Plant, Cell & Environment

Plant, Cell and Environment (2011) **34**, 52–64 doi: 10.1111/j.1365-3040.2010.02225.x

OsRAN2, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress

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ABSTRACT

With global climate change, abnormally low temperatures have affected the world's rice production. Many genes have been shown to be essential for molecular improvement of rice cold-tolerance traits. However, less is known about the molecular cellular mechanism of their response to cold stress. Here, we investigated OsRAN2 involved in regulation of cell division during cold stress in rice. Expression of *OsRAN2* was increased under cold treatment, but not during salt and drought stress. The mean root mitotic index was closely related to the expression level of *OsRAN2*. Knockdown transgenic rice lines showed an aberrant organization of spindles during mitosis and stunted growth during development. Overexpression of *OsRAN2* enhanced cold tolerance in rice. The transgenic rice overexpressing *OsRAN2* showed maintained cell division, decreased proportion of cells with intranuclear tubulin and formation of a normal nuclear envelope under the cold condition. Our study suggests a mechanism for OsRAN2 in regulating cold resistance in rice by maintaining cell division through promoting the normal export of intranuclear tubulin at the end of mitosis. This insight could help improve the cold-tolerance trait in rice.

Key-words: intact nuclear envelope; small GTPase.

INTRODUCTION

Rice, one of the world's most important crops, is normally grown in tropical and temperate climate zones. Low temperature is one of the major factors affecting rice development and yield (Kanada 1974; Andaya & Tai 2006; Suzuki, Nagasuga & Okada 2008). Rice is sensitive to even mild cold stress (chilling), especially at the early stages of seedling development (Cheng *et al*. 2007). Transgenic technique is an effective tool to enhance the cold stress tolerance.

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Cold tolerance is regulated by a series of genes in rice. Some of these are transcription factors such as *AP37*, *OsMYB3R-2*, *OsDREB1D*, *OsMYB4*, *OsiSAP1*, *OsiSAP8*, *OsCOIN* and *OsNAC6* (Mukhopadhyay, Vij & Tyagi 2004; Vannini *et al*. 2004; Liu *et al*. 2007; Nakashima *et al*. 2007; Kanneganti & Gupta 2008; Ma *et al*. 2009; Oh *et al*. 2009; Zhang *et al*. 2009). Protein kinases as signal transductors, such as *OsCIPK03*, *OsCIPK12*, *OsCIPK15*, *OsMEK* and *OsMAPK5*, also regulate the response to cold stress in rice (Wen, Oono & Imai 2002; Xiong & Yang 2003; Xiang, Huang & Xiong 2007). In addition, proteins responsible for compatible compound synthesis are involved in cold tolerance regulation in rice. These proteins include otsA and otsB, choline monooxygenase, and WFT1 and WFT2, which are responsible for the synthesis of trehalose, glycinebetaine and fructan, respectively (Garg *et al*. 2002; Shirasawa *et al*. 2006; Kawakami, Sato & Yoshida 2008).

Cell division plays an important role in plant growth and development. Our previous study suggests that the transcription factor OsMYB3R-2 targets and activates the expression of several G2/M-phase-specific genes such as cyclinB1:1 to increase the cell mitotic index and survival of rice plants under the cold condition (Ma *et al*. 2009). Therefore, maintenance of cell division should be essential for plants to survive and grow during cold stress. However, questions regarding the response to cold in the cell cycle remain. For example, the phase of cell division that is the most sensitive to cold stress is unknown.

The small GTPase superfamily is divided into the five families Ras, Rho, Rab, Arf and Ras-like nuclear GTPase (Ran) (Bischoff *et al*. 1999; Takai, Sasaki & Matozaki 2001). Ran is an abundant nuclear small GTPase (Drivas *et al*. 1990; Bischoff & Ponstingl 1991a). It cycles between GTP- and GDP-bound states. GTP hydrolysis occurs in the cytoplasm and is stimulated by RanGAP1 (Matunis, Coutavas & Blobel 1996), and exchange of GDP to GTP is catalysed by RCC1 in the nucleus (Bischoff & Ponstingl 1991a). This distribution ensures that Ran GTPases are bound with GTP in the nucleus and GDP in the cytoplasm, which is essential for their function. Evidence from yeast and animals has

shown that Ran controls several key cellular processes. Ran GTPase mediates the nuclear transport of RNA and proteins and guides spindle assembly at the onset of mitosis and nuclear envelope (NE) reassembly at the end of mitosis (Gorlich 1998; Hetzer *et al*. 2000; Zheng 2004; Ribbeck *et al*. 2006). Ran is encoded by a family of 4 genes in *Arabidopsis* (Haizel *et al*. 1997; Vernoud *et al*. 2003). The temperature-sensitive mutants of Pim1 were reported to enter mitosis without completing chromosomal DNA replication in Fission yeast, and overexpression of yeast Ran GTPase homolog Spi1 could suppress the *pim1-46* mutant phenotype (Matsumoto & Beach 1991). Similarly, overexpression of various plant Ran homologs, including those from tomato and tobacco, could also suppress *pim1-46* mutant phenotypes in yeast (Belhumeur *et al*. 1993; Ach & Gruissem 1994; Merkle *et al*. 1994). Wheat TaRAN1 is involved in regulation of cell division and alters primordial meristem and mitotic progress and sensitivity to auxin in rice and *Arabidopsis* (Wang *et al*. 2004a, 2006). Thus, RAN homologs are essential for the normal functioning of eukaryotic cells and have been well conserved in yeast, animals and plants.

Ran GTPase may be involved in the plant response to hormone or environment signalling. A decrease in ATP levels induced by oxidative stress causes a decrease in Ran-GTP levels and disordered Ran distribution (Yasuda *et al*. 2006). The expression of *OsRAN2* 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). RAN probably acts as a mediator of signalling molecules in gravitropism (Kriegs, Theisen & Schnabl 2006). However, little is known about how Ran GTPase works in regulating the cellular biological response of plants to cold stress.

In this study, we explored the function of OsRAN2 in the cell cycle and cold tolerance regulation in rice. We also observed the morphology of microtubules and NE integrity during the cold condition and analysed the possible mechanism of OsRAN2 in cold tolerance regulation. Our data suggest that plant Ran GTPase may have an important role in connecting cell division with cold stress signalling in rice.

MATERIALS AND METHODS

Plant materials

The plant material we used was rice (*Oryza Sativa* ssp. Japonica cv 'Zhonghua 10') and *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia). The transgenic lines overexpressing *TaRAN1* were described previously (Wang *et al*. 2006).

Plasmid construction and rice transformation

Total RNA of rice seedlings was isolated by use of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The cDNA of rice was synthesized by use of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in a 25 μL reaction system containing 2 μg total RNA. Reverse transcription reactions were carried out at 42 °C for 60 min followed by chilling on ice for 5 min.

Construction of pUN1301-*OsRAN2* for overexpression analysis: Full-length *OsRAN2* was amplified by RT-PCR with Pyrobest DNA Polymerase (TaKaRa, Kyoto, Japan), with the forward primer 5′-GGATCCTCGCGTCGCC GCCTTTT-3′ and reverse primer 5′-GGTACCCAGGCA CGCAGGAAACG-3′, then inserted into pGEM-T Easy vector (Promega) and sequenced. The *OsRAN2* fragment digested from pT Easy-*OsRAN2* was directionally cloned into the *Kpn*I-*BamH*I sites of a pUN1301 vector to obtain the pUN1301-*OsRAN2* construct. *OsRAN2* was driven by a ubiquitin promoter in the construct and a GUS marker was carried in the vector.

Construction of pTCK303-*OsRAN2* for RNAi analysis: The detailed protocols were as described (Wang *et al*. 2004b). The forward primer was 5′-GGGGTACCACTAG TCATGAGGCTGAGCTTGCG-3′ and the reverse primer 5′-CGGGATCCGAGCTCCACCGGATAAATAAATAC C-3′, with the digestion sites of *Kpn*I-*Spe*I and *BamH*I-*Sac*I, respectively.

The pUN1301-*OsRAN2* construct and pTCK303-*OsRAN2* construct were electroporated into *Agrobacterium tumefaciens* EHA105. Rice embryonic calli were induced and transfected with *A. tumefaciens* EHA105 containing the desired binary vector as described previously (Ge *et al*. 2004). *OsRAN2* transgenic plants were screened in half-strength Murashige and Skoog (MS) medium containing 75 mg L^{-1} hygromycin (Sigma, St Louis, MO, USA). Transgenic plants of the T0 generation were transplanted into soil and grown in the greenhouse.

RT-PCR and real-time PCR

The synthesis of cDNA was as described in the section 'Plasmid Construction and Rice Transformation'. An amount of 2 μL of fivefold-diluted cDNA was used as an RT-PCR template in a 20 μL reaction system. All PCR products were loaded onto a 0.8% agarose gel to visualize the amplified cDNAs. RT-PCR was repeated three times. Tubulin with the forward primer 5′-TCAGATGCCCAGTGACAGGA-3′ and the reverse primer 5′-TTGGTGATCTCGGCAACAGA-3′ was used as a control for 25 cycles. Fluorescence intensity of DNA bands were quantified using BIO-ID software after DNA bands were photographed by Vilber Lourmat UV light gel imaging system. The relative expression level of *OsRAN2* before treatment was set as 1 in different stress condition.

For real-time PCR, the cDNA samples were diluted to 2 and 8 ng μL^{-1}. Triplicate quantitative assays were performed with 1 μL of each cDNA dilution with the SYBR GreenMaster mix and an ABI 7900 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 actin was used as an internal control to normalize all data. Primers for *actin* were

forward 5′-GAACTGGTATGGTCAAGGCTG-3′ and reverse 5′-ACACGGAGCTCGTTGTAGAAG-3′; and *OsRAN2* forward 5′-ATTTCGTTGAAGCTGTTGC-3′ and reverse 5′-CAAGCTCAGCCTCATGCT-3′.

Southern blot assays

Genomic DNA was isolated from 2-week-old rice seedlings and digested with *Eco*RI or *Hind*III. DNA of 20 mg was used for Southern blot analysis. The digested DNA fragments underwent electrophoresis on a 0.7% agarose gel and were then blotted onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was prehybridized at 65 °C for 4 h and hybridized in the same solution containing [α-^{32}P]dCTP-labelled GUS for 20 h at 65 °C. The GUS of 680 bp was amplified by PCR with the forward primer 5′-CAACTGGACAAGGCACT AGC-3′ and reverse primer 5′-AGCGTCGCAGAACATT ACAT-3′. The membrane was washed with washing buffer [(2 × SSC plus 0.1% sodium dodecyl sulphate (SDS)] at 65 °C for 20 min after hybridization and then washed twice with 1 × SSC plus 0.1% SDS at 65 °C for 15 min. The membrane was stored at –70 °C for 3 to 7 d and then exposed to X-ray film.

Treatment of rice seedlings with cold stress

Transgenic plants of the T2 generation with positive GUS activity were used in this experiment. All seeds were germinated in a mixture of nutrimental soil and vermiculite (2:1). Transgenic and wild-type seedlings were grown under 12 h light/12 h dark (30 °C/25 °C). Two-week-old seedlings at the early period of the tetraphyllous leaf stage were treated at 4 °C for 72 h in a low-temperature Biochemical Incubator (BTI100; Lead Tech) under 12 h light/12 h dark. After treatment, the seedlings were moved to a greenhouse for recovery for 2 weeks.

Transformation of *OsRAN2* into *Arabidopsis*

The whole coding sequence was amplified with the primers for *OsRAN2*: 5′-CTCGAGATGGCGCTGCCG AATCAG-3′ with an *Xho*I digested site and 5′-GGTACCC TCGATCAGATCGTCATC-3′ with a *Kpn*I digested site. The PCR product was subcloned into the pBI121 vector to generate pBI121-OsRAN2-GFP containing an OsRAN2-GFP fusion protein under the control of a CaMV 35S promoter. The construct was electroporated into *A. tumefaciens* C58. *Arabidopsis* plants were transformed by the floral dip method (Clough & Bent 1998).

Colocalization analysis of OsRAN2 and tubulin in transgenic *Arabidopsis*

The construct pBI121-OsRAN2-GFP within a binary vector (pBI121) was used to transform *Arabidopsis*. The transgenic seeds were screened on MS medium containing kanamycin (50 μg mL^{-1}), and resistant seedlings were identified by green fluorescent protein (GFP) fluorescence with use of a fluorescence microscope for subsequent analysis.

Co-immunolocalization of OsRAN2 and tubulin in transgenic plants was as follows. Transgenic seedlings were double labelled. The primary polyclonal antibodies mouse anti-β-tubulin (Beyotime, Shanghai, China) and rabbit anti-GFP (CWBiotech, Beijing, China) were incubated together at 4 °C overnight, followed by 10 min washes with PBS for three times. The secondary antibodies CY3-conjugated goat anti-mouse IgG, shown in red, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (both Beyotime), shown in green, were added and incubated at 37 °C for 3 h. Fluorescence images were collected by use of a Zeiss LM 510 META (Zeiss, Berlin, Germany) confocal laser scanning microscope with a 100× oil-immersion objective.

Immunofluorescence analysis of rice root tips

The seeds of wild-type and transgenic rice plants overexpressing *OsRAN2* were generated and grown for 3 to 6 d at 28 °C. In each treatment, 10 root tips of 1 to 2 mm were fixed in 4% paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 0.1 mM EDTA, and 5 mM $MgCl_2$, pH 6.9) for 1 h, washed three times with PEM buffer for 10 min each, then digested with 1.5% cellulase and 1% pectolyase at room temperature for 50 min. After being washed once with PEM and twice with 1 × PBS, cells were fixed on coverslips with 1% polylysine and squashed between two coverslips with use of a pencil eraser, then stored at –20 °C for 5 min and at room temperature for 2 min. The coverslips were incubated with 1% Triton X-100 for 30 min, washed three times with 1 × PBS for 10 min each, then blocked with 1% BSA for 10 min. The solution was incubated with the primary antibody anti-β-tubulin at 4 °C overnight, then FITC-conjugated secondary antibody anti-mouse IgG at 37 °C for 3 h. The coverslips were then sealed with clear nail polish. Cells were viewed with use of a Zeiss microscope.

Mean root mitotic index was calculated by the following formula: number of cells in mitosis/total number of cells; proportion of cells with abnormal spindles: number of cells with aberrant spindles/number of cells with normal and abnormal spindles. The decreasing rate of proportion of cells with different microtubules under the low temperature condition was calculated by the following formula: percentage of cells with different microtubules under low temperature treatment/percentage of cells with different microtubules under normal condition (100%).

Observation of NE structure

The transgenic and wild-type seeds were germinated for 3 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, then fixed in 1% osmic acid under 4 °C overnight. The materials were

washed three or four times with 0.1 M PBS, then 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 resin: acetone : resin = 2:1; 1:1; 1:2, for 3 h and 100% resin for 12 h. The materials were embedded, then polymerized under 60 °C for 24 h. Root tips were cut into 50–70 nM with use of an ultramicrotome (Leica Ultracut R, Leica, Berlin, Germany), and the NE was observed by transmission electron microscopy (JEM1230).

RESULTS

Expression pattern of *OsRAN2* in response to cold, salt and drought stress

First, we explored the amino acid similarity of plant Ran GTPases by phylogenetic analysis (Fig. 1a). Plant Ran GTPases have more than 95% amino acid identity. However, Ran GTPases of monocotyledons such as maize, wheat and rice show higher similarity. TaRAN1 has been shown to promote the cell mitosis transition in rice (Wang *et al.* 2006). Phylogenetic analysis revealed that of the two rice RAN proteins, OsRAN2 is nearer to TaRAN1 than is OsRAN1.

We used RT-PCR to monitor the expression pattern of *OsRAN2* in response to different stresses. Under cold stress, the transcript level of *OsRAN2* began to increase after 24 h cold treatment and gradually accumulated up to 96 h, with peak level at 72 h (Fig. 1b,e). For salt stress, expression of *OsRAN2* was decreased slightly for 1 and 4 h and then increased slightly from 8 to 24 h (Fig. 1c,f). The level of *OsRAN2* was not significantly increased in response to PEG, a mimic for drought stress (Fig. 1d,g). These data suggest that *OsRAN2* predominantly responded to low temperature as compared with salt and drought stress.

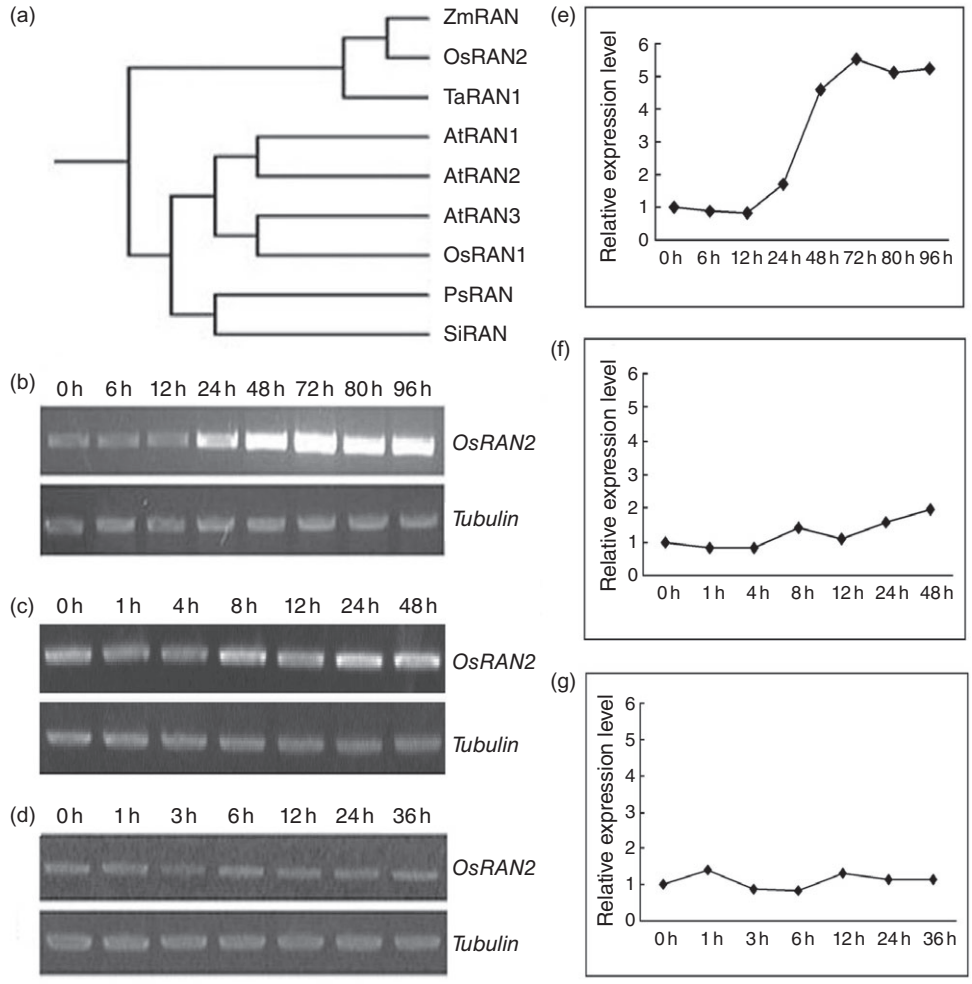

Figure 1. Phylogenetic analysis of plant Ran GTPase and semi-quantitative RT-PCR analysis of *OsRAN2* expression in response to stress. (a) Phylogenetic tree of plant Ran GTPases. The tree was constructed with the DNAMAN tree program with amino acid sequences of rice OsRAN1, OsRAN2 and other Ran GTPases isolated from maize (ZmRAN), wheat (TaRAN1), *Arabidopsis* (AtRAN1, AtRAN2, AtRAN3), pea (PsRAN) and tomato (SlRAN). (b, e) Time course of *OsRAN2* expression during cold treatment (4 °C); (c, f) Time course of *OsRAN2* expression during treatment with 250 mM NaCl. (d, g) Time course of *OsRAN2* expression during treatment with 20% PEG6000, a mimic for drought stress. The rice seedlings were germinated and grew for 10 d before they were treated with cold, salt and drought stresses. *Tubulin* was used as an internal control.

Overexpression of *OsRAN2* and *TaRAN1* increased cold tolerance in rice

To study the function of OsRAN2 in plants, we obtained transgenic rice overexpressing *OsRAN2*. Real-time PCR analysis revealed that *OsRAN2* transcripts increased more than 10-fold in the seven T2 transgenic lines tested (Fig. 2a). Southern blot analysis with DNA digested with *Eco*RI or *Hind*III and *β-glucuronidase (GUS)* gene as the probe revealed the three selected transgenic lines with different hybridization patterns. In contrast, no signals were detected in the wild type under the same conditions (Fig. 2b). Therefore, the three transgenic lines are independent.

To test the possible function of OsRAN2 overexpression on cold tolerance of rice, the seedlings of T2 transgenic lines and wild-type seedlings at trefoil stage were exposed to 4 °C for 72 h for cold stress. Then the plants were removed to the greenhouse to recover at 28 °C. After 14 d recovery under normal conditions, the survival rates of three transgenic lines were 50.3, 80.0 and 62.5%, whereas the survival rate for the wild type was only 14.3% (Fig. 2d). Phenotypically, more than 50% of transgenic seedlings were green and

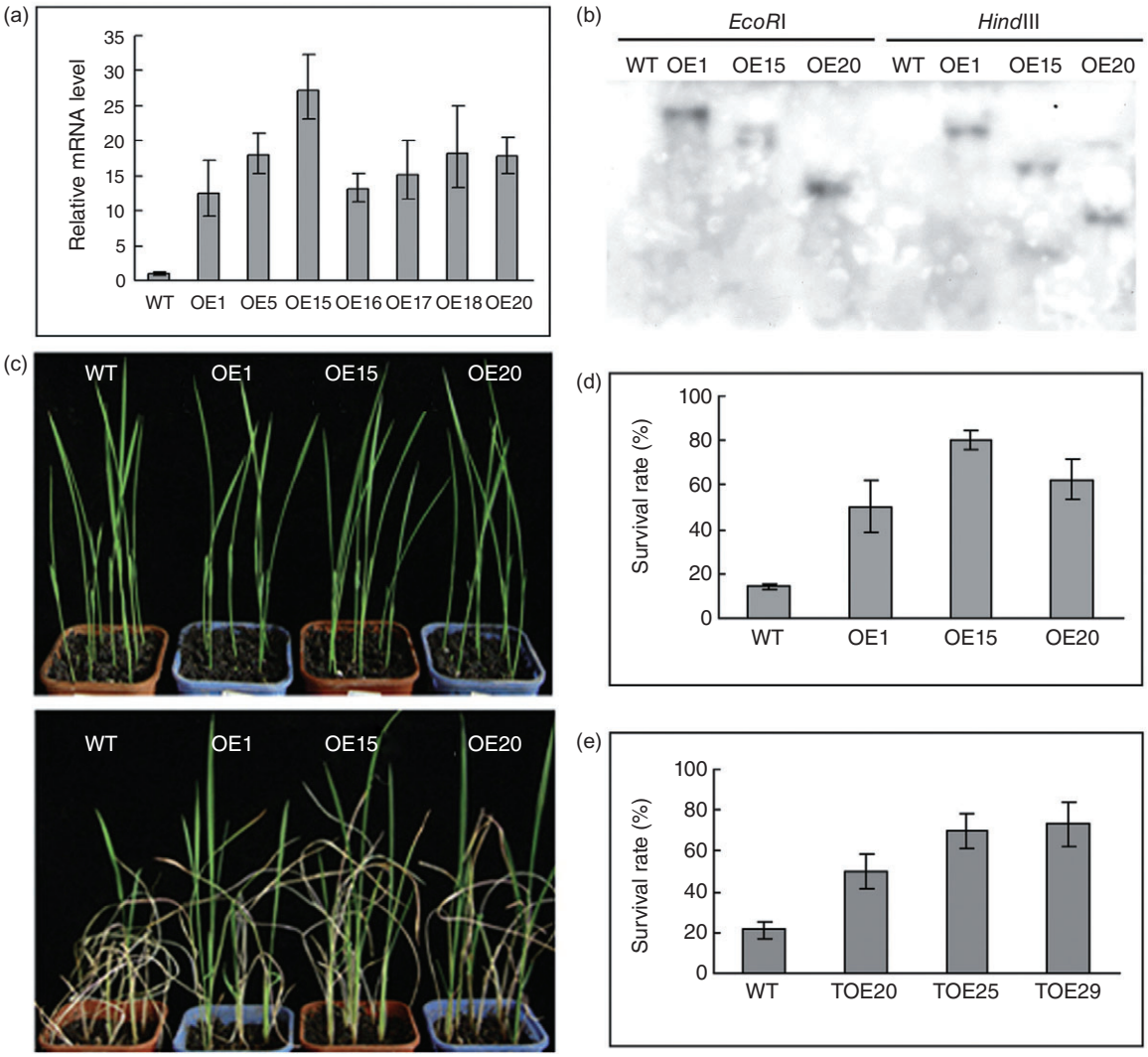

Figure 2. Molecular characterization and cold tolerance analysis of transgenic rice overexpressing *OsRAN2*. (a) Real-time RT-PCR analysis of the expression of *OsRAN2* in transgenic rice relative to that of *Actin*. Data represent means and SE of three replicates. OE1, OE5, OE15, OE16, OE17, OE18, OE20 represent transgenic lines overexpressing (OE) *OsRAN2*. (b) Southern blot assay of rice transgenic plants. Genomic DNA isolated from the transformed lines or wild type (WT) was digested with *Eco*RI or *Hind*III. The blot was hybridized with the encoded region of the GUS gene labelled with ^{32}P-dCTP as described in the Materials and Methods section. (c) Two-week-old OE transgenic and WT plants (top photographs) were cold stressed at 4 °C for 72 h and then transferred back to the normal condition for recovery. Bottom photographs of representative seedlings of WT and three transgenic lines were taken after 14 d of recovery. (d) Survival analysis of WT and *OsRAN2* transgenic plants 14 d after cold treatment. (e) Survival analysis of WT and *TaRAN1* transgenic plants 14 d after cold treatment. TOE20, TOE25, TOE29 represent transgenic lines overexpressing (TOE) *TaRAN1*. Error bars indicate standard deviation and results are from three independent replications of the same experiment. The phenotype was confirmed by further experiments that were repeated more than three times.

could regrow after recovery under normal conditions, whereas about 85% of wild-type seedlings became white and died (Fig. 2c). Thus, *OsRAN2* overexpression enhanced the tolerance to cold stress in rice.

Wheat TaRAN1, with 98.19% identity to OsRAN2, was transformed into rice plants. The survival rate of the wild type was about 20% under cold stress, but that of the T2 transgenic lines was about 70% (Fig. 2e). These data suggest that Ran GTPase was conserved in the regulation of cold stress in rice and wheat.

OsRAN2 is essential for cell division in rice

We obtained knockdown transgenic rice lines with RNAi approach. Because we could not acquire seeds from RNAi transgenic rice, we used T0 transgenic lines. The T0 seedlings with undetectable glucuronidase activity, which were at the same developmental stage with RNAi transgenic lines, were used as a control. Real-time quantitative PCR revealed significantly decreased expression of *OsRAN2* in seedlings of the two RNAi T0 transgenic lines as compared with control seedlings (Fig. 3A). After growing to the 8 week tassel stage in the greenhouse, the RNAi transgenic rice plants developed slowly (Fig. 3B) and began to die as compared with the control. Thus, *OsRAN2* has a fundamental role in the normal growth of rice.

To study the function of the *OsRAN2* transgene in the cell cycle, we examined the mean root mitotic index of transgenic lines. The mean mitotic index of transgenic lines overexpressing *OsRAN2* was increased to 7–8% as compared with the wild type (about 5.8%) (Fig. 3C). In contrast, the mean mitotic index for RNAi transgenic lines was a little lower (<4%) than that for the control with undetectable glucuronidase activity (about 5%) (Fig. 3D). These results are consistent with those of TaRAN1 in transgenic yeast and rice (Wang *et al*. 2004a, 2006).

We also observed the spindle morphology of transgenic lines (Fig. 3F). Transgenic lines overexpressing *OsRAN2* showed no morphological changes of spindles as compared with the wild type (data not shown). In contrast, RNAi transgenic lines showed abnormal spindle structures (Fig. 3F). About 50% of cells displayed segmentary [Fig. 3F(b,d)] or punctuate [Fig. 3F(c)] morphology of spindle microtubules as compared with only about 15% of the transgenic control (Fig. 3E), which may explain the stunted phenotype of the RNAi transgenic rice (Fig. 3B). Thus, OsRAN2 may be essential for spindle formation in the mitotic process in rice.

Overexpression of OsRAN2 maintained cell division under cold stress

We monitored the mean root tip mitotic index at temperatures from 4 to 28 °C (Fig. 4a). In the overexpression lines, the mitotic index was insensitive to temperatures ranging from 10 to 28 °C. However, during cold treatment (10 °C) in the wild type, the mitotic index was only half that under the normal condition (28 °C). Even at 4 °C, the index for the transgenic lines was higher than that for the wild type, although both the transgenic lines and the wild type showed a significantly decreased index at this temperature.

Preprophase bands, spindles and phragmoplasts represent preprophase, metaphase and telophase, respectively, in the cell mitotic process. To determine the phase in the cell cycle that is affected during the cold condition, we calculated the decreasing rate of the proportion of cells with preprophase bands, spindles or phragmoplasts (Fig. 4b). The proportion of cells with the three mitotic apparatuses under normal temperature (28 °C) was set as 1 (100%). At 10 °C, the overexpression lines showed no decrease in proportion of cells with preprophase bands and spindles. In contrast, the wild type showed significantly decreased percentage of cells with these two kinds of microtubules. At 4 °C, wild type and transgenic line 15 showed a decreased proportion of cells with preprophase bands and spindles, with no difference between the wild type and transgenic line in proportion of cells with phragmoplasts (Fig. 4b). The results mentioned earlier indicated that the proportion of cells in preprophase and metaphase decreased more slowly in *OsRAN2* overexpression lines than in the wild type, which suggests that OsRAN2 may promote the transition of cells from interphase to preprophase and preprophase to metaphase under cold stress.

OsRAN2 partially co-localized at spindle microtubules in the mitotic phase

To understand the localization of OsRAN2 during cell mitosis, we obtained a stable transformed *Arabidopsis* line with the construct harboring the *OsRAN2-GFP* fusion gene. Transgenic *Arabidopsis* root tips were analysed by immunofluorescence assay with anti-GFP and anti-β-tubulin antibody. The fluorescence of OsRAN2-GFP appeared mainly in the nucleus and slightly in the cytoplasm when the cells were in interphase (Fig. 5). Cells entering the mitotic phase showed enriched fluorescence of OsRAN2-GFP at spindles and tubulin in metaphase and anaphase, which suggests that OsRAN2 may have functions during cell mitotic process.

OsRAN2 is involved in the regulation of the accumulation of tubulin into the nucleus during cold stress

With prolonged 0 °C treatment, tobacco BY2 microtubules were depolymerized and entered the nuclei (Schwarzerova *et al*. 2006). We analysed wild-type and *OsRAN2* T2 transgenic rice root tips by immunofluorescence assay with anti-β-tubulin antibody to observe the microtubule morphology under the cold condition. Cortical microtubules in most root tip cells were depolymerized into fragments with 1 h treatment at 4 °C, with no difference between transgenic line 15 and the wild type in depolymerization degree (middle images in Fig. 6a).

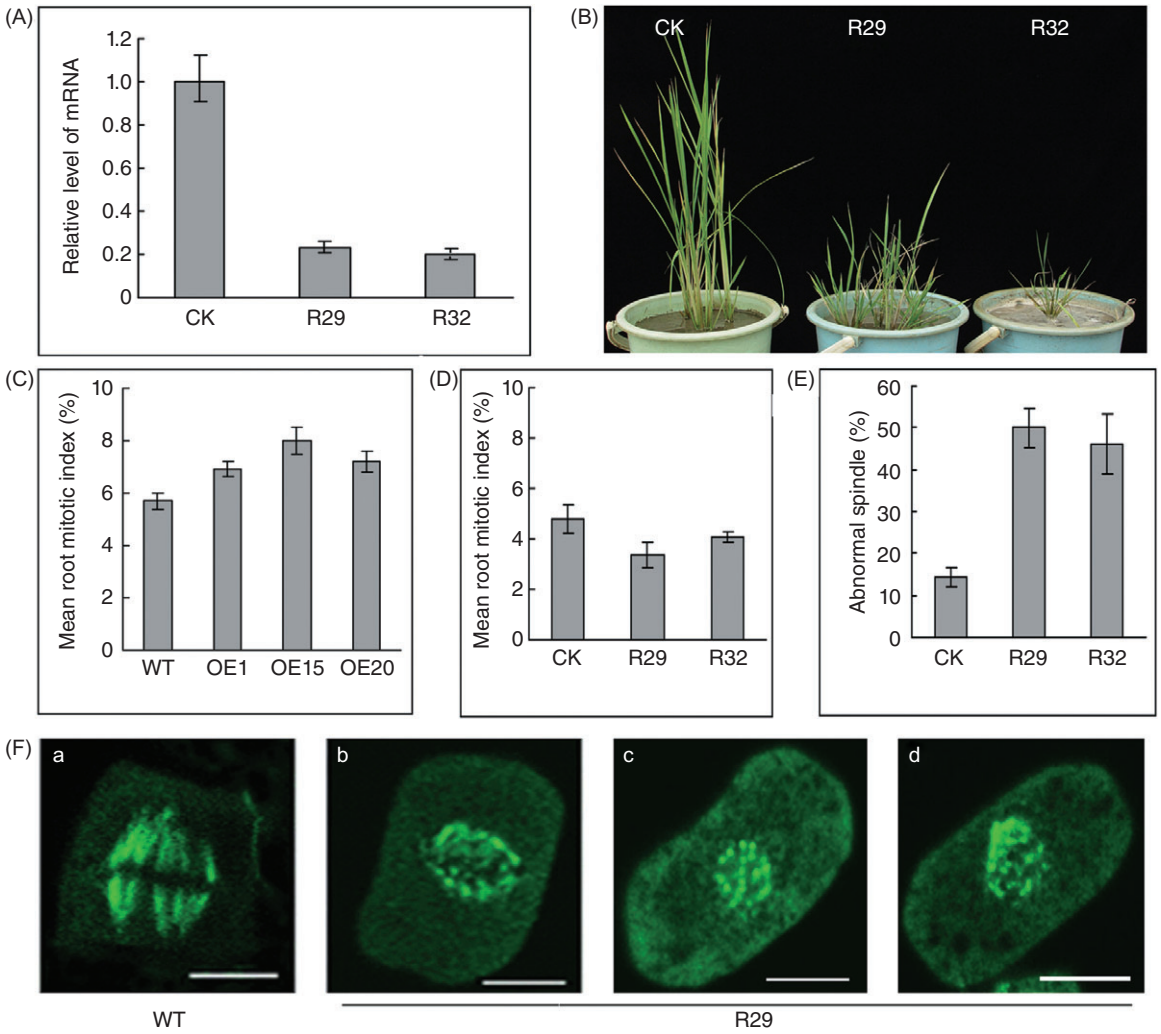

Figure 3. Phenotype, mean root mitotic index and spindle morphology of *OsRAN2* RNAi transgenic plants. (A) Real-time RT-PCR analysis of the expression of *OsRAN2* in transgenic rice relative to that of *Actin*. Data represent means and SE of three replicates. (B) Stunted phenotype of the T0 generation of *OsRAN2* knockdown transgenic rice. Seedlings with undetectable glucuronidase activity of the T0 transgenic line were used as a control. R29 and R32 represent *OsRAN2* RNAi transgenic lines. Control, R29 and R32 were grown for 2 months in the greenhouse. (C) Mean root mitotic index of wild-type (WT) and transgenic rice overexpressing *OsRAN2*. About 2000 cells of 10 root tips were analysed in every replicate. Data represent means and standard deviation of three replicates. (D) Mean root mitotic index of *OsRAN2* RNAi transgenic rice lines; seedlings of T0 transgenic plants with undetectable glucuronidase activity are a control (CK). About 1000 cells of five root tips were used in every replicate. Data represent means and standard deviation of three replicates. (E) Proportion of cells with abnormal spindles in control (CK) and *OsRAN2* RNAi transgenic lines. More than 20 spindles were observed in every replicate. Data represent means and standard deviation of three replicates. (F) Spindle morphology of WT and *OsRAN2* RNAi transgenic plants. Spindles were visualized by immunofluorescence with anti-β-tubulin antibody and fluorescein isothiocyanate-conjugated secondary antibody. (a) Normal spindle morphology in the WT; (b–d) Abnormal spindle structures in R29 transgenic line. Bars = 5 μm.

Microtubules in some cells depolymerized completely and accumulated into the nuclei during cold treatment (last images in Fig. 6a). DAPI staining revealed that the depolymerized microtubules indeed entered the nucleus (Fig. 6b). The proportion of cells with intranuclear tubulin was increased progressively during prolonged cold treatment in wild-type and transgenic rice but was always higher in the wild type than in the transgenic line (Fig. 6c). To investigate whether the difference between the wild-type and transgenic rice was induced by the stability of microtubules, we used a microtubule-depolymerizing compound, oryzalin, to treat rice roots. Under normal conditions (28 °C) and all oryzalin concentrations, transgenic lines and the wild type did not differ in depolymerization degree of root microtubules, and no depolymerized microtubules accumulated in the nuclei at any concentration (Fig. 6d). In contrast, at 4 °C and all oryzalin concentrations, transgenic lines showed lower proportion of cells with intranuclear tubulin than did the wild type, with the accumulation seemly not related to oryzalin concentration

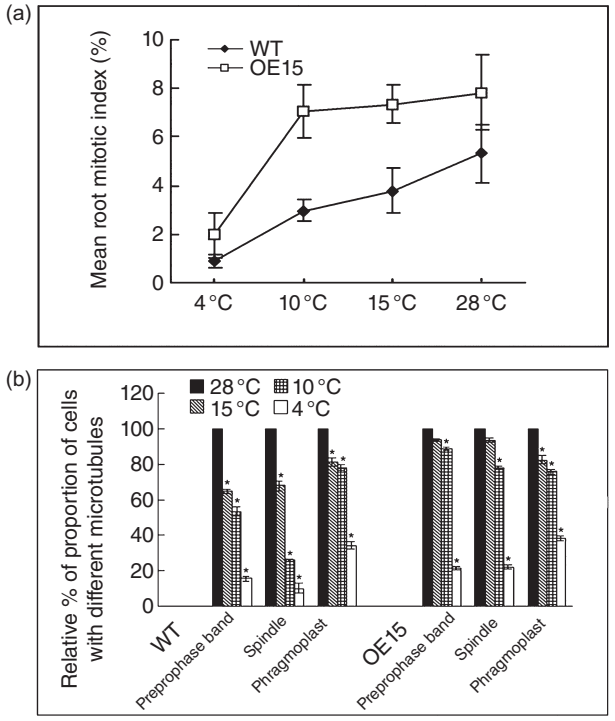

Figure 4. Mean root tip mitotic index of wild-type (WT) and transgenic rice overexpressing *OsRAN2* during the cold condition. (a) Mean root tip mitotic index under cold treatment at different temperatures in the WT and one transgenic line overexpressing *OsRAN2*. (b) Decreasing rate of the proportion of cells with preprophase bands, spindles or phragmoplasts under cold treatment at different temperatures in the WT and transgenic line. Ten root tips were analysed in every replicate. Data represent means and standard deviation of three replicates. The calculation of relative percentage of cells with different microtubules in (b) were as described in the Materials and Methods section. Asterisk (*), Significant difference, $P < 0.01$.

(Fig. 6e). These results suggest that OsRAN2 may affect nucleocytoplasmic transport of tubulin only under cold stress.

Overexpression of OsRAN2 promoted the formation of an intact NE under cold stress

We observed NE of root tip cells of the overexpression line 15 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 and had no obvious difference in morphology (Fig. 7a,b). With 3 h treatment at 4 °C, about 60.7% cells showed completely dissociated double membranes in wild type (Fig. 7c,d). However, most cells in the overexpression line could form an intact NE under cold stress (Fig. 7e), only about 28.3% cells showed partially dissociated NE (Fig. 7f). These results suggested that overexpression of OsRAN2 may promote the formation of an intact NE under cold stress.

DISCUSSION

OsRAN2 functions in cell cycle regulation similar to its homologs in yeast and other plants

Small GTPase Ran is becoming increasingly known to be involved in the coordination of mitosis in animals and yeasts (Drivas *et al*. 1990; Bischoff & Ponstingl 1991a,b) and have functions in centrosome duplication, microtubule dynamics, chromosome alignment, kinetochore attachment of microtubules and NE dynamics (Clarke & Zhang 2008). Overexpression of various wild-type Ran homologs of plants, including tomato and tobacco *Ran*, suppressed the *pim1* mutant phenotypes in yeast (Matsumoto & Beach 1991; Belhumeur *et al*. 1993; Ach & Gruissem 1994; Merkle *et al*. 1994), which suggests that Ran GTPase may have a conserved function in cell cycle regulation.

Our study reveals that overexpression of *OsRAN2* enhanced the root tip mitotic index of transgenic rice (Fig. 3C), whereas knockdown of *OsRAN2* expression had an opposite effect (Fig. 3D). In addition, knockdown of the expression of *OsRAN2* induced severe abnormalities in spindle assembly (Fig. 3E,F). Because the control has 15% showing abnormal spindle morphologies, they might be normal morphologies occurring at specific stages of a cell cycle for the transgenic seedlings. The increase of such morphologies in RNAi transgenic lines might reflect an extension of these specific stages because of the knockdown expression of OsRAN2. Investigations in animals and yeast indicate that Ran localizes to the mitotic chromosomes, spindle microtubules and centrosomes to provide spatial signals directing cell mitosis (Clarke & Zhang 2008). Our immunofluorescence results also confirmed that OsRAN2 co-localizes with spindles and may provide spatial signals for organization of the mitotic microtubular structures during cell division (Fig. 5). Thus, plant Ran homologs may have well-conserved functions in plants, yeast and animals during evolution.

OsRAN2 enhanced cold tolerance by maintaining active cell division through regulating the formation of an intact NE under cold stress in rice

We found the expression of *OsRAN2* upregulated under cold stress (Fig. 1). This expression pattern suggests that OsRAN2 may function as a regulator in the cold signalling pathway in rice. Both OsRAN2 and TaRAN1 increased the cold resistance of transgenic rice (Fig. 2c,d). Ran GTPase in plants was shown to be involved in cell cycle regulation and response to phytohormones such as indoleacetic acid, jasmonic acid, and even to gravitropism (Kriegs *et al*. 2006; Miche *et al*. 2006; Wang *et al*. 2006). Our data suggest a new role for this versatile molecular switch in cold tolerance regulation.

OsRAN2 overexpression could maintain a high mean root mitotic index under cold stress (Fig. 4a). Further analysis indicated that OsRAN2 may promote the transition of

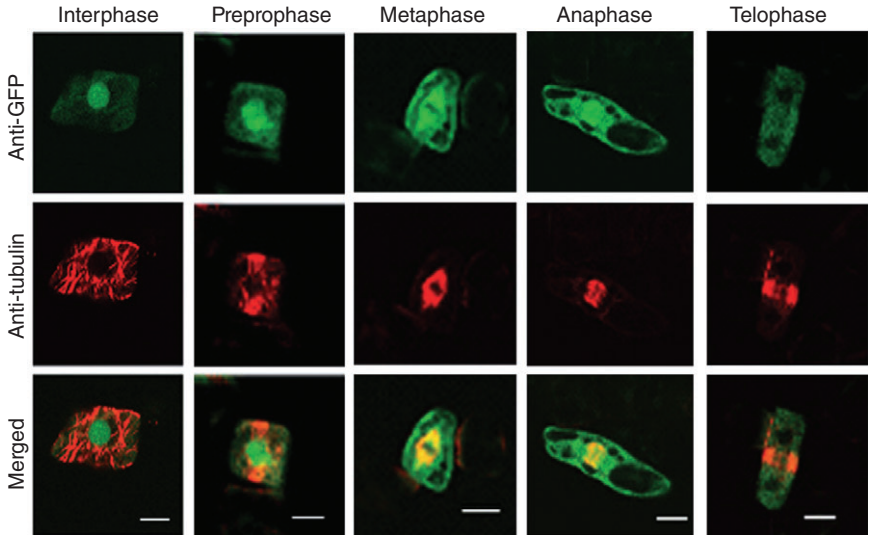

Figure 5. Immunolocalization of OsRAN2 and tubulin in *Arabidopsis* stably expressing OsRAN2-GFP. Microtubules were visualized by immunofluorescence with anti-β-tubulin antibody and Cy3-conjugated secondary antibody. OsRAN2-GFP was visualized by immunofluorescence with anti-green fluorescent protein (GFP) antibody and fluorescein isothiocyanate-conjugated secondary antibody. Confocal images (GFP in green and β-tubulin in red) were from various cell phases. Bars = 10 μm.

cells from interphase to mitosis and organization of spindles during cold treatment (Fig. 4b). Many investigations have shown that cell division is closely related to stress tolerance in plants. *Arabidopsis* plants constitutively overexpressing *HAL3a* showed improved growth, as well as salt and osmotic tolerance in *Arabidopsis* and rice (Espinosa-Ruiz *et al*. 1999; Sun *et al*. 2009). *Arabidopsis* STT3 functions in salt stress adaptation to maintain the mitotic activity of root cells (Koiwa *et al*. 2003). Overexpression of *Arabidopsis SKP2A* maintains active cell division longer than in the wild type under high sucrose stress; therefore, *SKP2A*-overexpressed seedlings are able to survive high sucrose stress (Jurado *et al*. 2008). Transgenic rice lines overexpressing *OsMYB3R-2* and *OsCycB1;1* exhibited enhanced cold tolerance, which indicates that maintaining cell division activity could enhance cold resistance in plants (Ma *et al*. 2009). Our results suggest that a Ran GTPase-activated cell cycle also mediates the cold stress response of rice.

We found that OsRAN2 functions in the regulation of tubulin accumulation into nuclei (Fig. 6a). Reports of intranuclear tubulin in plants are scarce. Intranuclear microtubule-like structures have been reported in *Aesculus hippocastanum* L. (Barnett 1991). Cortical microtubules of tobacco BY-2 cells depolymerized and accumulated into the nuclei, and the proportion of cells with intranuclear tubulin increased steadily during chilling treatment, which indicates that the proportion of cells with intranuclear tubulin may be a marker reflecting the degree of cell injury under cold stress (Schwarzerova *et al*. 2006). This phenomenon was interpreted as impaired integrity of the NE and normal transport of tubulin through the nuclear pores during cold treatment, which results in intranuclear accumulation of tubulin. OsRAN2 had no effect on the depolymerization of cortical microtubules in rice roots treated with microtubule-depolymerization compound oryzalin (Fig. 6d), which suggests that the difference in tubulin accumulation between the overexpression line and wild type should have no relation to the stability of microtubules. Tubulin accumulated into the nuclei and OsRAN2 overexpression reduced the proportion of cells with intranuclear tubulin only under the cold condition (Fig. 6), which suggests that OsRAN2 may function in the regulation of tubulin transport through the nuclear membrane during cold condition.

The meristematic zone of the rice root undergos constitutive cell division. Depending on the phase of the cell cycle, microtubules are organized in different structures: cortical microtubules at G1, preprophase bands at G2 phase, spindles at metaphase and phragmoplasts at telophase. The cortical microtubules and preprophase band of microtubules are always confined to the cytoplasm. When the NE disintegrates at the onset of mitosis, the organized microtubules disappear and the spindle is established. After the completion of cell division and reformation of the NE, a new cytoplasmic interphase array of microtubules is re-established (Hasezawa & Kumagai 2002). The export of tubulin from the nucleus at the end of mitosis is important for all organisms to ensure proper cell cycle progression (Schwarzerova *et al*. 2006). Studies of tobacco BY2 cells and our present work showed inhibited exclusion of tubulin from the nucleus during the cold condition. We supposed that the retention of tubulin in the nucleus induced disorders of cell cycle regulation and inhibited the normal cell division process during cold stress.

As mentioned earlier, impaired integrity of the NE and active transport of tubulin through the nuclear pores during cold treatment could result in intranuclear accumulation of tubulin (Schwarzerova *et al*. 2006). Tubulin molecules contain 5 putative nuclear export signals (NESs), and its exclusion from nuclei possibly involves the conventional transport mechanism, including the components of importin, exportin, Ran and RanGAP (Schwarzerova *et al*. 2006). So from our results, *OsRAN2* overexpression could promote the normal transport of tubulin through the NE for a normal cell cycle under cold stress. Ran GTPase promotes the NE assembly and nuclear pore organization at the end of mitosis in *Xenopus* egg extracts (Zhang & Clarke

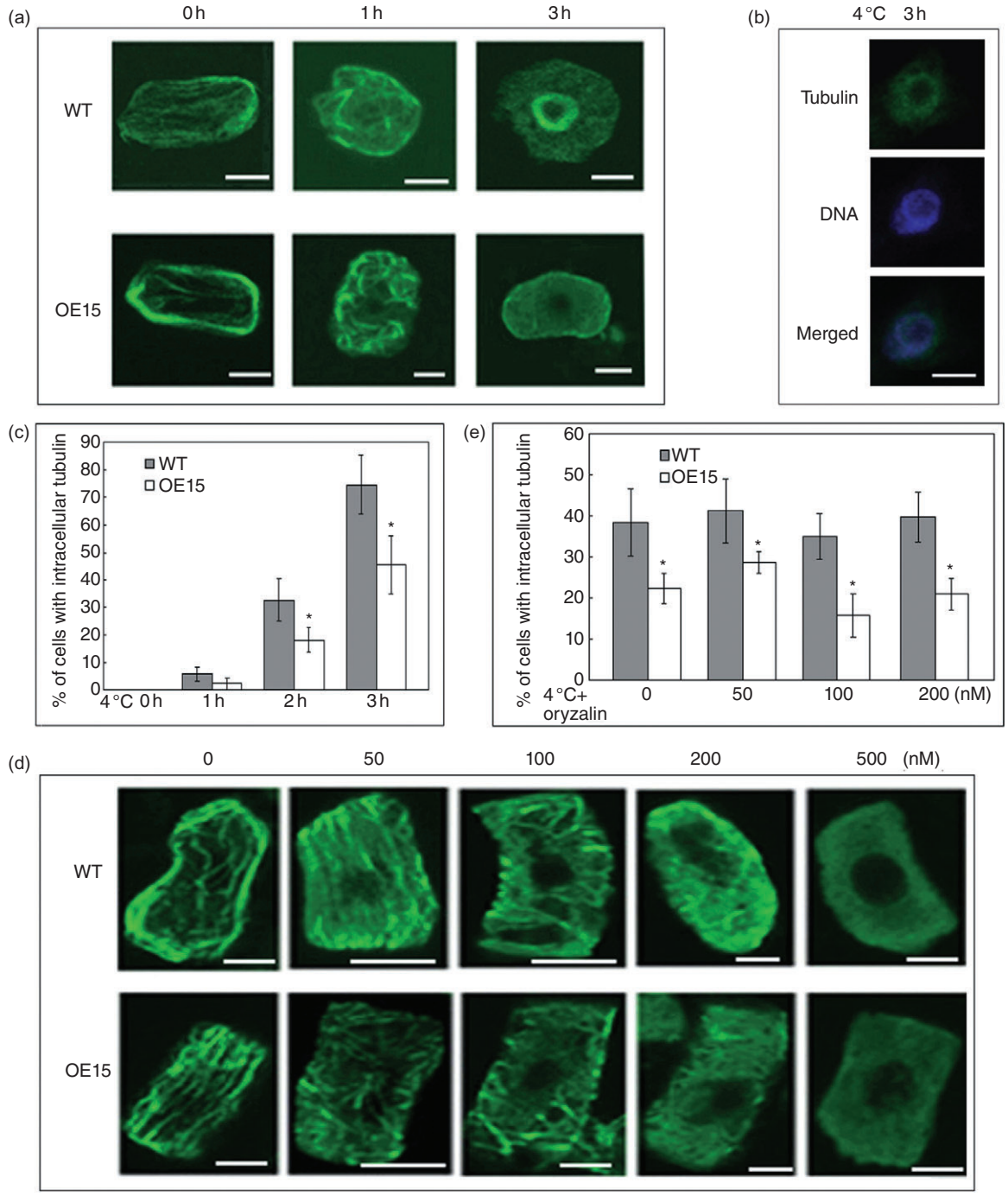

Figure 6. Nuclear accumulation of β-tubulin in the wild type and transgenic rice overexpressing *OsRAN2* under cold and oryzalin treatment. (a) Accumulation of β-tubulin in the nuclei during chilling (4 °C) in the wild type (WT) and transgenic line 15. 0, 1 and 3 h represent time courses of 4 °C treatment. (b) DAPI staining of DNA showing tubulin accumulated in nuclei. (c) Proportion of cells with intranuclear tubulin under cold treatment. Three-day-old seedlings were used, and 10 root tips were observed in every replicate. Data are the mean proportion of cells with intranuclear tubulin of three replicates. Error bars indicate standard deviation. (d) Depolymerization of β-tubulin in the WT and transgenic line 15 treated with different concentrations of oryzalin for 4 h. Six-day-old seedlings were used. (e) Proportion of cells with intranuclear tubulin under the cold condition combined with concentrations of oryzalin for 4 h. Six-day-old seedlings were used, and 10 root tips were observed in every replicate. Error bars indicate standard deviation. Data are the mean proportion of cells with intranuclear tubulin of three replicates. Microtubules (a,b,d) were visualized by immunofluorescence with anti-β-tubulin antibody and fluorescein isothiocyanate-conjugated secondary antibody. Asterisk (*), Significant difference, $P < 0.01$. Bars = 5 μm.

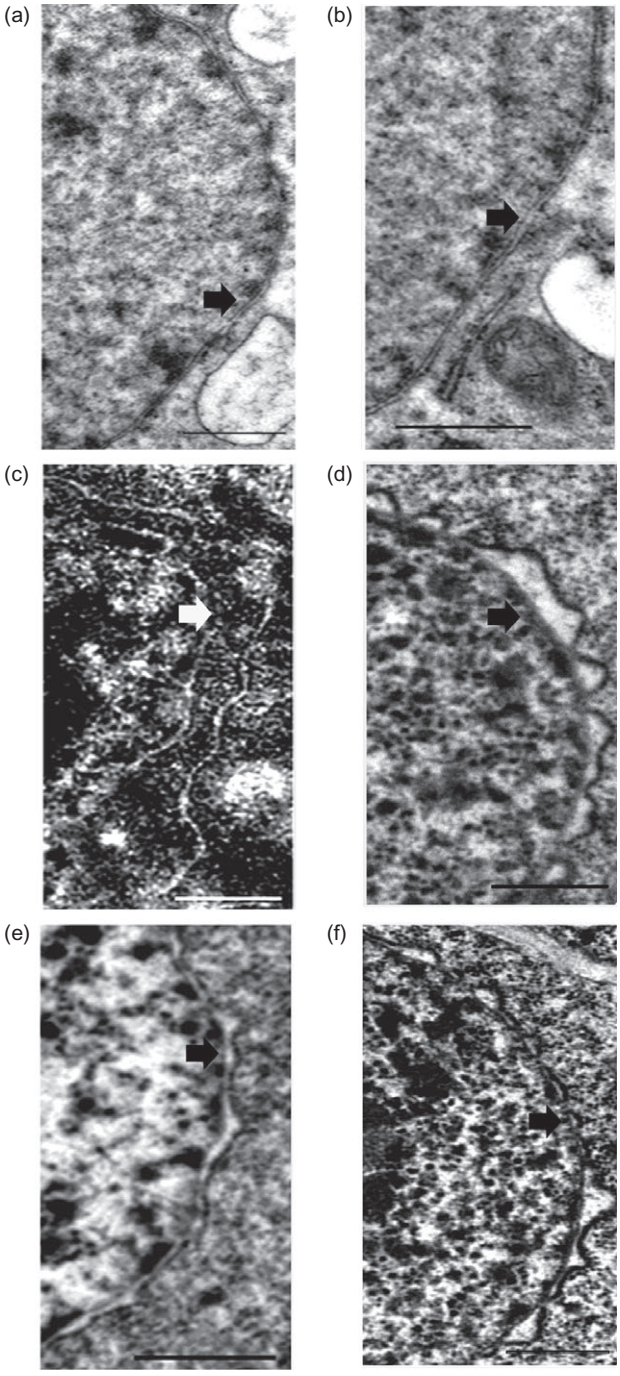

Figure 7. Morphology changes of nuclear envelope in the wild-type (WT) and transgenic line under cold stress. Nuclear envelope of the WT (a) and *OsRAN2*-overexpression line (b) under normal conditions (28 °C). Nuclear envelope of the WT (c,d) and *OsRAN2*-overexpression line (e,f) after 3 h treatment at 4 °C. Five root tips were observed in every condition. The root tips were transversely cut in the meristematic zones. Arrows indicate the nuclear envelope. Bars = 0.5 μm.

2000, 2001). Despite no report of the function of plant Ran GTPase in NE assembly, our NE observations indicated that *OsRAN2* overexpression promoted the formation of an intact NE under cold stress (Fig. 7). Combining our results with those of previous studies, we speculate that OsRAN2 may be involved in the organization of normal NE structures at the end of mitosis during the cold condition, which is essential for normal transport of depolymerized microtubules.

In conclusion, we found the expression of *OsRAN2* induced by cold treatment and led to an increased mitotic index in rice. *OsRAN2* overexpression could enhance cold tolerance by maintaining cell division ability under cold stress in rice. The regulation of OsRAN2 in cell division may be through the following mechanism: in the mitotic phase, OsRAN2 co-localizes with the spindle and provides a spatial signal for cell division, and at the end of mitosis, OsRAN2 may also have a role in promoting the assembly of an intact NE under cold stress. Our studies established a new mechanism for OsRAN2 in regulating cold tolerance in rice.

ACKNOWLEDGMENTS

We thank Rongxi Jiang and Yuan Zhao (Institute of Botany, Chinese Academy of Science) for rice transformation and Hong Cao for help with RT-PCR. We are grateful to the National Basic Research Program of China (2006CB100104) and the National Natural Science Foundation of China (30821007) for the financial support.

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Received 23 June 2010; received in revised form 23 August 2010; accepted for publication 29 August 2010