**RAN1** is involved in plant cold resistance and development in rice (*Oryza sativa*)

Peipei Xu and Weiming Cai*

Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Graduate School of Chinese Academy of Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China

* To whom correspondence should be addressed. E-mail: wmcai@sibs.ac.cn

Received 31 January 2014; Revised 20 March 2014; Accepted 21 March 2014

Abstract

Of the diverse abiotic stresses, low temperature is one of the major limiting factors that lead to a series of morphological, physiological, biochemical, and molecular changes in plants. Ran, an evolutionarily conserved small G-protein family, has been shown to be essential for the nuclear translocation of proteins. It also mediates the regulation of cell cycle progression in mammalian cells. However, little is known about Ran function in rice (*Oryza sativa*). We report here that Ran gene *OsRAN1* is essential for the molecular improvement of rice for cold tolerance. Ran also affects plant morphogenesis in transgenic *Arabidopsis thaliana*. *OsRAN1* is ubiquitously expressed in rice tissues with the highest expression in the spike. The levels of mRNA encoding *OsRAN1* were greatly increased by cold and indoleacetic acid treatment rather than by addition of salt and polyethylene glycol. Further, *OsRAN1* overexpression in *Arabidopsis* increased tiller number, and altered root development. *OsRAN1* overexpression in rice improves cold tolerance. The levels of cellular free Pro and sugar levels were highly increased in transgenic plants under cold stress. Under cold stress, *OsRAN1* maintained cell division and cell cycle progression, and also promoted the formation of an intact nuclear envelope. The results suggest that *OsRAN1* protein plays an important role in the regulation of cellular mitosis and the auxin signalling pathway.

Key words: Cold tolerance, cell division, *OsRAN1*, reduced apical dominance, intact nuclear envelope.

Introduction

Ran is a small GTPase that is essential for nuclear transport, nuclear assembly, mRNA processing, and cell cycle control, and is also the only known member of the family of small GTP-binding proteins primarily localized inside the nucleus (Ciciarello et al., 2007; Di Fiore et al., 2004). Ran can switch between a GDP- and GTP-bound state, and the transition from RanGDP to RanGTP can occur by nucleotide exchange. Although the intrinsic rate of nucleotide exchange and hydrolysis of Ran is slow, it is stimulated by the regulator of chromosome condensation 1 (RCC1) and the GTPase-activating protein (RanGAP1) (Gorlich and Kutay, 1999; Sazer and Dasso, 2000). As RanGAP and RanBP1 (Ran binding protein 1) are excluded from the nucleus, they cooperate in the cytoplasm to deplete RanGTP (Bischoff and Ponstingl, 1991; Bischoff and Ponstingl, 1995; Ramdas et al., 1991). In plants, the identification of RanBP1 and RanGAP enabled the study of Ran function (Ach and Gruissem, 1994; Rose and Meier, 2001). Owing to the high similarity in amino acid sequence and subcellular localization, plant Ran proteins are probably highly conserved with their mammalian and yeast counterparts in nucleo-cytoplasmic trafficking and mitotic processes (Wang et al., 2006).

Ran is encoded by a family of four genes in *Arabidopsis* and three genes in rice (Haizel et al., 1997; Vernoud et al., 2003). The temperature-sensitive mutants of *Pim1* (pre-mature initiation of mitosis 1) in fission yeast enter mitosis without completing chromosomal DNA replication, and overexpression of yeast Ran GTPase homologue *Spil* suppresses the *pim1-46* mutant phenotype (Matsumoto and Beach, 1991). Wheat *RAN1* is involved in regulation of cell
division and alters primordial meristem, mitotic progress, and sensitivity to auxin in rice and Arabidopsis (Wang et al., 2006). Ran GTPase may be involved in the plant response to hormone or environment signalling. Decreased ATP levels induced by oxidative stress lead to decrease in Ran–GTP levels and disordered Ran distribution (Yasuda et al., 2006). The expression of OsRAN1 is induced by jasmonic acid in rice (Miche et al., 2006); overexpression of OsRAN2 affects the sensitivity to salt stress in rice (Zang et al., 2010). OsRAN2 also plays an important role in cold tolerance (Chen et al., 2011). Therefore, Ran protein not only plays an important role in plant development but also mediates plant response to the environment.

Rice is a cold-sensitive plant that has its origin in tropical or sub-tropical areas. Unpredictable cold snaps at the booting stage delay heading and result in pollen sterility owing to the failure of microspore development under low-temperature conditions, which was thought to be one of the key factors responsible for reduced grain yield (Imin et al., 2004). Screening for genes involved in cold tolerance is an important initial step for crop improvement strategy using genetic engineering (Andaya and Tai, 2006; Dai et al., 2004). Maintenance of cell division is essential for plant survival and growth during cold stress. However, the cell cycle-associated cold response requires further research. In this study, we explored the role of OsRAN1 in the cell cycle and cold tolerance regulation in rice. We also observed the nuclear envelope integrity during cold stress. Our results suggest that plant Ran GTPase may have an important and conserved role in cold stress signalling in plants.

Materials and methods

Plant materials

Rice plants (Oryza sativa L. ssp. japonica) were germinated and grown in a Hoagland nutrient solution and soil substrate at a photoperiod of 12/12 h day/night cycle at 28 °C in a phytootron. Arabidopsis (ecotype Columbia) and tobacco (Nicotiana tabacum L. cv. Gexin No.1) were grown under long-day conditions (16/8 h light/dark cycle) with a fluence rate of 120 μmol m−2 s−1, 60–80% relative humidity, 28 °C/25 °C. To search the expression pattern of OsRAN1, one-week-old transgenic rice seedlings were germinated in water (as control) or 1 μmol l−1 propidium iodide solution and observed under a confocal laser scanning microscope (Zeiss LSM510; Jena, Germany). In addition, the GFP signal was also observed in roots of 1-week-old transgenic Arabidopsis seedlings.

Plasmid constructions and generation of transgenic plants

The full-length cDNA of OsRAN1 was amplified from rice plant Zhonghua11 (Oryza sativa L. ssp. japonica) according to GenBank accession No. AB015971. The modified green fluorescent protein (GFP) gene was amplified using the pCMBIA1302 vector. To construct the plasmid for gene overexpression, OsRAN1 or OsRAN1:GFP were cloned into a pHB vector (Mao et al., 2005) to generate double 35S:OsRAN1 and 35S:OsRAN1:GFP transgens. OsRAN2 was inserted using the floral-dipping method (Clough and Bent, 1998). Japonica rice cv. Zhonghua11 plants were transformed with the double 35S:OsRAN1:GFP using an Agrobacterium-mediated transformation as described (Hiei et al., 1994). Hygromycin (Roche)-resistance was used to screen positive transgenic plants. Genomic PCR was used to confirm the transgenic plants with specific primers for the hygromycin phosphotransferase (HPT) gene. Semi-quantitative RT-PCR and qPCR were conducted to detect gene expression level in transgenic Arabidopsis and rice plants. Primers used for plasmid constructions are shown in Supplementary Table S1 available at JXB online.

Subcellular localization study

Tobacco epidermal cells were injected with the construct of double 35S:OsRAN1:GFP construct and analysed by confocal microscopy (Zeiss LSM510; Jena, Germany). In addition, the GFP signal was also observed in roots of 1-week-old transgenic Arabidopsis seedlings.

RT-PCR and real-time PCR

The synthesis of cDNA using ReverTra Ace qPCR Master Kit (FSQ-201) was performed as described previously (Zang et al., 2010). An aliquot of 2 μl of tenfold-diluted cDNA was used as an RT-PCR template in a 20 μl reaction system. All PCR products were loaded onto a 1% agarose gel to visualize the amplified cDNAs. RT-PCR was repeated three times. OsUbiquitin was used as a control for 25 cycles. Fluorescence intensity of DNA bands was quantified using Bio-Rad’s ChemiDoc MP System. The relative expression level of OsRAN1 before treatment was set at 1 in different stress condition. For real-time PCR, the cDNA samples were diluted to 2ng μl−1. Triplicate quantitative assays were performed with 1 μl of cDNA dilution with the SYBR GreenMaster mix and an ABI fast sequence detection system according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). The relative quantification method (Delta-Delta CT) was used to evaluate quantitative variation. The amplification of actin2 was used as an internal control to normalize all data. The primers for gene expression are listed in Supplementary Table S2 available at JXB online.

Stress treatment of rice seedlings

Homologous T2 transgenic rice (OsRAN1 overexpression) seeds were used for the stress tolerance assay. All seeds were germinated in a mixture of nutrient soil and vermiculite (2:1). Transgenic and wild-type seedlings were grown under 12 h light/12 h dark (28 °C/25 °C). To search the expression pattern of OsRAN1, one-week-old transgenic rice seeds were germinated in water (as control) or in water containing 150 mM NaCl, 10% PEG 6000 (polyethylene glycol, average Mn 6000), or 1 mM indoleacetic acid (IAA). For cold treatment, two-week-old seedlings at the trefoil stage were treated at 4 °C for 84 h under 12 h light/12 h dark. After treatment, the seedlings were moved to a greenhouse for recovery in 2 weeks. Photographs were taken at the indicated times.

Imaging of root cell size

To examine cell arrangement and size, root tips were stained with 100 μg μl−1 propidium iodide solution and observed under a confocal laser scanning microscope (Zeiss) with an argon laser.

Measurement of Pro and soluble sugar contents

Transgenic plants at the four-leaf stage were used for biochemical analysis. Free Pro content in leaves was determined by established techniques (Troll and Lindsey, 1955). About 50mg of leaves were homogenized in 10ml of sulfosalicylic acid (3%) and the homogeneous mixture was centrifuged at 13 000rpm for 15min at 4 °C. The extract (2ml) was transformed to a microcentrifuge and mixed with 2ml of acid ninhydrin (0.1g ninhydrin dissolved in 24ml of glacial acetic acid and 16ml of 6-motho-phosphoric acid) and 2ml of acetic acid and 16 ml of 6-mortho-phosphoric acid) and 2 ml of acetic acid and 16 ml of 6-mortho-phosphoric acid for analysis.
acid. The reaction mixture was boiled in a water bath at 100 °C for 30 min and cooled down at 4 °C for 30 min. This was followed by addition of 4 ml of toluene to the leaf extract, which was then thoroughly mixed. Finally, 1.2 ml of the toluene phase was removed for absorbance measurement at 520 nm in a UV 2800 spectrophotometer (Unicron). Total soluble sugars in leaves were determined by the modified phenolsulfuric acid method (Dubois et al., 1951). About 0.1 g of leaves were homogenized in 8 ml of double-distilled water, and boiled twice in a water bath at 100 °C for 30 min. The extract (about 5 ml) was transferred to a new microcentrifuge, mixed with 1.5 ml of double-distilled water, 1 ml of 9% (v/v) phenol, and 5 ml of sulfuric acid added, and kept at room temperature for 30 min. The absorbance was measured at 485 nm in a UV 2800 spectrophotometer (Unicron).

Flow cytometry of cell cycle progression
T2 generation seeds of OsRAN1 transgenic rice were sterilized with 0.15% mercuric chloride and germinated on the filter with sterilized water at 28 °C in the dark for 7 d. All of the seedlings of wild-type or OsRAN1 transgenic rice were assigned in equal quantity and subjected to 28 °C or 4 °C for 12 h. Samples of cell nuclei were prepared as described by (Galbraith et al., 1999). Root apical tips (3 mm) were excised, immediately chilled on ice, and chopped with a single-edged razor blade in a glass petri dish (diameter, 10 cm). Chopping buffer (45 mM MgCl2, 30 mM sodium citrate, 20 mM 4-morpholine propane sulfonate, and 1 mg μl-1 Triton X-100, pH 7.0) was used to release the cells from the chopped tissues. The DNA content of individual transgenic cells was determined by flow cytometry. Cell nuclei were stained with 2 μg μl-1 2-(4-aminidophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Ma et al., 2009). Each sample was prepared three times and subjected to BECKMAN COULTER MoFlr-XDP three times. A total of 10 000 nuclei were designed to be measured per analysis.

Nuclear envelope observation
The transgenic and wild-type seeds were germinated for 7 d and then treated for 0 and 3 h at 4 °C. The root tips (2–3 mm) were fixed for 5–6 h in the fixation buffer (3% glutaraldehyde in 0.1 m PBS, pH 7.2). The materials were washed three or four times with 0.1 m PBS, and fixed in 1% osmic acid under 4 °C overnight. The materials were washed three or four times with 0.1 m PBS, dehydrated with an ethanol series of 30, 50, and 70% (4 °C, overnight), and then 80, 90, 95, 100–100% (30 min for every concentration). Ethanol was replaced with acetone (1:1) and infiltrated with acetone and a mixture of resin:acetone:resin=2:1; 1:1; 1:2, for 3 h and 100% resin for 12 h. The materials were embedded, and polymerized under 60 °C for 24 h. Root tips were cut into 50–70 nm using an ultramicrotome, the nuclear envelope (NE) was observed by transmission electron microscopy (HITACHI H-7650, Japan).

Analyses of auxin effects
Col-0 Arabidopsis seeds were surface sterilized in 75% ethanol for 1 min, followed by 50% (v/v) NaClO solution for 8 min, and rinsed in sterile water. Seeds were then placed on plates and vernalized for 72 h to synchronize germination. After vernalization, all plates were placed in the same growth chamber and allowed to grow for 10 d to determine the effect of various concentrations of auxin (IAA) on root length and lateral root production (Kim et al., 2001)

Results
Identification and characterization of the OsRAN1 gene
The OsRAN1 gene was cloned using primers designed according to Accession no. AB015971. OsRAN1 cDNA was isolated as a full-length coding region and encoding the predicted protein of 221 amino acids. A nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov) was performed against the Oryza sativa genome matching the isolated gene to the Oso1g0611100 gene. The predicted protein sequence of OsRAN1 was aligned with related sequences from Arabidopsis (AtRan1, AtRan2, AtRan3, and AtRan4), wheat (OsRAN1), human (Ran/TC4), mouse (MusRan), Zea mays (ZmRan), and rice (OsRAN2, OsRAN3) (Fig. 1A). The alignment showed 87% sequence homology between OsRAN1 and its human counterpart, and 95% homology between OsRAN1 and OsRAN2 at the amino acid level. The characteristic domains of the Ran proteins, which are known to be involved in GTP-binding and hydrolysis, in addition to the acidic C-terminal domain and the effector-binding domain, are highly conserved in most Ran proteins of various organisms (Ma et al., 2009).

The expression pattern of OsRAN1 in leaves, root, stem, sheath, and panicle was investigated using quantitative real-time PCR. The expression level was higher in panicle than in other organs examined (Fig.1B). The subcellular localization of OsRAN1:GFP was traced to root cells of transgenic Arabidopsis steadily overexpressing OsRAN1 and tobacco epidermal cells expressing transiently. The green fluorescent signal of OsRAN1:GFP was detected mainly within the nucleus, with some signals in the hypocotyl cytoplasm (Fig. 1C), whereas the green fluorescent signal was randomly distributed in the cell under the GFP vector control. These
findings were consistent with mammalian counterparts, which indicates that Ran is GDP-bound in the cytoplasm during interphase, and GTP-bound in the nucleus (Quimby and Dasso, 2003). In addition, this localization pattern was also observed through transient expression of OsRAN1:GFP in tobacco epidermal cells with identical results (Fig. 1D).

Expression pattern of OsRAN1 in response to cold, salt, polyethylene glycol, and indoleacetic acid treatment

We investigated the effects of abiotic stress on OsRAN1 expression using semi-quantitative RT-PCR to monitor the expression pattern of OsRAN1 in response to different stresses. The transcript level of OsRAN1 began to increase after 18 h of cold treatment and further accumulated up to the peak level at 48 h (Fig. 2A, B). Saline stress was induced with NaCl (150 mM) (Verslues et al., 2006) increasing the expression of OsRAN1 after 6 h and only slightly thereafter (Fig. 2C, D). We then investigated the effect of osmotic stress on the expression of OsRAN1, using 10% polyethylene glycol (Mn 6000; PEG 6000) to mimic osmotic stress. The expression of OsRAN1 was nearly 2-fold higher than that in the control 48 h after treatment, when it decreased again to normal level (Fig. 2E, F). Approximately 1 μM indoleacetic acid (IAA) was used for 84 h, with OsRAN1 levels starting to increase after 24 h of treatment and peaking at 72 h, with a 5-fold increase (Fig. 2G, H). In conclusion, the data suggest that OsRAN1 predominantly responds to low temperature and IAA treatment compared with salt and drought stress.

Overexpression of OsRAN1 increased tiller number and later flowering, and reduced apical dominance and abnormal roots in transgenic Arabidopsis

To analyse the roles of OsRAN1 in plants further, OsRAN1 was overexpressed in rice and Arabidopsis under the control of the double constitutive cauliflower mosaic virus (CaMV) 35S promoter. Stable inherited homozygous transgenic lines were obtained at the T2 generation. In addition, semi-quantitative reverse transcription (RT-PCR) and real-time quantitative PCR (qPCR) were conducted to examine exogenous OsRAN1 expression in the transgenic Arabidopsis and rice lines. As expected, it was found that OsRAN1 was overexpressed in both transgenic Arabidopsis and rice plants (Fig. 6C and Supplementary Fig. S1 available at JXB online). Furthermore, we observed the development of the phenotypes. We obtained 25 highly overexpressed lines from 32 independent transgenic lines (Supplementary Fig. S1 available at JXB online). Compared with the wild type, all the

**Fig. 2.** Semi-quantitative RT-PCR analysis of OsRAN1 expression in stress response. (A, B) Time course of OsRAN1 expression during cold treatment (4°C). (C, D) Time course of OsRAN1 expression during treatment with 150 mM NaCl. (E, F) Time course of OsRAN1 expression during treatment with 10% PEG 6000, a mimic for drought stress. (G, H) Time course of OsRAN1 expression during treatment with 1 μM IAA. The rice seedlings were germinated and grew for 10 d before they were treated with cold, salt, drought, and IAA stresses. OsUbiquitin was used as an internal control.
highly overexpressed transgenic *Arabidopsis* showed distinct phenotypes, such as excess rosette leaves, increased tiller number, longer hypocotyl in the white light, weak apical dominance, and abnormal root development (Fig. 3A–J). The flowers emerged about 4 d later in *OsRAN1* transgenic plants on long days (Fig. 3K). *Arabidopsis* plants overexpressing *OsRAN1* were shorter, with more lateral floral branches, and partly abortive flowers compared with wild-type plants (Fig. 3C–H). Overall, the apical dominance of transgenic *Arabidopsis* was reduced. Other distinct phenotypes among *OsRAN1* transgenic *Arabidopsis* seedlings related to root development (Fig. 3L–P). Many lines of transgenic seedlings showed a similar phenotype, such as greatly reduced number of lateral roots and stunted primary roots. The number of lateral roots was only 4.4 per plant on average in the transgenic seedlings. In contrast, the wild type showed 10.1 per plant under the same condition. Exogenous applications of IAA addressed the phenotype of fewer lateral roots (Fig. 4). These results demonstrated that *OsRAN1* controls development of shoots and the roots probably by affecting IAA signalling in the transgenic *Arabidopsis*.

**Overexpression of OsRAN1 increased cell division in rice roots**

We further examined the phenotypes in the OsRAN1 overexpressed lines of rice. The transgenic rice plants showed a tiller number up to 9.3 per plant on average. In contrast, wild-type rice had fewer tillers, about 7.3 per plant (Table 1). The *OsRAN1* overexpressed rice plants were shorter with...
more tillering branches. These results suggest that OsRAN1 expression affected tiller initiation in the shoot meristem. Furthermore, the roots of transgenic rice contain many small and tightly arrayed cells emerging from the meristem (Fig. 5). This information, combined with the results of the increased 4C DNA in the OsRAN1 overexpression line (Fig. 8A–D), indicates that OsRAN1 is involved in meristem cell proliferation in the root. OsRAN1 probably regulates cell division, particularly in the root tip meristem.

Overexpression of OsRAN1 increased cold tolerance in transgenic rice and Arabidopsis

To test the possible role of OsRAN1 overexpression in cold tolerance of rice, the seedlings of T2 transgenic lines and wild type at trefoil stage were exposed to cold stress at 4 °C for 84 h. The plants were then removed to the greenhouse to recover at 28 °C. After 14 d recovery under normal conditions, the survival rates of three transgenic lines were between 71% and 78%, whereas the survival rate for the wild type was only 26% (Fig. 6). We further examined the freezing tolerance of Arabidopsis overexpressing OsRAN1. The results showed that transgenic lines 3, 5, and 15 have higher freezing tolerance after 3 d cold acclimation. The survival rate of these three lines after 2 weeks of recovery was 52%, 68%, and 60%, whereas the survival rate of the wild type was 32% (Supplementary Fig. S2 available at JXB online). Therefore, we concluded that OsRAN1 overexpression increases the freezing tolerance of transgenic rice and Arabidopsis.

Increased Pro and soluble sugar contents in OsRAN1 transgenic plants under cold stress

Plant adaptation to environmental stresses is often associated with metabolic adjustment, such as accumulation of Pro and soluble sugars (Abraham et al., 2003). To investigate the physiological basis for the improved stress tolerance in transgenic rice, we measured the Pro and soluble sugar content in plants that overexpressed the OsRAN1 gene and the wild type under normal growth and stress conditions. Under normal growth conditions (28 °C), the levels of cellular free Pro did
OsRAN1 maintains cell proliferation under cold stress

not differ between wild-type and transgenic rice, at quantities ranging between 92 and 98 mg fresh weight (Fig. 7A). In contrast, after cold treatment (4 °C), the levels of free Pro in OsRAN1 transgenic rice increased substantially, with more than 178 mg g⁻¹ (207 mg g⁻¹, 198 mg g⁻¹) fresh weight compared with 146 mg g⁻¹ fresh weight in the wild-type plants. The levels of free sugar in OsRAN1 transgenic rice increased to 1.1 mg g⁻¹ (1.6 mg g⁻¹, 1.4 mg g⁻¹) fresh weight compared with 0.85 mg g⁻¹ fresh weight in the wild-type plants after cold treatment, although the levels of cell-free sugar did not differ between the two types of rice (Fig. 7B).

We further measured the expression levels of two putative carboxylate synthetase genes (AK102633 and AK101230) and two putative Pro transporter genes (AK067118 and AK0666298) in the OsRAN1 transgenic plants subjected to cold stress. As shown in Figure 7C, AK067118, AK0666298, and AK101230 had higher expression level (3- to 5-fold) in the OsRAN1 overexpressed lines than in the wild type after cold treatment. We also measured the expression level of putative sugar synthetase relative genes Os01g0205700 and OsSPS1 and two putative sugar transporter genes (Os08g0178200 and Os12g0641400) in the OsRAN1 transgenic plants under cold stress. Genes in the OsRAN1 overexpressed lines had higher expression level (2-fold–4.5-fold) than the wild type (Fig. 7D).

Cell cycle progression in transgenic rice lines

We monitored the mitotic index of AtRAN1-overexpressed lines under cold conditions. Mitotic index is defined as the ratio between the number of cells in mitosis and the total number of cells, which is used as a measure for the proliferation status of a cell population. Flow cytometry showed that the 4C DNA content of OsRAN1-overexpressed lines increased in comparison with the wild type under normal (28 °C) and cold (4 °C) conditions (Fig. 8A–D). Under the normal conditions, the index for the transgenic lines was 61–78% compared with that of the wild-type 46%. Even at 4 °C, the index for the transgenic lines was 21–31% compared with that of the wild type 14% (Fig. 8E), although both the transgenic lines and the wild type showed a significantly decreased index at this temperature. Thus, the overexpressing lines possessed more cells in the proliferation, especially under the cold condition. Therefore, we conclude that OsRAN1 overexpression increased mitosis.

Overexpression of OsRAN1 promoted the formation of intact nuclear envelope under cold stress

Next, we observed the nuclear envelope (NE) of root tip cells of the overexpressed transgenic line 47 and wild-type rice under...
normal and cold conditions. Under the normal condition (28 °C), NEs of the transgenic line and wild type were all intact with no obvious difference in morphology (Fig. 9A, B). After treatment at 4 °C for 4 h, about 70% cells in wild type showed obscured or partly dissociated double membranes (Fig. 9E, F). However, most cells in the overexpressed lines formed an intact NE under cold stress (Fig. 9C, D), with cells showing partially dissociated NE. The results suggested that overexpression of OsRAN1 may promote the formation of an intact NE under cold stress.

Discussion

OsRAN1 in cell cycle regulation

Several studies indicate that the small GTPase called Ras-related nuclear protein (Ran) is a central regulator of several cell cycle events. In the 1990s, scientists first discovered that Ran was an essential factor for protein import into the nucleus during interphase. In subsequent studies, they discovered that Ran played a role in spindle assembly and nuclear envelope formation during mitosis. Ran seems to modulate these vastly different cellular processes through a gradient mechanism, wherein active Ran is more concentrated around its primary site of action and less concentrated elsewhere (Clarke and Zhang, 2001). Overexpression of various wild-type Ran homologues from plants, including tomato and tobacco Ran, suppressed the pim1 mutant phenotypes in yeast (Lee et al., 1993; Merkle et al., 1994), which suggests that the role of Ran GTPase may be conserved in cell cycle regulation. The precise details associated with specific processes during cell cycle progression have yet to be delineated (Rossi and Varotto, 2002). Plant meristematic cells do not undergo cellular expansion and are relatively uniform in size. The size of the meristematic cells of the primary root tip was reduced on average in our OsRAN1-overexpressed plants. The root-tip meristem of OsRAN1-overexpressed plants contained smaller cells than those in the wild type, thus increasing the total number of meristematic cells. In brief, our study reveals that OsRAN1 was involved in cell proliferation in the root. OsRAN1 overexpression enhanced the root tip mitotic index of transgenic rice (Fig. 8E), further increasing the cold tolerance. Previous research demonstrated that OsRAN1 and TaRAN1, sharing high identity at the amino acid level, were also increased in cold tolerance in transgenic rice plants (Chen et al., 2011). Another study conducted by our laboratory in Arabidopsis showed that atran1 atran3 double mutants have increased freezing sensitivity, whereas the atran1 or atran3 signal mutant in Arabidopsis did not have increased freezing sensitivity (data not shown). These results indicate that Ran GTPase regulates cold stress redundantly. Thus, Ran homologues may have well-conserved functionality in plants, yeast, and animals during evolution.
Functional analysis of OsRAN1 suggests a role in auxin signal transduction; exogenous application of IAA partly repairs the transgenic root defects

Response to IAA in the lateral root initiation involves multiple root and shoot phenotypes commonly associated with auxin mutants (Berleth et al., 2000). We tested whether OsRAN1 transgenic Arabidopsis root development defects were affected by auxin by supplementing the growth medium with exogenous IAA. The results indicate that IAA typically promotes the lateral root initiation but have no obvious effects
on primary root length in transgenic Arabidopsis (Fig. 4). In the auxin-signalling pathways, suppressors of auxin action block the expression of auxin-induced genes in the nucleus (Ulmasov et al., 1999). Ran proteins play an important role in nuclear transport. Overexpression of OsRAN1 protein might result in an abnormal or reduced rate of transport of important protein modulators to the nucleus. The OsRAN1-overexpressed transgenic plants are partly recovered by auxin, supporting the hypothesis that Ran was regulated by auxin and was involved in the auxin-mediated signalling pathway.

OsRAN1 enhanced cold tolerance by maintaining cell division and regulating the intact NE under cold stress in rice

Studies have shown that cell division is closely related to stress tolerance in plants. Arabidopsis plants constitutively overexpressing HAL3a (Halotolerance gene 3a) showed improved growth, as well as salt and osmotic tolerance in Arabidopsis and rice (Espinosa-Ruiz et al., 1999; Rubio et al., 2006). Transgenic rice lines overexpressing OsMYB3R-2 (R1R2R3 MYB gene) and OsCycB1;1 (Cyclin B gene 1:1) exhibited enhanced cold tolerance, which indicates that ongoing mitosis enhances cold resistance in plants (Ma et al., 2009). Our results indicate that the expression of OsRAN1 was up-regulated under cold stress (Fig. 2). Also, cellular Pro and free sugar level are involved in OsRAN1 transgenic rice cold tolerance. These results were similar to the alterations observed in other transgenic rice overexpressing cold resistance genes, such as OsNAC6, OsCIPK03, and OsCIPK12 (Nakashima et al., 2006; Xiang et al., 2007). OsRAN1 overexpression could maintain a high mean root mitotic index under cold stress and cell cycle progression (Fig. 8), suggesting that OsRAN1 functions as a regulator in the cold signalling pathway in rice.

Ran plays a pivotal role in the regulation of the nuclear envelope assembly under the cooperation of its binding proteins RanGAP1, RCC1, importins, etc. LBR (lamin B receptor), a chromatin and lamin B binding protein in the inner nuclear membrane, targets the membrane precursor vesicles to the chromatin mediated by importin β during NE assembly. Ran GTPase also promotes nuclear pore organization at the end of mitosis in Xenopus egg extracts (Zhang et al., 2002). Despite the previous report of the function of plant Ran GTPase OsRAN2 in NE assembly, our NE observations indicated that OsRAN1 overexpression also promoted the formation of an intact NE under cold stress (Fig. 9). By combining our results with those of previous studies, we speculate that both OsRAN1 and OsRAN2 may be involved in the organization of normal NE structures at the end of mitosis during the cold condition. In conclusion, OsRAN1 overexpression could enhance cold tolerance by maintaining cell division ability and NE assembly under cold stress in rice.

Supplementary data

Supplementary data are available at JXB online

- **Table S1.** Primers used in plasmid constructions transgenes Primers.
- **Table S2.** Primers used in RT-PCR in detecting gene expression level in transgenic plants.
- **Table S3.** Gene-specific primers used in qPCR experiments.
- **Figure S1.** Transgenic OsRAN1 Arabidopsis expression pattern.
- **Figure S2.** Transgenic OsRAN1 Arabidopsis increased freezing tolerance after cold acclimation.

Acknowledgements

This work was supported by National Natural Science Foundation of China (31070237), the National Basic Research Program of China (2011CB710902), the National Scientific Program (2012AA101103-04), Strategic Priority Research Program of the Chinese Academy of Sciences (XDA04020202-15), and the China Manned Space Flight Technology Project.

References


Andaya VC, Tai TH. 2006. Fine mapping of the qCTS12 locus, a major QTL for seedling cold tolerance in rice. Theoretical and Applied Genetics 113, 467–475.


