering, drought tolerance via increased water use efficiency and desiccation tolerance, and drought avoidance via reduced transpiration and increased water uptake (Levitt 1980). Xerophytes are a category of plant species that has adapted to survive in water-scarce environments, and they often exhibit highly advanced systems for survival by employing the above-mentioned strategies (Wickens 1998; Akashi et al. 2008).

Optimization of root architecture to maximize soil water uptake from deep soil layers is an advantageous trait under water deficit conditions. Wild watermelon (*Citrullus lanatus* sp. no. 101117-1) in the Kalahari Desert, Botswana, is a xerophyte that adopts this strategy (Yoshimura et al. 2008). In the early stage of drought stress, root development of this plant is significantly stimulated, indicating the activation of a drought avoidance mechanism for absorbing water from deep soil layers. As a first step to elucidate the molecular mechanisms underlying this physiological response, drought-induced changes in the root proteome were monitored by two-dimensional gel electrophoresis (2DE)-based proteome analysis (Yoshimura et al. 2008). The results revealed dynamic changes in the root proteomes under drought conditions. The expression levels of proteins associated with root morphogenesis and primary metabolism were upregulated in the earlier phase of the stress response and were correlated with enhanced root development and increasing energy demands. Among the upregulated proteins, two types of Ran GTPase were detected from two independent spots on the 2DE gel (Yoshimura et al. 2008).

Ran GTPase is an abundant nuclear protein and comprises one of the five small

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GTPase superfamilies (Ras, Rho, Rab, Arf and Ran) in eukaryotes (Bischoff and Ponstingl 1991; Takai et al. 2001). Ran proteins cycle between GTP- and GDP-bound forms, which is mediated via GTP hydrolysis in the cytoplasm by RanGAP1, and GDP/GTP exchange in the nucleus by RCC1. This system ensures an intracellular gradient of the GTP-bound form in the nucleus and GDP-bound form in the cytoplasm, which is essential for Ran function (Dasso 2002; Clarke and Zhang 2008). Ran GTPase has multiple roles in key cellular processes, such as a mediator of nucleocytoplasmic transport of macromolecules including RNAs and proteins, as an initiator of spindle assembly during mitosis, and as a regulator of reassembly of the nuclear envelope at the end of mitosis (Dasso 2002; Zheng 2004; Clarke and Zhang 2008).

In plants, Ran GTPase is encoded by small gene family in Arabidopsis (Haizel et al. 1997; Vernoud et al. 2003; Ma et al. 2007). Ran GTPase genes from various plant species, including those from tomato and tobacco, were able to complement the yeast *pim1-46* mutant phenotype (Belhumeur et al. 1993; Ach and Gruissem 1994; Merkle et al. 1994), suggesting functional exchangeability among the Ran GTPase homologs. Overexpression of Ran GTPase genes from wheat (TaRAN) and rice (OsRAN2) resulted in an increase in the mitotic index in root apical meristems and enhanced growth in transgenic rice and Arabidopsis (Wang et al. 2006; Chen et al. 2011), indicating that the function of Ran GTPase is strongly associated with meristem activity and cellular proliferation in plants. External stimuli such as cold stress (Chen et al. 2011) and jasmonic acid (Miche et al. 2006) are known to induce Ran GTPase expression in plants, but to the best of our knowledge, a connection between drought response and Ran GTPase in plants has not been described thus far.

In the present study, we attempted to elucidate the physiological role of drought-induced Ran GTPase in the xerophyte wild watermelon, via physiological, molecular biology, immunological and transgenic approaches. Our data suggested that Ran GTPase may have an important role in root growth and drought avoidance mechanisms in the wild watermelon.

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Materials and Methods

Plant materials and growth conditions

Seeds of wild watermelon (*Citrullus lanatus* sp. no. 101117-1) and domesticated watermelon (cv. Sanki) were soaked in water at 30°C in the dark overnight and planted onto Isolite^{TM} granular soil Type-I (2 mm-diameter, Isolite Insulating Products, Osaka, Japan) in small pots. Germinated seedlings of 1 cm height were transplanted onto cylindrical pots (37 cm depth and 12 cm diameter) that were filled with the same soil. Plants were grown in a growth chamber under fluorescent light of 200 μmol photons m^{-2} s^{-1} with a 16-h light/8-h dark illumination regime, and with a temperature and humidity cycle of 35°C/25°C and 50%/60%, respectively. Plants were fertilized with a 2,000-fold dilution of HYPONex;N:P:K=6:10:5 nutrient solution (HYPONeX Japan, Osaka, Japan), applied daily 1 h after the start of the light period. When the first true leaf had expanded, water-deficit stress was applied by withholding water as described previously (Yoshimura et al. 2008).

For agar culture of watermelon, the outer coat of the seeds was removed and surface-sterilized using 5% sodium hypochlorite and 0.05% Tween-20 for 5 min, and rinsed five times with sterile water. The seeds were placed on basal medium (BM) containing Murashige and Skoog (MS) salts, 10 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 0.8% agar, and the plates were placed vertically under fluorescent light of 200 μmol photons m^{-2} s^{-1} and a 16-h light/8-h dark illumination regime at 25ºC. When the primary roots reached 1 cm in length, osmotic stress was imposed by transferring the seedlings onto BM supplemented with various concentrations of polyethylene glycol (PEG) 6000 (Sigma-Aldrich, St. Louis, MO). Primary root length was measured 5 d after the onset of stress imposition.

Analysis of nucleotide sequences and transcript levels

Total RNA of wild watermelon roots was extracted using TRizol Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The first-strand cDNA was generated from 1 μg of total RNA with oligo(dT) primer by using a ReverTra-Plus Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. A partial sequence of Ran GTPase

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from wild watermelon was amplified using KOD-Plus polymerase (Toyobo) with plant Ran GTPases-specific primers (RAN-int-F and -R; Supplementary Table S1) designed for highly conserved sequences among Ran GTPase cDNA sequences from Arabidopsis (*AtRan1*, NP_197501.1; *AtRan2*, NP_197502.1; *AtRan3*, NP_200330.1), tomato (*Solanum lycopersicum SLRan*, NP_001234020.1), and wheat (*Triticum aestivum TaRan*, AF433653.1). The missing 5'- and 3'-end sequences were obtained by 5'- and 3'-RACE, respectively, using specific primers (CLRAN5'-R1 and -R2, and CLRAN3'-F1 and -F2, respectively) using a Marathon™ cDNA Amplification Kit (Takara, Shiga, Japan) in accordance with the manufacturer's instructions. Full-length cDNAs of Ran GTPases from wild watermelon (*CLRan1* and *CLRan2*) were amplified using KOD-Plus polymerase (Toyobo) with specific primers (CLRAN1FL-F and CLRAN1FL-R, and CLRAN2FL-F and CLRAN2FL-R, respectively). The amplified fragments were cloned into pZErO-2 (Invitrogen). Nucleotide sequences of the obtained clones were determined using an ABI PRISM310 Genetic Analyzer (Applied Biosystems, Foster City, CA). BLAST search was performed online with the Cucurbit Genomics Database (http://www.icugi.org/) and NCBI (http://blast.ncbi.nlm.nih.gov/). Alignments of nucleotide and amino acid sequences, and construction of the phylogenetic tree were performed as described previously (Akashi et al. 2011).

For quantitative RT-PCR analysis, 1 μg of total RNA was used for reverse transcription using ReverTra Ace-α and a random 9-mer primer (Toyobo). Real-time PCR was performed using a LightCycler 480 (Roche, Basel, Switzerland) with CYBR Premix Ex Taq II (Takara) and pairs of oligonucleotide primers, as listed in Supplementary Table 1. The thermal program was set as described previously (Akashi et al. 2011). Actin mRNA was used as an internal standard in all experiments, and relative expression levels were normalized to actin mRNA levels. A set of conserved primers for watermelon actin genes (Kajikawa et al. 2010) was used, and each assay was repeated at three times.

To identify genomic sequences corresponding to the *CLRan1* and *CLRan2* cDNAs, the 5'-flanking regions of the *CLRan1* and *CLRan2* genes were amplified by TAIL-PCR essentially as described previously (Liu et al. 1995; Liu and Whittier 1995), and the obtained

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fragments were cloned and sequenced. Genomic PCR was carried out using PrimeSTAR GXL polymerase (Takara) with the same primers as used for the amplification of full-length of the cDNAs, as listed in Supplementary Table S1. Genomic DNA was isolated from seedlings of wild watermelon using a DNeasy Plant mini kit (Qiagen, Germantown, MD) in accordance with the manufacturer's instructions, and used as a template.

Promoter-GUS analysis

The 5'-flanking regions of *CLRan1* (711-bp) and *CLRan2* (1,350-bp) were amplified by PCR using pairs of primers (Ran1-U-F and Ran1-U-R for CLRan1, and Ran2-U-F and Ran2-U-R for CLRan2) as listed in Supplementary Table S1. The amplified genome fragments were cloned into the Gateway cloning sites upstream of a GUS gene in a pGWB3 vector (Nakagawa et al. 2007). Generation of transgenic hairy root in wild watermelon, and histochemical GUS assays were performed as described previously (Kajikawa et al. 2010).

Immunoblotting analyses

An open reading frame of *CLRan1* was amplified by PCR using a pair of primers (CLRAN1-NdeI-F and CLRAN1-XhoI-R, as listed in Supplementary Table S1), and cloned into the *Nde*I and *Xho*I restriction sites of a pET15b vector (Novagen, Madison, WI) to generate pET-CLRan1. The pET-CLRan1 was introduced into *Escherichia coli* BL21 (DE3), and the His-tagged CLRan1 recombinant protein was expressed and purified on a HisBind resin (TALON™, Clontech, Mountain View, CA) in accordance with the manufacturer's instructions. A rabbit polyclonal antibody was raised against the purified recombinant protein, and immunoblotting analysis was done by a standard method as described previously (Hoshiyasu et al. 2013). Total proteins were extracted as described previously (Yoshimura et al. 2008), and used for immunoblotting analysis. Visualization of the immunoblotting images and quantification of signal intensity were carried out using an ECL-Plus Kit (GE Healthcare, Waukesha, WI) with LAS 4000 mini and Multi Gauge ver 3.0 software (Fuji Film, Tokyo, Japan).

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Generation and analysis of transgenic plants

A full-length cDNA of *CLRan1* was amplified by PCR using a pair of primers (attB1-CLRAN-F and attB2-CLRAN1-R, as listed in Supplementary Table S1), and cloned into a region downstream of the CaMV35S promoter in a pGWB2 vector (Nakagawa et al. 2007) using the GATEWAY system (Invitrogen). The obtained pGWB2-CLRan1 vector was introduced into *Agrobacterium tumefaciens* strain EHA105 and used for Arabidopsis transformation by the vacuum infiltration method, as described previously (Bechtold and Pelletier. 1998). T_1 seedlings were selected on MS medium containing 100 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin, and transferred to soil. T_3 seeds were harvested and used for experiments.

T_3 seeds were germinated and grown for 3 d on sucrose-free, half-strength MS solid medium, on vertically-positioned plates under continuous fluorescent light of 90 μmol photons m^{-2} s^{-1} at 23ºC. The seedlings were then transferred to the same media supplemented with 1 or 5% PEG 6000 to impose osmotic stress. Primary root length was measured 6 d after the onset of stress imposition.

Results

Response of wild watermelon roots to water deficit and osmotic stress

To examine the change in total root biomass under water stress conditions, seedlings of wild and domesticated watermelon were grown in a pot until their first true leaves had expanded, and water deficit stress was imposed by withholding watering. The use of a long cylindrical pot allowed vertical growth of the root system of the seedlings, especially for wild watermelon (Figure 1A). Upon onset of drought stress, more vigorous root growth was observed in wild watermelon at day 3 compared with its irrigated control (Figure 1A). On day 5, the dry weight of wild watermelon seedlings was 88 ± 18 mg/plant, which was significantly higher than the 49 ± 2 mg/plant for the irrigated wild watermelon control (Figure 1B). In contrast, domesticated watermelon did not show significant root growth enhancement under water deficit conditions

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(Figure 1A, B). This observation confirmed our previous study that water deficit stimulated root growth in wild watermelon (Yoshimura et al. 2008).

To gain more information on the response of wild watermelon roots to varying environmental conditions, osmotic stress was imposed by adding PEG in the medium, and seedling growth was monitored. In domesticated watermelon, primary root growth in the presence of 1~5% PEG was not significantly different from that in unstressed control seedlings (Figure 1D). In contrast, the length of primary roots of wild watermelon increased 23 ± 14% and 54 ± 40% on 1% and 5% PEG media, respectively, showing that PEG-induced osmotic stress also stimulated root growth in wild watermelon (Figure 1C, D).

Structure of wild watermelon Ran GTPase

Our previous study on the root proteome of wild watermelon identified that Ran GTPase was induced in the early phase of drought stress (Yoshimura et al. 2008). We attempted to elucidate more information on the molecular entity of Ran GTPase in this plant. Based on the detected peptide fragments in liquid chromatography-tandem mass spectrometry analysis, a pair of primers for conserved plant Ran sequences was designed as described in the *Materials and Methods*. RT-PCR followed by 5'- and 3'-RACE led to the isolation of two distinct full-length cDNA from wild watermelon roots, designated *CLRan1* and *CLRan2* (DDBJ/Genbank/EMBL accession numbers AB930123 and AB930124, respectively). BLAST2 homology searches showed that *CLRan1* and *CLRan2* were 80% and 95% identical to each other at the nucleotide and protein sequence levels, respectively (Figure 2A). The theoretical molecular masses for the CLRan1 and CLRan2 proteins were both 25.1 kDa, with expected isoelectric points (p*I*) of 6.44 and 6.38, respectively. A BLASTN search of the recently published draft genome sequence of domesticated watermelon of Chinese elite inbred line 97103 (Guo et al. 2013) revealed that the *CLRan1* cDNA had significant match with three watermelon coding sequences (CDSs) *Cla012278* (99% and 100% nucleotide and amino acid identity, respectively), *Cla012277* (93% and 100%, respectively), and *Cla012279* (94% and 99%, respectively) (data not shown), all of which were located in tandem on chromosome 6. The *CLRan2* cDNA sequence perfectly

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matched with watermelon CDS *Cla022303* (100% nucleotide identity) on chromosome 8. The genomic sequences of *CLRan1* and *CLRan2* obtained from wild watermelon (DDBJ/Genbank/EMBL accession numbers AB935249 and AB935250, respectively) showed 8 exons and 7 introns, respectively, and were structurally most homologous to the Arabidopsis AtRan3 gene (Supplementary Figure S1). Except for the highly divergent AtRan4 sequence, the sequence alignment showed high amino acid sequence homology of CLRan sequences with other plant Ran proteins (91~97%, Figure 2A). Hallmark sequence motifs of plant Ran-GTPase, such as the RanGAP-binding motif and C-terminal acidic tail (Ma et al. 2007), were well-conserved in CLRan1 and CLRan2 (Figure 2A). Phylogenetic analysis showed a close association of *CLRan* genes with other plant Ran sequences (Figure 2B).

mRNA expression profiles of CLRan genes

The mRNA transcript levels of *CLRan1* and *CLRan2* in the roots were investigated by quantitative RT-PCR using gene-specific primers. In this analysis, two different parts were harvested from the roots, the root apical region at 0~5 mm from the root apex (hereafter designated as zone I), and the penultimate region spanning 5~20 mm from the apex (designated as zone II). In unstressed conditions, the expression level of *CLRan1* was approximately twofold higher in zone I than in zone II, but similar expression levels were observed for *CLRan2* in both zones (Figure 3A). Comparison of the expression levels between unstressed and drought-stressed roots showed that the mRNA levels of both *CLRan1* and *CLRan2* were unchanged in zone I during drought stress progression (Figure 3B).

The tissue specificity of *CLRan* gene expression was further examined by promoter-reporter assays. For this analysis, the 5'-upstream genomic regions of *CLRan1* and *CLRan2* were obtained by TAIL-PCR, which led to the isolation and cloning of *CLRan1* (711-bp) and *CLRan2* (1350-bp) promoter fragments (Genbank/EMBL/DDBJ accession numbers AB930123 and AB930124, respectively). The 711-bp *CLRan1* promoter fragment had 95% sequence identity with that of the upstream region of the Cla012278 gene (chromosome 6) from domesticated watermelon inbred line 97103, whereas the 1350-bp *CLRan2* promoter

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fragment showed a 97% sequence match with that of the upstream regions of the Cla022303 gene (chromosome 8) from line 97103 (Guo et al. 2013). The obtained promoter fragments were fused to the GUS coding sequence, and the resultant CLRan1pro::GUS and CLRan2pro::GUS fusion genes were introduced into wild watermelon using a hairy root transformation system (Kajikawa et al. 2010).

When the CLRan1pro::GUS fusion gene was introduced into hairy roots, blue-colored signals were observed preferentially in the apex region of the roots, together with strings of small dots alongside the elongation zone of the roots (Figure 3C). Higher magnification images showed that strong blue-colored signals were observed in the region spanning 0~1 mm from the root apex (Figure 3E), and also in lateral root primordia that were emerging from the roots (Figure 3F). In contrast, using the CLRan2::GUS construct, the signals in the apex region were relatively weaker in hairy roots (Figure 3D). In addition, more intense signals were observed in the basal region of roots that were distant from the apex.

Changes in the protein abundance of CLRan

To examine the abundance of CLRan protein, we first raised an antibody against CLRan1. A cleavable hexa-His-tagged recombinant protein of the full-length coding sequence of CLRan1 was expressed in *E. coli*, and purified to homogeneity. A polyclonal antibody raised against the purified protein recognized a single band of apparent molecular mass of approximately 26 kDa in immunoblotting assays using total protein lysates from wild watermelon roots, which was consistent with the theoretical molecular mass of CLRan1 (25.1 kDa) deduced from the cDNA sequence (Figure 4A). Because CLRan1 and CLRan2 are 95% identical in amino acid sequence, and the predicted molecular masses are 25.1 kDa for both polypeptides, the polyclonal antibody used in this study should recognize CLRan2 as well.

Using the anti-CLRan antibody, changes in the abundance of Ran proteins were examined in lysates of wild watermelon roots. In zone I of the root (0~5 mm from the root apex), a 42 ± 7.1% increase in the abundance of Ran protein was observed on day 4 of drought stress in comparison with the unstressed control (Figure 4A, B). A statistically significant increase was

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also observed in zone II of the root (5~20 mm from the apex), although the increase was relatively modest (21 ± 9.7% relative to the unstressed control). Overall, these observations were consistent with our previous proteome study, in which water deficit induced Ran-GTPase protein in wild watermelon roots (Yoshimura et al. 2008).

Enhancement of root growth in transgenic Arabidopsis plants overexpressing CLRan1

As one of the steps to elucidate the function of drought-induced CLRan proteins, transgenic Arabidopsis plants overexpressing *CLRan1* were generated and analyzed. The *CLRan1* cDNA sequence was cloned downstream of the 35S promoter in a binary vector, and introduced into Arabidopsis plants. The T_3 generation of three independent transgenic lines (designated as T2, T6 and T11) was investigated in this study. Aerial parts of these transgenic lines were phenotypically normal and indistinguishable from those of vector-transformed control transgenic plants (data not shown). Immunoblotting analysis showed that roots of the established overexpressing lines had modest (1.9-fold increase for T2) or higher (4.1- and 3.7-fold for T6 and T11, respectively) increases in Ran GTPase protein abundance in comparison with those of the vector-transformed control transgenic plants (Figure 5A). An immunoblotting signal from the control plants was probably derived from intrinsic Ran proteins in Arabidopsis, which have 74~97% amino acid identity with CLRan1 (Figure 2A).

Using these transgenic plants, root growth was compared on MS agar plates. We observed that the two transgenic lines with higher CLRan1 expression levels (lines T6 and T11) showed enhanced growth of primary roots in standard MS-agar conditions (Figure 5B, C). The primary root length at 9 d after germination was enhanced by 54 ± 25% and 26 ± 18% for transgenic lines T6 and T11, respectively, in comparison with control plants (black bars in Figure 5C). Root growth in line T2, which had a modest level of CLRan1 expression (Figure 5A), was not statistically different from the control plants (Figure 5C).

The transgenic seedlings were subjected to PEG-induced osmotic stress in MS-plates. When 1~5% concentration of PEG was applied to the medium, growth retardation of primary

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roots was statistically significant in the control plants (Figure 5B, C, N=18, P<0.01). By contrast, PEG-induced growth retardation was not observed in the transgenic plants.

Discussion

Small GTPase Ran is ubiquitous protein among eukaryotes and has multiple functions in cell cycle regulation, nucleo-cytoplasmic macromolecule transport and organelle dynamics (Dasso 2002; Clarke and Zhang 2008). Complementation of yeast *pim1* mutant phenotypes by Ran homologs from various plants has been reported (Ach and Gruissem 1994; Merkle et al. 1994), suggesting a conserved function of plant Ran homologs in the regulation of cellular proliferation. However, the physiological roles of Ran proteins in plants, especially in responses to environmental conditions, are not fully understood. In this study, we present evidence that the CLRan protein, a drought-induced Ran GTPase from the xerophyte wild watermelon, is associated with the maintenance of root growth under environmental stress conditions.

We isolated two cDNA clones, *CLRan1* and *CLRan2*, from drought-resistant wild watermelon (*Citrullus lanatus* sp. no. 101117-1), which had significant sequence similarities with *Cla012278* and *Cla022303* from domesticated watermelon inbred line 97103 (Guo et al. 2013). The draft sequence of the watermelon inbred line 97103 revealed four genes in the genome; *Cla02277*, *Cla02278* and *Cla02279* are closely related each other in coding sequence (99~100% deduced amino acid identity among them) and are located in tandem on chromosome 6, whereas *Cla022303* is relatively divergent in sequence (95~96% amino acid identity with *Cla02277~02279*) and is present independently in chromosome 8. These observations suggested that the three Ran genes on chromosome 6 may have triplicated relatively recently in watermelon evolution, but divergence of the Ran gene on chromosome 8 might be dated earlier. Although the detailed genomic structure of CLRan genes in drought-resistant wild watermelon (*Citrullus lanatus* sp. no. 101117-1) awaits further investigation, significant sequence matches between *CLRan1* and *Cla02278*, and between *CLRan2* and *Cla022303*, indicated that *CLRan1* and *CLRan2* may be derived from chromosome 6 and 8, respectively, and possibly diverged relatively earlier in watermelon

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evolution. Nevertheless, the deduced amino acid sequence of CLRan1 and CLRan2 had 91~97% amino acid identity with other plant Ran-GTPase (Figure 2), suggesting the conservation of physicochemical properties of CLRan with other Ran polypeptides in the plant kingdom.

Quantitative RT-PCR analysis showed that the mRNA for the *CLRan1* gene was more abundant in the apical region of roots (zone I; 0~5 mm from the root apex) than in more the basal region (zone II; 5~20 mm from the apex) (Figure 3A). This observation indicated that *CLRan1* gene expression may be spatially associated with the root apex in wild watermelon. Consistent with this scenario, promoter-GUS assays demonstrated that *CLRan1* promoter activity was observed in root apex regions and lateral root primordia (Figure 3C, E, F), where higher levels of cellular proliferation were expected. In contrast, the levels of *CLRan2* mRNA were similar in both zone I and II, and *CLRan2* promoter activity was detected in more basal regions of the root (Figure 3A, D). These observations suggested a difference in the transcriptional regulation of *CLRan1* and *CLRan2* in terms of cell/tissue-type specificity.

Quantitative RT-PCR and immunoblotting analyses suggested that the mRNA levels of *CLRan1* and *CLRan2* were not changed under drought stress conditions in zone I of the roots, whereas a statistically significant increase in the protein abundance of CLRan was observed (Figure 3B, Figure 4A, B). One plausible scenario explaining this difference is that CLRan protein abundance may be regulated post-transcriptionally during drought stress. Alternatively, a possibility of transcriptional upregulation of other Ran genes upon drought stress cannot be excluded. Nevertheless, immunological analysis in the present study confirmed the previous proteome analysis results (Yoshimura et al. 2008) that Ran protein levels increased under drought stress in wild watermelon roots. In the previous 2DE-based proteome analysis, two upregulated Ran spots were detected, which had the same molecular weight but slightly different apparent p*I* values of 6.87 and 6.73 (Yoshimura et al. 2008). It is possible that these two protein spots might be derived from CLRan1 and CLRan2, which have slightly different theoretical p*I* values of 6.44 and 6.38, respectively (Figure 2A). Factors underlying the difference between the observed and theoretical p*I* values should be resolved in future studies.

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The present study demonstrated that transgenic Arabidopsis plants overexpressing *CLRan1* gene had enhanced primary root growth in the seedling phase, especially under PEG-induced osmotic stress conditions (Figure 5). Although the effects of *CLRan1* overexpression on plant development and biomass production have to be examined in more detail, the present observations suggest that enhanced *CLRan1* expression may be positively correlated with growth promotion and maintenance under environmental stress conditions. Our data are consistent with a previous report that *OsRan2*-overexpressing transgenic rice acquired cold resistance by maintaining cell division under stress conditions (Chen et al. 2011). We observed that transgenic Arabidopsis with higher *CLRan1* transgene expression (lines T6 and T11) exhibited enhanced root growth in both unstressed and PEG-stressed conditions (Figure 5C). The effects of *CLRan1* overexpression were observed also in the transgenic line with lower *CLRan1* transgene expression (line T2), which sustained root growth under PEG-stress conditions. We speculated that there may be a certain threshold level of Ran-GTPase protein accumulation in order to exert growth promotion in transgenic plants. In this respect, it is noteworthy that the degree of cold resistance and root mitotic index among transgenic lines were positively correlated with the relative expression level of *OsRan2* transgene among the lines in the previous study (Chen et al. 2011).

The molecular mechanism linking Ran-GTPase and growth maintenance under stress conditions remains elusive. Wheat Ran-GTPase TaRAN1 was shown to regulate mitotic progress and alter primordial meristems in transgenic rice and Arabidopsis (Wang et al. 2006). Because Ran-GTPase localizes to the mitotic chromosomes and other cell division machinery, such as spindle microtubules and centrosomes, to provide spatial signals for cell division (Clarke and Zhang 2008), fortification of Ran-GTPase abundance may ensure proper organization of the mitotic microtubular structure during division cycles. In fact, overexpression of OsRan2 was shown to rescue the impairment of intranuclear tubulin export and sustain cell division under cold stress in transgenic rice (Chen et al. 2011). Furthermore, the levels of plant Ran-GTP were shown to respond to external physicochemical stimuli such as auxin, jasmonic acid, and gravitropism (Wang et al. 2006; Kriegs et al. 2006; Miche et al. 2006), raising a possibility that

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Ran-GTPase may function as one of the factors integrating various intrinsic and extrinsic information for the regulation of cell cycle progression, which is a susceptible cellular processes under environmental stress condition (Ma et al. 2009; Chen et al. 2011).

In conclusion, we found that the abundance of CLRan protein increased in the roots of xerophyte wild watermelon under drought stress conditions. CLRan overexpression could enhance primary root growth in seedlings of transgenic Arabidopsis, especially under osmotic stress conditions. Our study suggests a new role of plant Ran-GTPase in maintaining root growth under drought stress conditions.

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Figure legends

Figure 1. Response of watermelon roots to water stress. (A) A representative picture showing differential root development of wild (plants 2, 4, 5 and 7) and domesticated (plants 1, 3 and 6) watermelon plants under drought stress. At 11 d after germination, when the first true leaf was expanded, water deficit stress was imposed by withholding watering, and plants before the stress (plant 1 and 2), stressed for 3 d (plants 3, 5), and stressed for 5 d (plants 6, 7) were examined. A control wild watermelon plant under irrigation for 3 d is also shown (plant 4). Scale bar = 3 cm. (B) Change in root dry weight of wild (circles) and domesticated (triangles) watermelon plants under drought stress (closed symbols and bold lines) and irrigated control (open symbols and dashed lines). Data are average and standard deviation of three independent plants. Asterisks represent statistically significant differences between the values of drought stressed and irrigated controls (Student's t-test, $P < 0.05$). (C) Root growth of wild watermelon under polyethylene glycol (PEG)-induced osmotic stress on agar cultures for 5 d. Two seedlings are shown for each condition. (D) Effect of osmotic stress on the primary root length of watermelon seedlings. The root length was compared between wild (closed bar) and domesticated (open bar) watermelon seedlings. Data are the average of 10 independent plants. Asterisks indicate statistically significant differences compared with unstressed plants ($P<0.05$).

Figure 2. Deduced amino acid sequence of watermelon CLRan. (A) Alignment of the watermelon sequences (CLRan1 and CLRan2) with Ran sequences from other organisms. Red boxes indicate that the amino acid residue is identical in at least five out of nine sequences. The sequence names and their accession numbers are as follows: *C. lanatus* CLRan1 (AB930123), CLRan2 (AB930124), Arabidopsis AtRan1 (NP_197501.1), AtRan2 (NP_197502.1), AtRan3 (NP_200330.1), AtRan4 (NP_200319), *Solanum lycopersicum* SLRan (NP_001234020.1), *Triticum aestivum* TaRan (AF433653.1), and *Homo sapiens* HsRan (NP_006316.1). Conserved motifs for plant RanGAP-binding and acidic tails are boxed. (B) Phylogenetic analysis of Ran sequences. The amino acid alignment in Figure 2A was used for

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ClustalW analysis (http://www.ddbj.nig.ac.jp) and visualized using the NJplot program (http://doua.prabi.fr/software/njplot).

Figure 3. The mRNA transcription profiles of *CLRan* genes in root tissues. (A) Quantitative RT-PCR analysis of *CLRan* mRNA expression levels in different regions of watermelon root tissues. Roots of unstressed wild watermelon grown in pot culture were harvested, and root apex regions were dissected into zone I (0~5 mm from the root apex) and zone II (5~20 mm from the root apex). The abundance of *CLRan1* and *CLRan2* mRNAs in these zones was compared using specific primers. Data are the average and standard deviation of three experimental replicates. Asterisks represent statistically significant differences between the values of zones I and II ($P < 0.05$). (B) Change in *CLRan* mRNA abundance under drought stress in zone I of wild watermelon roots. CT; unstressed control plants. D2 and D4; plants stressed for 2 and 4 d, respectively. Data are the average and standard deviation of three experimental replicates. (C-F) Histochemical localization of GUS activity in transgenic hairy roots of wild watermelon transformed with CLRan1pro::GUS (C, E, F) and CLRan2pro::GUS (D) constructs. In (C) and (D), two independent hairy roots are shown. Enlarged views of a primary root apex (E) and a lateral root primordium (F) are also shown for CLRan1pro::GUS hairy roots. Primary root apex regions are indicated by arrows. Bar = 10 mm in (C) and (D), 1 mm in (E), 0.2 mm in (F).

Figure 4. Drought-induced changes in the abundance of CLRan protein in the roots of wild watermelon. (A) Immunoblotting analysis of CLRan proteins in different regions of wild watermelon root tissues. Wild watermelon plants were subjected to drought stress, and the root apex regions were harvested and dissected into zone I (0~5 mm from the root apex) and zone II (5~20 mm from the root apex). Total protein (30 μg) samples were loaded in each lane. Apparent molecular weight (26-kDa) of the detected signal for CLRan proteins is indicated by an arrow. CT; unstressed control plants. D2 and D4; plants stressed for 2 and 4 d, respectively. (B) Quantitative change in the relative abundance of CLRan proteins in the root of wild watermelon under drought stress. Data are the average and standard deviation of three

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independent experiments. Asterisks represent statistically significant differences between control and drought conditions ($P < 0.05$).

Figure 5. Effect of overexpression of *CLRan1* in transgenic Arabidopsis. (A) *Top panel.* Immunoblotting analysis of Ran-GTPase abundance in transgenic Arabidopsis plants expressing *CLRan1*. Independently transformed lines T2, T6 and T11 were used for the analysis. Apparent molecular weight (26-kDa) of the detected signal for Ran proteins is indicated by an arrow. CT, control Arabidopsis plants transformed with an empty vector. *Bottom panel.* Quantitative estimation of Ran protein abundance in the roots of transgenic plants. The values are expressed relative to that for the control transformant. (B) Representative images of the phenotypes of control plants and CLRan1-overexpressing line T11 under PEG-induced osmotic stress. The concentrations of PEG in the medium are shown above the images. (C) Primary root length of control transformants and CLRan1-overexpressing lines under PEG-induced osmotic stress. Seedlings (3 d old) were placed on MS agar plates containing various concentrations of PEG and grown vertically for 6 d. Asterisks represent statistically significant differences between PEG-free and PEG-containing medium ($N>18$, $P < 0.01$).

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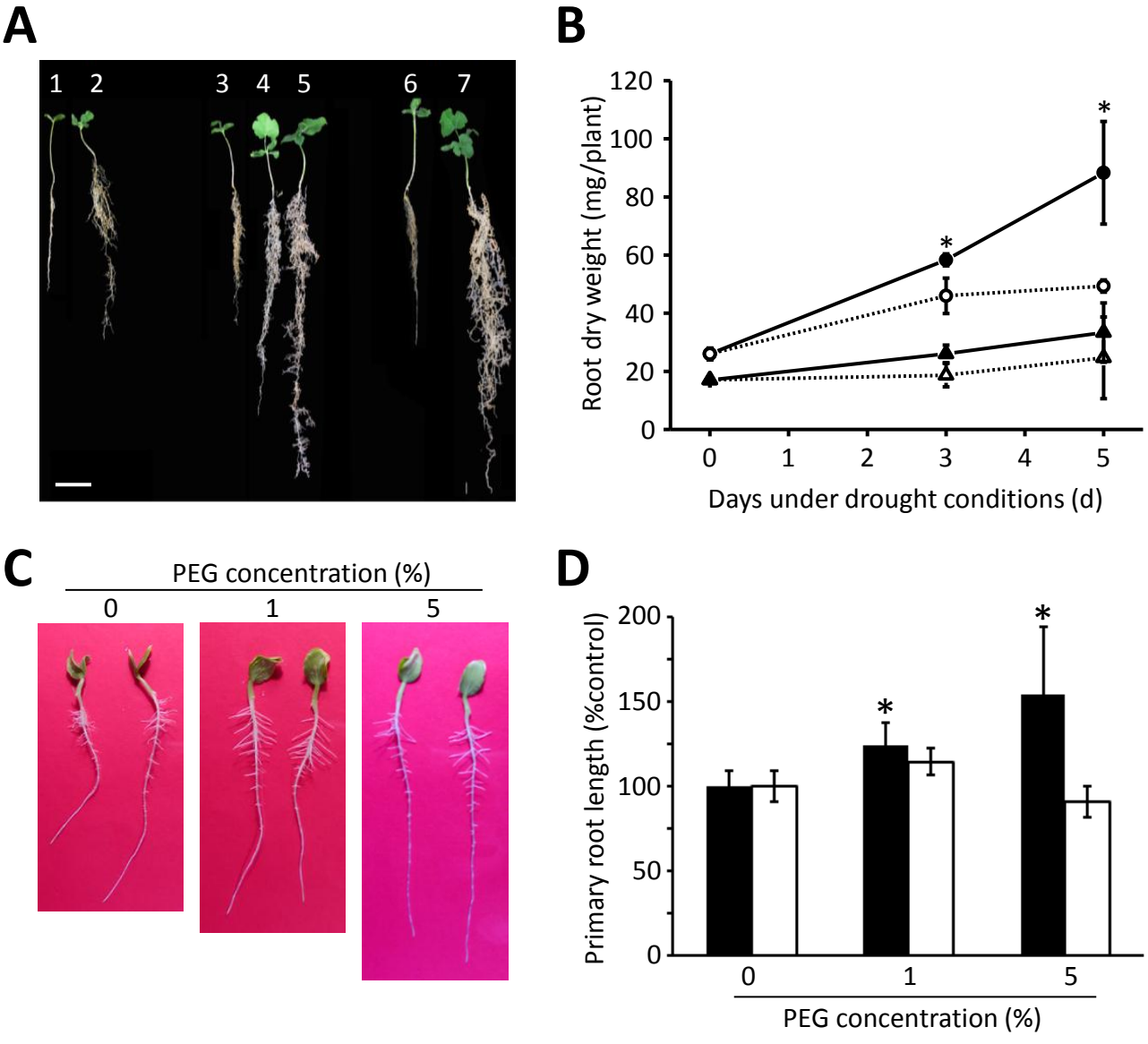

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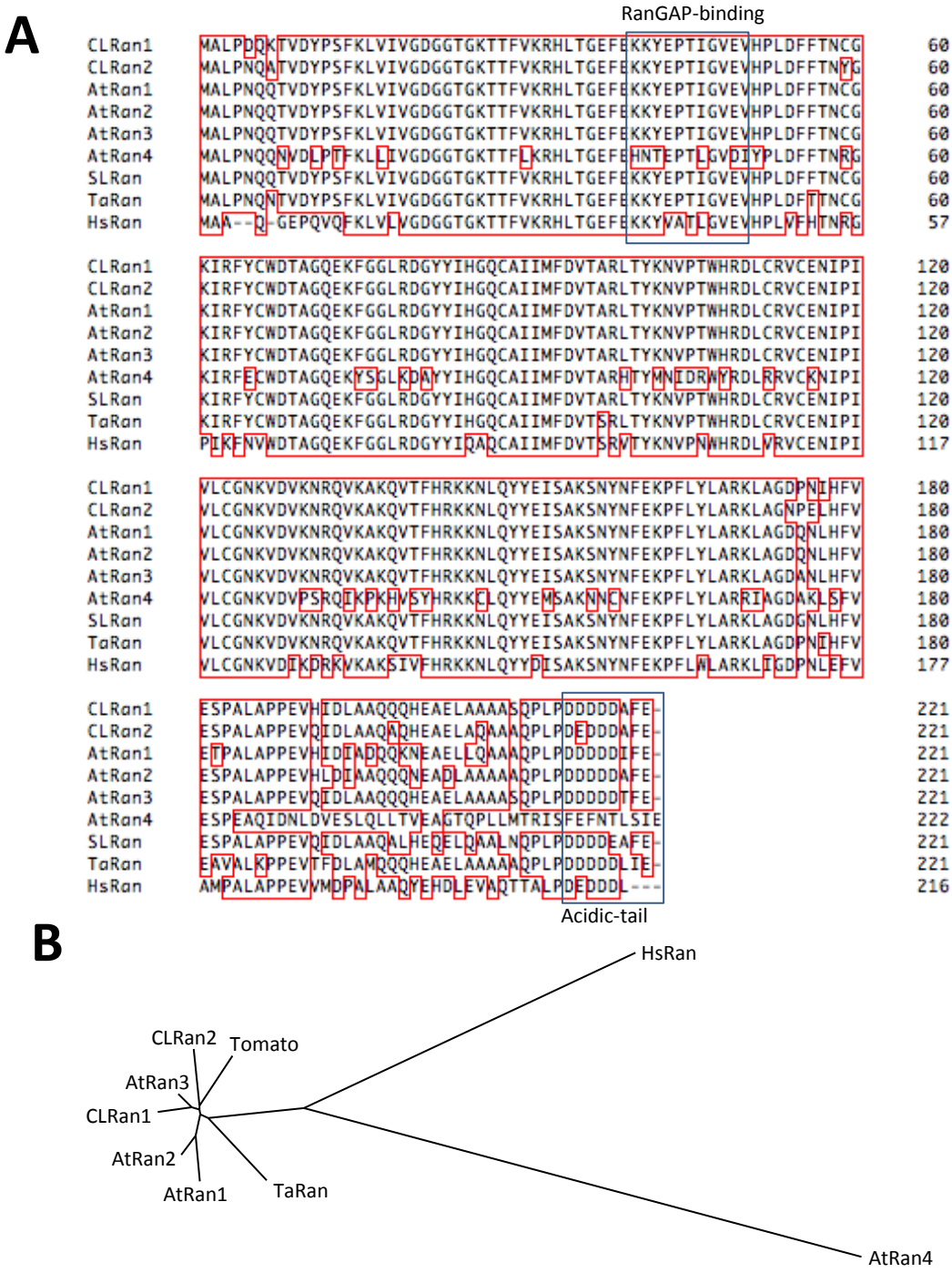

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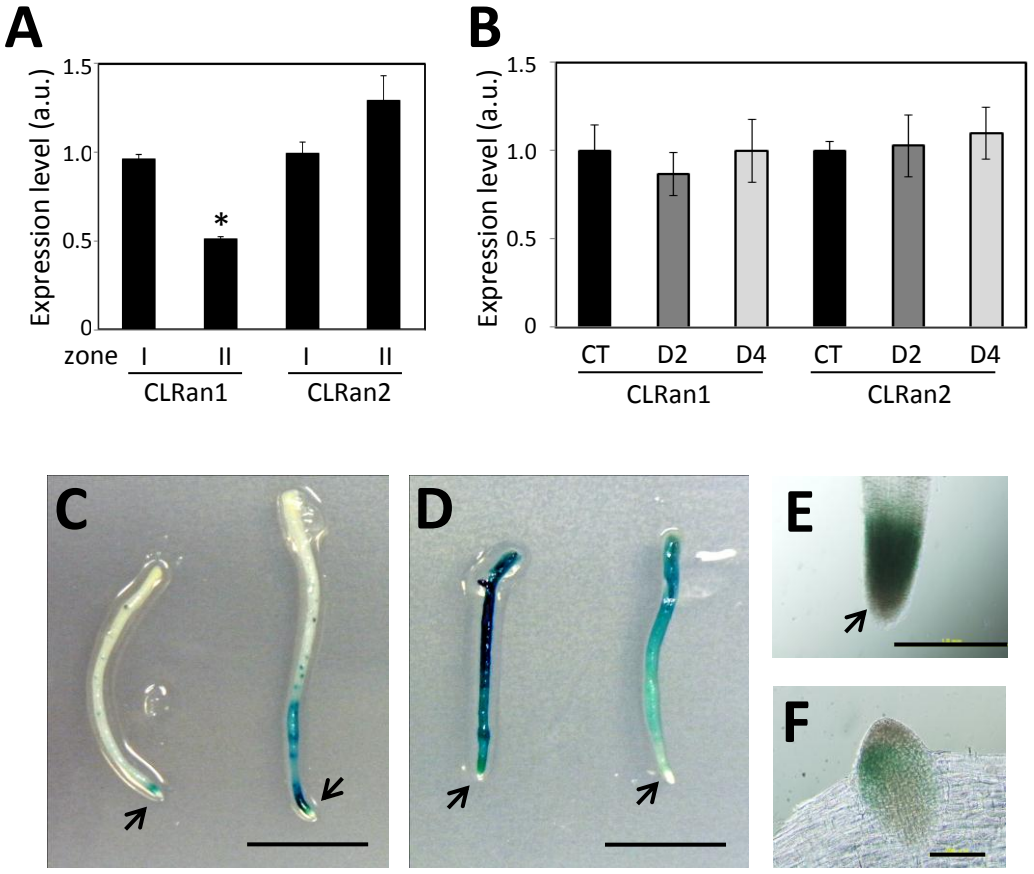

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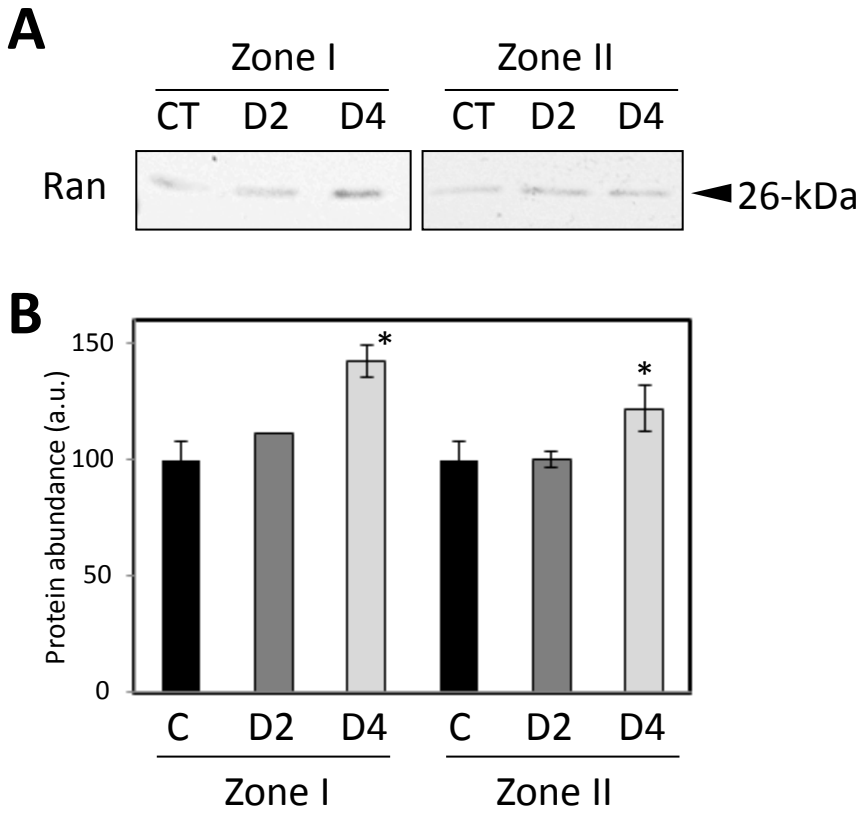

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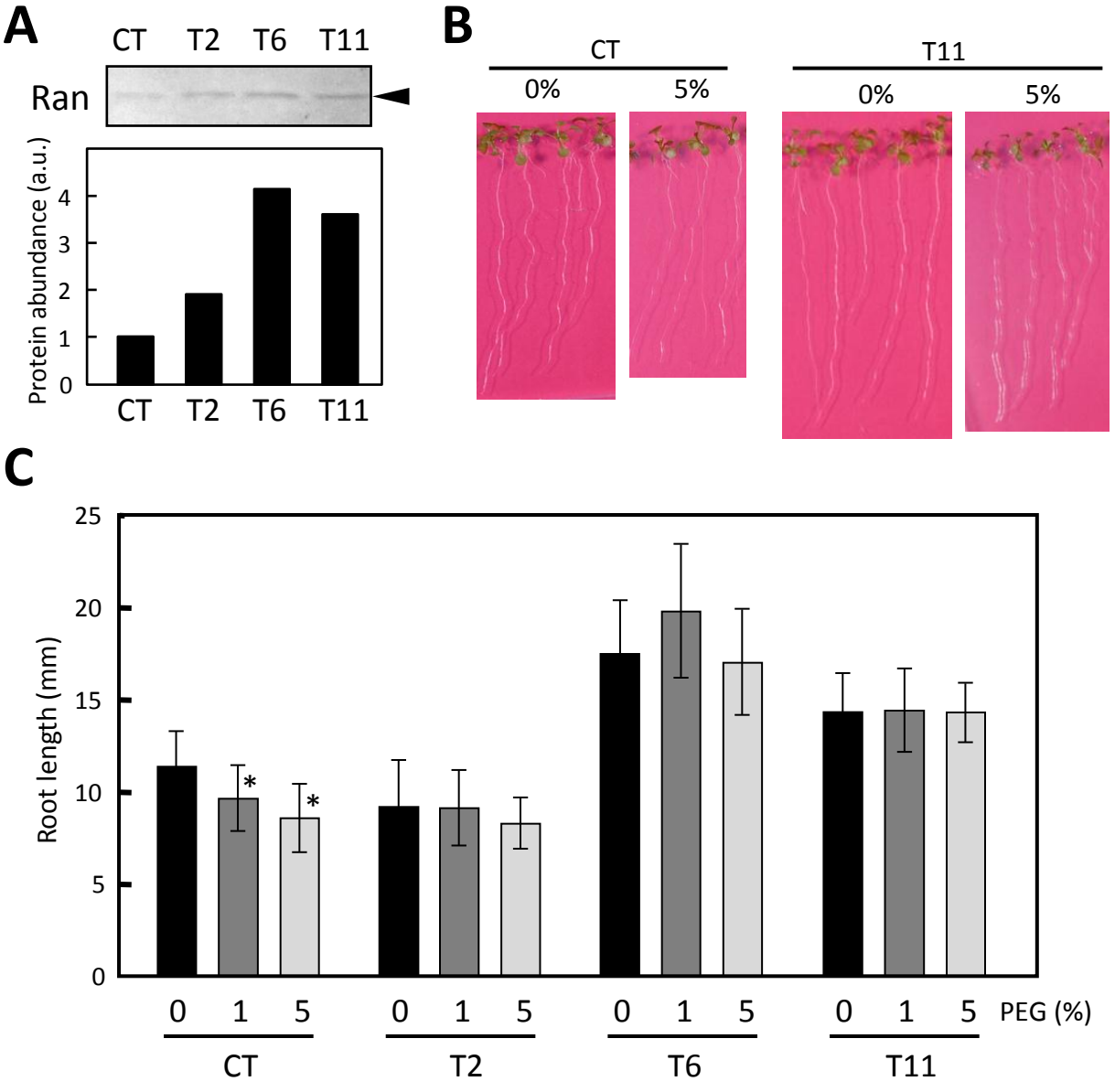

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