Overexpression of OsRAN2 in rice and Arabidopsis renders transgenic plants hypersensitive to salinity and osmotic stress

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Abstract

Nucleo-cytoplasmic partitioning of regulatory proteins is increasingly being recognized as a major control mechanism for the regulation of signalling in plants. Ras-related nuclear protein (Ran) GTPase is required for regulating transport of proteins and RNA across the nuclear envelope and also has roles in mitotic spindle assembly and nuclear envelope (NE) assembly. However, thus far little is known of any Ran functions in the signalling pathways in plants in response to changing environmental stimuli. The OsRAN2 gene, which has high homology (77% at the amino acid level) with its human counterpart, was isolated here. Subcellular localization results showed that OsRan2 is mainly localized in the nucleus, with some in the cytoplasm. Transcription of OsRAN2 was reduced by salt, osmotic, and exogenous abscisic acid (ABA) treatments, as determined by real-time PCR. Overexpression of OsRAN2 in rice resulted in enhanced sensitivity to salinity, osmotic stress, and ABA. Seedlings of transgenic Arabidopsis thaliana plants overexpressing OsRAN2 were overly sensitive to salinity stress and exogenous ABA treatment. Furthermore, three ABA- or stress-responsive genes, AtNCED3, AtPLC1, and AtMYB2, encoding a key enzyme in ABA synthesis, a phospholipase C homologue, and a putative transcriptional factor, respectively, were shown to have differentially induced expression under salinity and ABA treatments in transgenic and wild-type Arabidopsis plants. OsRAN2 overexpression in tobacco epidermal leaf cells disturbed the nuclear import of a maize (Zea mays L.) leaf colour transcription factor (Lc). In addition, gene-silenced rice plants generated via RNA interference (RNAi) displayed pleiotropic developmental abnormalities and were male sterile.

Key words: Abiotic stress, Arabidopsis, nuclear-cytoplasmic trafficking, OsRAN2, rice.

Introduction

The protein Ran is a member of an important family of small GTPases that control multiple cellular processes, including the trafficking of proteins and RNAs into and out of the nucleus (Görlich and Kutay, 1999) and the assembly of the mitotic spindle and nuclear envelope (NE) (Quimby and Dasso, 2003; Di Fiore et al., 2004; Cicciarella et al., 2007). Ran is GDP-bound in the cytoplasm during interphase and GTP-bound in the nucleus. This asymmetric distribution of Ran-GTP and Ran-GDP is essential for its roles (Bischoff and Ponstingl, 1991; Bischoff et al., 1994, Izaurralde et al., 1997; Kalab et al., 2002). The guanine exchange factor (GEF) termed RCC1 (regulator of chromosome condensation 1) loads GTP onto Ran in the nucleus (Bischoff and Ponstingl, 1991), while the GTP hydrolysis-activating protein RanGAP1 hydrolyses GTP on Ran to generate Ran-GDP in the cytoplasm (Bischoff et al., 1994). Ran binding protein 1 (RanBP1) acts an essential accessory factor to increase RanGAP1-mediated nucleotide hydrolysis and by inhibiting the GEF activity of RCC1 (Bischoff et al., 1995).

In plants, the identification of RanBP1 and RanGAP (Ach and Gruissem, 1994; Haizel et al., 1997; Rose and Meier, 2001; Pay et al., 2002), has allowed Ran’s function to be
Materials and methods

Plant materials

Rice plants (Oryza sativa L. ssp. japonica) were germinated and grown in nutrient solution (Ni, 1985) at a photo flux density of 350–400 μmol m⁻² s⁻¹, 60–80% relative humidity, 12/12 h day/night cycle at 28 °C in a phytotron. Experiments were performed with 3-week-old seedlings. Arabidopsis (ecotype Columbia) and tobacco (Nicotiana tabacum L. cv. Gexin No.1) was grown in a growth room under long-day conditions (16/8 h light/dark cycle) with a fluence rate of 120 μmol m⁻² s⁻¹ of white light (produced by cool-white fluorescent lamps) at 22 °C.

Exogenous ABA and stress treatments

Three-week-old rice seedlings were placed so that their roots were submerged in nutrient solution (control) or nutrient solution containing 50 μM or 100 μM ABA (Sigma), 100 mM or 200 mM NaCl, 100 mM or 200 mM KCl, or 20% polyethylene glycol (PEG 6000) (SanYo, Tokyo, Japan). At different time points (0, 1, 2, 4, 8, 12, and 24 h in NaCl and KCl treatments; 0, 1, 2, 3, 5, 10, and 24 h in PEG 6000 treatment; 0, 0.5, 1, 2, 5, 10, and 24 h in ABA treatments), leaves were cut with a razor blade, then immediately frozen in liquid nitrogen and stored at −70 °C. One-week-old Arabidopsis plants (wild-type and transgenic lines) were transferred onto plates containing 150 mM NaCl, 150 mM KCl, or 100 μM ABA. 6 h later, seedlings were collected and frozen in liquid nitrogen and stored at −70 °C.

Quantitative real-time PCR analysis

Total RNA was isolated from rice tissues as described by Mamiatis et al. (1982). The cDNA was synthesized using oligo (dT)18 primers and ReverTra Ace M-MLV Rase (Toyobo, Japan) according to the manufacturer’s recommendations. Quantitative real-time PCR (qPCR) of OsRAN2 and stress- or ABA-related genes, including AtPLC1, AtMYB2, and AtNCED3 was performed using gene-specific primers. Ubiquitin5 and ACTIN7 were used as the reference genes for rice and Arabidopsis, respectively. The relative gene expression was evaluated using a comparative ratio of the examined gene over the reference gene. Gene-specific primers for qPCR are listed in Supplementary Table S1 at JXB online.

Plasmid constructions and generation of transgenic plants

The full-length cDNA of OsRAN2 was amplified according to GenBank accession No. AB015972. And the modified green fluorescent protein (GFP) gene was amplified according to the pCMBIA1302 vector which contains the modified GFP gene. For gene overexpression plasmid construction, OsRAN2 or OsRAN2:GFP were cloned into a pHB vector (Mao et al., 2005) to generate double 35S:OsRAN2 and 35S:OsRAN2-GFP. For gene-silencing plasmid construction, OsRAN2 was introduced into the binary vector pFGC1008 (http://www.chromdb.org/fgc1008.html) in the sense orientation using AscI and Swa sites and in the antisense orientation using BamHI and SpeI sites. In the final plasmid, the sense and antisense sequences were separated by a spacer originating from a beta-glucuronidase (GUS) gene. Expression in rice was controlled by the CaMV 35S promoter. The transgenes were confirmed by DNA sequencing. All the reagents and enzymes used here for PCR amplification or restriction digestion were purchased from Takara, Japan. Primers used for plasmid constructions are shown in Supplementary Table S2 at JXB online.

Arabidopsis plants (ecotype Columbia) were transformed with double 35S:OsRAN2 and 35S:OsRAN2:GFP transgenes using the floral-dipping method (Clough and Bent, 1998). Japonica rice cv. Zhonghua 11 plants were transformed with the double 35S: OsRAN2-GFP and OsRAN2-dsRNA RNAi transgenes using an Agrobacterium-mediated transformation method as described by Hei et al. (1994).

Hygromycin (Roche)-resistance was used to screen positive transgenic plants. Genomic PCR were used to confirm the transgenic plants with specific primers for the hygromycin phosphotransferase (HPT) gene: 5′-TGCTCTCCGGTAAATAGC-3′ and 5′-TGCTCCATAAAGGCAAACC-3′ (AY836546). Semi-quantitative RT-PCR and qPCR were conducted to detect gene expression level in transgenic Arabidopsis and rice plants. The primers used here are listed in Supplementary Tables S3 and S1 at JXB online. The experiments were conducted using homozygous T2 plants.

studied. Because of 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 process (Ach and Gruissem, 1994; Haizel et al., 1997). Overexpression of plant Ran proteins, including the tomato and the tobacco Ran genes, suppresses the phenotype of the cell cycle regulatory mutant pm1-46 fission yeast (Ach and Gruissem, 1994; Merkle et al., 1994). Overexpression of wheat Ran1 (TaRAN1) in Arabidopsis and rice resulted in an elevated mitotic index and prolonged life cycle (Wang et al., 2006).

In addition, it has been shown that Ran and RanBP are considered to be involved in the regulation of hormone sensitivities, light signalling, and resistance to pathogens. TaRAN1 overexpression stimulated hypersensitivity to exogenous auxin. Anti-sense expression of AtRanBP1c in transgenic plant roots resulted in hypersensitivity to auxin (Kim et al., 2001). Virus-induced gene silencing (VIGS) of NbRanBP1 in Nicotiana benthamiana showed stress responses, such as reduced mitochondrial membrane potential and excessive production of reactive oxygen species (Cho et al., 2008). Lee et al. (2008) reported that Ran gene expression was differentially regulated by various light sources via a phytochrome-mediated signalling pathway.

Abiotic stresses such as drought, salinity, and temperature extremes pose severe limitations to plant growth and development, thus limiting agricultural productivity. Modulation of transcriptional activity of stress-related genes is critical to the survival and reproduction of plants in response to stress (Xiong and Zhu, 2001). Environmental signals must be transduced into the nucleus to switch on gene transcription by means of specific regulatory proteins. Post-transcriptional regulation of gene expression occurs by pre-mRNA processing, mRNA stability, nuclear RNA export from the nucleus, and finally translation (Mazzucotelli et al., 2008). Thus the nucleo-cytoplasmic trafficking of proteins and non-coding RNA molecules through Ran-dependent karyopherin (importin β) proteins may provide an alternative regulatory way in response to abiotic stresses (Mossammaparast and Pemberton, 2004; Harel and Forbes, 2004; Mazzucotelli et al., 2008; Chinnusamy et al., 2008). In this paper, OsRAN2 expression was determined under different abiotic stress (salinity and osmotic stresses) and exogenous ABA treatment. OsRAN2 overexpressed Arabidopsis and rice plants were generated and the function of OsRAN2 in response to abiotic stresses was characterized.
Subcellular localization study

Onion epidermal cells were bombarded with the construct of double 35S:OsRAN2:GFP transgene using a particle gun-mediated system (PDS-1000/He; Bio-Rad) and analysed by confocal microscopy (Carl Zeiss; LSM 510 Meta). In addition, the GFP signal was analysed in roots of 1-week-old transgenic rice seedlings and Arabidopsis.

Stress tolerance assays

Homologous T2 transgenic Arabidopsis (transformed with double 35S:OsRAN2) and transgenic rice (OsRAN2 overexpression) seeds were used for stress tolerance assays. Arabidopsis seeds were sterilized and grown on Murashige and Skoog (MS) plates containing 100 mM NaCl, 100 mM KCl, 0.25 or 0.5 μM ABA (MS0 as control). Transgenic rice seeds were germinated in water (as control) or in water containing 100 mM NaCl, 100 mM KCl, 10% PEG 6000 or 10 μM ABA. Photographs were taken at the indicated times.

Pollen viability and scanning electron microscope assay

Mature anthers (before flowering) of RNAi lines and wild-type plants were harvested and anthers before fertilization were dissected out from transgenic or wild-type plants. To evaluate pollen viability, pollen grains of each anther were stained using a 1% iodine-potassium iodide (I2-KI) solution, as described by Shinjyo (1969), and observed under an optical microscope. The dark-blue pollen grains were viable and the reddish ones were not. For the scanning electron microscope (SEM) assay, mature spikelets (before flowering) were harvested and fixed in a fixative solution of buffered glutaraldehyde (3%) for 3 d, dehydrated in a series of ethanol-water (30%, 50%, 70%, 80%, 90%, 95% and 100%), and incubated in an ethanol–isoamyl acetate mixture for 1 h. The samples were then dried, nipped to let pollen grains spout out, coated with gold powder, and finally examined in a JEOL JSM-6360LV (JEOL).

Transient expression of Lc and OsRAN2 in tobacco leaf epidermal cells

As a reporter gene for nuclear import, maize (Zea mays) Lc was fused to yellow fluorescent protein (YFP) in the pA7-YFP plasmid to generate pA7-Lc-YFP (35S promoter-driven). Lc was cloned from the Lc-containing vector (kindly provided by Dr Xiangling Dasso, 2003). In addition, this localization pattern was also observed in root cells of transgenic plants (rice and Arabidopsis) overexpressing OsRAN2 (Fig. 2B, C).

Subcellular localization of OsRan2

The subcellular localization of OsRAN2:GFP was examined through transient expression of OsRAN2:GFP in onion epidermal cells. The green fluorescent signal of OsRAN2: GFP was detected mainly within the nucleus, with some signal in the cytoplasm (Fig. 2A). This was consistent with the mammalian counterparts’ subcellular localization, 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 in root cells of transgenic plants (rice and Arabidopsis) overexpressing OsRAN2 (Fig. 2B, C).

The effects of salinity stress on OsRAN2 expression

To investigate the effects of salinity stress on OsRAN2 expression, different concentrations of NaCl (100 or 200 mM) and KCl (100 or 200 mM) were used to induce salinity stress (Verslues et al., 2006a) in treated 3-week-old rice seedlings. Decreased expression levels of OsRAN2 were observed in both NaCl and KCl treatments (Fig. 3A, B, C, D). In time-course experiments with both treatments, OsRAN2 expression declined at first until it reached its lowest level, then recovered to some degree compared with the control. These effects were dose-related. In NaCl treatment, expression of OsRAN2 decreased to nearly 75% of the control 1 h after 200 mM NaCl treatment and reached 36% of the control, the lowest level, 4 h after 200 mM NaCl treatment, then it began to rise again and it reached 59% of the control 12 h later (Fig. 3B). In response to 100 mM NaCl it showed about 91% of the control at the 1 h point and 43% of the control at 4 h after treatment; after that it decreased still further to 29% of the control 24 h after 100 mM NaCl treatment (Fig. 3A). In the KCl treatment, the lowest level of OsRAN2 expression appeared 4 h after the 200 mM KCl treatment being 22% of the control, while almost 78% of the control was observed in 100 mM KCl treatment at this same time point (Fig. 3C, D). OsRAN2 expression reached

Results

Isolation of OsRAN2 and comparison with related sequences

Using GenBank Accession no. AB015972, primers were designed to isolate OsRAN2 cDNA which contained the full-length coding region and encoded the predicted protein of 221 amino acids as with other Ran proteins. A nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast/) was performed against the Oryza sativa genome showing that the gene matched exactly to the gene Os05g0574500. The predicted protein sequence of OsRAN2 (corresponding to the nucleotide sequence of AB015972) was aligned with related sequences from Arabidopsis (AtRan1, AtRan2, AtRan3, and AtRan4), wheat (TaRan1), and human (Ran/TC4) (Fig. 1). The alignment showed high sequence homology, 77% at the amino acid level, between OsRan2 and its human counterpart. The characteristic domains of the Ran proteins known to be involved in GTP-binding and hydrolysis, as well as the acidic C-terminal domain and the effector-binding domain have been highly conserved in most Ran proteins of different organisms. Only AtRan4 lacks the acidic domain and the effector-binding domain, suggesting this Ran may have a distinct role or subcellular localization (Ma et al., 2007).
its lowest level of 27% of the control 12 h after 100 mM KCl treatment, at which time the expression level had come back to almost 50% of the control from its lowest expression point in 200 mM KCl (Fig. 3C, D).

The effect of osmotic stress on OsRAN2 expression

To investigate the effect of osmotic stress on the expression of OsRAN2, 20% PEG 6000 was used to mimic osmotic stress in 3-week-old rice seedlings. The response was similar to the salinity stress treatments. The expression of OsRAN2 reached its lowest level of nearly 39% of the control 10 h after treatment, then it increased again to almost 68% of the control 24 h after treatment (Fig. 3E).

The effect of exogenous ABA on OsRAN2 expression

ABA is well-known as a key signal during plant responses to environment extremes such as salt and osmotic stresses. 50 or 100 μM ABA was applied to 3-week-old rice seedlings to determine the effect of ABA on OsRAN2 expression. It was found that ABA reduced the OsRAN2 expression level and the effect was dose-related. OsRAN2 expression was much lower at the same time point under 100 μM ABA treatment than under 50 μM ABA treatment (Fig. 4). At 0.5 h after treatment, the OsRAN2 expression level was about 76% of the control under the 50 μM ABA treatment, while
it was 45% of the control under the 100 μM ABA treatment (Fig. 4). The lowest level was observed 10 h after treatment in both concentrations, about 21% or 18.5% of the control under 50 μM and 100 μM ABA treatments, respectively. After that, the expression level began to rise again 24 h after treatment in both concentrations.

OsRAN2 overexpression in rice and Arabidopsis caused enhanced sensitivity to ABA, salinity, or osmotic stress treatments

To analyse the roles of OsRAN2 in plants further, OsRAN2 was overexpressed in rice and Arabidopsis under the control
of the double constitutive cauliflower mosaic virus (CaMV) 35S promoter. Hygromycin-resistance was used to select the transformants. Specific primers for the hygromycin gene were designed to perform PCR on genomic DNA from transgenic lines or the wild type (negative control) (data not shown). 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 OsRAN2 expression in the transgenic Arabidopsis or rice lines, respectively. As expected, it was found that OsRAN2 was over-expressed in both transgenic rice and Arabidopsis plants (Figs 5A, 6A).

Homologous transgenic T2 or Zhonghua11 (control) seeds were immersed in water with or without 100 mM NaCl, 100 mM KCl, 10% PEG 6000, or 10 μM ABA at 28 °C. Although transgenic plants were smaller than control plants in water, seedling growth in transgenic lines was severely

![Graph](https://example.com/graph.png)

**Fig. 5.** Stress or ABA sensitivity of wild-type and transgenic rice seedlings. (A) Expression level of OsRAN2 in transgenic rice, with Zhonghua 11 as control. Data were means ±SE (n=3); (B) photographs were taken after 5 d of growth in water containing 0 (control), 100 mM NaCl or 100 mM KCl, 10% PEG 6000 or 10 μM ABA; (C) photographs were taken after 10 d of growth in water containing 0 (control), 100 mM NaCl or 100 mM KCl, 10% PEG 6000 or 10 μM ABA.

![Images](https://example.com/images.png)

**Fig. 6.** Stress or ABA sensitivity of wild-type and transgenic Arabidopsis seedlings. (A) OsRAN2 expression level in transgenic Arabidopsis, wild-type plants as control; (B) NaCl or KCl stress sensitivity of wild-type or transgenic seedlings. Photographs taken following 2 weeks of growth on media containing 0 (control), 100 mM NaCl or 100 mM KCl; (C) ABA sensitivity of wild-type or transgenic seedlings. Photographs taken following 3 weeks of growth on media containing 0 (control), 0.25 μM ABA or 0.5 μM ABA; (D) sensitivity of wild-type or transgenic seedlings following 3 weeks of growth on media containing 0 (the left side of the photo) or 0.25 μM ABA (the right side of the photo).
suppressed, much more than that of control by salinity, osmotic stress, or ABA after 5 d under stress conditions or ABA application. Even after 10 d, transgenic lines still showed shortened and fewer roots and their leaves were smaller than those of control plants (Fig. 5B, C).

When overexpression and wild-type Arabidopsis seeds were grown in MS medium with or without NaCl, KCl or ABA, salinity stress (100 mM NaCl and 100 mM KCl) or exogenous ABA (0.5 μM) clearly suppressed seedling growth of the overexpressed lines more than those of wild-type plants (Fig. 6B, C). Under 0.25 μM exogenous ABA treatment, growth-suppression was not as severe as that under 0.5 μM ABA treatments, with only transgenic line 1 showing obviously suppressed seedling growth and some individual plants of the other transgenic lines showing a recovery of seedling growth (Fig. 6D).

Plants transformed with OsRAN2-dsRNA display pleiotropic developmental abnormalities

To silence the OsRAN2, a dsRNA approach was applied. Full-length cDNA was placed in both antisense and sense orientations in a binary plasmid under the control of a constitutive promoter (Fig. 7A). An RNA molecular marker transcribed from the fused fragments is capable of forming a dsRNA stem with a single-stranded terminal loop (Fig. 7A), a structure that can potentially induce silencing of the OsRAN2 by the RNAi mechanism. Eleven transgenic rice lines were generated and confirmed by genomic PCR (Fig. 7B). OsRAN2 expression was determined to be efficiently reduced in differently transgenic lines by semi-quantitative RT-PCR (Fig. 7C, D). Thus the dsRNA strategy was effective in simultaneously silencing the expression of OsRAN2. In addition, OsRAN1 was also detected to be reduced (Fig. 7C, D).

Compared to Zhonghua 11 plants, RNAi plants had notable developmental defects (Fig. 8). In the vegetative growth stage, Lines 10 and 11 had severely arrested seedling growth compared to Zhonghua 11 plants (Fig. 8A–C). They became etiolated and died. Abnormal tillering of the transformants was observed in Lines 2, 4, and 9 compared to Zhonghua 11 plants (Fig. 8D–G). In the generative phase, RNAi plants showed abnormal inflorescence development including brown spikelets, mouth-open spikelets, abnormal lateral branching development, and incorrect lateral meristem identity (Fig. 8H–M). Seed set rate of RNAi transformants was nearly zero so that no successive T1 seeds were harvested. The pollen viability of RNAi plants was tested by the I2-KI staining method and it was found that not only did RNAi plants have fewer pollen grains but also pollen grains had very low viability compared with the wild-type plants (Fig. 9A–F). Abnormal pollen development was confirmed by SEM assay of pollens from RNAi plants (Fig. 9G–I). All the results indicated that RNAi plants were male sterile.

OsRAN2 overexpression altered ABA- or stress-responsive gene expression in Arabidopsis

Transcriptional regulation of gene expression is relatively well understood during the molecular response of plants to abiotic stresses (Zhu, 2002; Chinnusamy et al., 2005, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Thus it was questioned whether OsRAN2 overexpression resulted in a sensitive response to abiotic stresses via the regulation of stress-responsive gene expression. To examine this hypothesis, the expression levels of five ABA- or stress-responsive genes, AtRAB18, AtCOR15A, AtNCED3 (Verslues et al., 2006b), AtPLC1, and AtMYB2 (Liu and Zhu, 1997) were determined in transgenic Arabidopsis plants with or without stress treatments by qPCR. AtCOR15A and AtRAB18 displayed no difference in expression levels between

![Fig. 7.](https://academic.oup.com/jxb/article-abstract/61/3/777/475625) Strategy for silencing OsRAN2 and gene expression in transgenic plants assayed by semi-quantitative RT-PCR. (A) Strategy for silencing OsRAN2; (B) genomic PCR using HTP specific primers. Lanes 1–11 refer to the 11 transgenic lines, respectively, and lane 12 was the negative control with Zhonghua 11 DNA as template. (C) Transcriptional level of genes assayed in transgenic lines. UBI was used as positive control; CK=control; (D) Densitometric ratio, Zhonghua 11=control.
transgenic plants and wild-type plants under normal conditions, stress, and exogenous ABA application conditions (data not shown). Increased expression levels of \( \text{AtNCED3} \) were induced by NaCl and ABA treatments in transgenic plants (Fig. 10A). In addition, \( \text{AtPLC1} \) had a higher expression level in transgenic plants under normal condition, and an increased expression level was induced only after NaCl treatment, while after treatments with KCl and ABA, \( \text{AtMYB2} \) had higher expression in transgenic plants (Fig. 10B, C).

**Overexpression of OsRAN2 in plants disturbs nuclear import**

The role of OsRAN2 in plant nuclear import was investigated by testing the effect of \( \text{OsRAN2} \) overexpression on the localization of a nuclear protein. The maize transcription factor Lc was used as a nuclear import reporter (Shieh et al., 1993). Lc was fused with YFP and transient expression based on ballistic transient transformation was applied as an assay. First, \( \text{Nicotiana tabacum} \) L. cv. Gexin No. 1 was transiently transformed with pA7-Lc-YFP and Lc-YFP was found to be localized exclusively in the nucleus (Fig. 11A). Subsequent co-expression of Lc-YFP with CFP-OsRAN2 showed CFP-OsRAN2 localization mainly in the nucleus with some detected in the cytoplasm, consistent with our previous subcellular localization results. However, Lc-YFP, with its primary localization in the nucleus was also present at low levels in the cytoplasm (Fig. 11B).

**Discussion**

OsRan2 shares high amino acid identity with its animal counterpart and similar subcellular localization

The roles of Ran in plants remain elusive (Yang, 2002), but its function in protein and RNA trafficking in and out of the nucleus is well understood in mammalian and yeast cells (Görlich and Kutay, 1999). In addition, Ran has been reported to have functions in mitotic spindle and nuclear envelope (NE) assembly (Quimby and Dasso, 2003; Di Fiore et al., 2004; Ciciarello et al., 2007). The high sequence homology at amino acid level (Ach and Gruissem, 1994; Haizel et al., 1997; Ma et al., 2007) and the similar pattern of intercellular localization (Ach and Gruissem 1994) suggests that plant Ran proteins may have similar roles to their animal counterparts. In the present study, our results showed that, at the amino acid level, OsRan2 shared 77% sequence identity with its human counterpart Ran/TC4. The subcellular localization results indicated that OsRan2 is mainly localized in the nucleus but with some in the cytoplasm (Fig. 2), consistent with its animal counterparts. Former studies indicated that Ran is predominantly a nuclear protein in mammalian tissue and cultured Saccharomyces cerevisiae cells (Bischoff and Ponstingl, 1991; Belhumeur et al., 1993; Ren et al., 1993). Consequently, it is predicted that OsRan2 might be involved in nucleo-cytoplasmic trafficking similar to that of animals, in addition to roles in mitotic process.
Fig. 9. Reduced pollen amount and viability in RNAi plants. I$_2$-KI staining of pollen grains from Zhonghua 11 (A, B) and RNAi plants (C, D for Line 1; E, F for Line 6). The stainable dark-blue grains are viable grains while unstainable reddish ones are unviable ones. The whole pollen grains of each anther were stained and photographs were taken. Bars=1 mm. SEM of pollen grains from Zhonghua 11 (G), showing the presence of normal and round grains; SEM of pollen grains from RNAi plants (H, I for Line 1 and Line 6, respectively), showing the presence of abnormal grains.
OsRAN2 negatively responds to abiotic stress stimulus and OsRAN2 overexpression caused transformants hypersensitive to abiotic stress

Chinnusamy et al. (2008) reported that nucleo-cytoplasmic trafficking through Ran-dependent karyopherin proteins might provide an alternative regulatory way in response to abiotic stress. However, very few reports regarding Ran or its specific binding proteins involved in abiotic stress response are available. The present study showed that OsRAN2 expression was reduced under salinity and osmotic stresses (Fig. 3) as well as exogenous ABA treatment (Fig. 4). Furthermore, transgenic rice and Arabidopsis overexpressing OsRAN2 displayed hypersensitivity to abiotic stresses compared to wild-type plants (Figs 5, 6). All these results indicated that Ran, at least in part, influences the abiotic stress response. Mutation in SAD2 in Arabidopsis caused hypersensitivity to ABA, salt, and PEG treatments (Verslues et al., 2006b). SAD2 encodes an importin β, which is a transporter receptor in nucleo-cytoplasmic trafficking. Ran is a required factor in importin β-mediated nucleo-cytoplasmic transport. Thus it is hypothesized that OsRan2' roles in abiotic stress might be somehow related to importin β' roles in abiotic stresses.

**Induced ABA- or stress-related genes in transgenic Arabidopsis plants under stresses**

Transcriptional regulation of gene expression belongs to the main mechanisms during abiotic responses (Zhu, 2002; Chinnusamy et al., 2005, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Three ABA- or stress-related genes (AtNCED3, AtPLC1, and AtMYB2) (Liu and Zhu, 1997; Xiong et al., 2002; Verslues et al., 2006b) detected here were found to have induced increased expression in transgenic Arabidopsis seedlings compared to wild-type ones. The induction patterns were unique among each treatment, where NaCl treatment induced both AtNCED3 and AtPLC1 expression, KCl treatment induced AtMYB2 expression, and ABA application induced AtNCED3 and AtMYB2 (Fig. 10).

Phospholipase C (PLC) catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(2)) to produce inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), which are second messengers in stress signalling (Mahajan and Tuteja, 2005). Nine AtPLC genes have been characterized in the Arabidopsis genome. AtPLC1 is strongly induced by various environmental stimuli, including salt, drought, and cold (Hirayama et al., 1995; Tasma et al., 2008). Exogenous ABA can also induce its expression (Tasma et al., 2008). AtMYB encodes a putative transcriptional factor related to the MYB protein and is induced by drought and salt stress as well as by ABA treatment (Abe et al., 1997; Liu and Zhu, 1997; Zhu et al., 1998). However, little is known about the mechanisms inherent in AtPLC and AtMYB stress signalling. It is speculated that OsRAN2 is at least partially involved in NaCl or KCl stress signalling, through regulation of the expression of AtPLC1 and AtMYB2.

ABA plays a critical role in response to various stress responses. The 9-cis-epoxycarotenoid cleavage reaction, catalysed by NCED, is considered to be a rate-limiting step in ABA biosynthesis (Mahajan and Tuteja, 2005). Xiong et al. (2002) reported that AtNCED3 expression was induced by NaCl treatment in wild-type Arabidopsis (C24, Landsberg, and Columbia backgrounds). Some induction of NCED3 by

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**Fig. 10.** Gene expression in wild-type and transgenic Arabidopsis plants in response to NaCl and KCl stress, or ABA treatment. One-week-old wild-type and transgenic seedlings were transferred to plates containing 0 (control, CK), 150 mM NaCl, 150 mM KCl, and 100 μM ABA for 6 h. Real-time PCR was used to analyse AtNCED3, AtPLC1, and AtMYB2 expression. Data were means ± SE (n=3). Asterisks indicate a significant difference (P ≤0.05) between transgenic and wild-type plants for the indicated treatment.

**Fig. 11.** Overexpression of OsRAN2 in plants disturbs nuclear import. (A) YFP fluorescence of Lc-YFP in tobacco leaf epidemic cells. (B) YFP fluorescence of Lc-YFP coexpressed with OsRAN2. DIC, differential interference contrast. Bars=50 μm.
ABA application was also observed in wild-type Arabidopsis (Landsberg and Columbia). Xiong et al. (2002) found that AtNCED3 expression levels under stress were significantly reduced in ABA-deficient mutants (los5 and los6) compared with wild-type seedlings, demonstrating that ABA is required for full AtNCED3 activation during osmotic stress (Xiong et al., 2002). The present study showed that AtNCED3 expression was up-regulated more in transgenic plants by NaCl and ABA applications. Therefore, under NaCl stress, the greater expression level of AtNCED3 might cause more ABA accumulation in OsRAN2-overexpressed seedlings, potentially resulting in enhanced NaCl sensitivity.

**Disturbed nuclear import by OsRAN2 overexpression**

Nucleo-cytoplasmic partitioning of regulatory proteins serves an integral role in pathways critical for environmental and developmental signalling (Merkle, 2003) in plants, including light responses (Stacey et al., 1999; Yang et al., 2000; Liu et al., 2008), hormone signalling (Hutchison and Kieber, 2001; Itoh et al., 2002), disease resistance (Kinkema et al., 2000), and abiotic stress responses (Lee et al., 2001). Recent research on Ran and/or its binding proteins revealed that Ran might participate in light responses, auxin sensitivity, and pathogen resistance (Wang et al., 2006; Cho et al., 2008; Lee et al., 2008); and these responses may, in part, result from protein import regulation and/or RNA export (Wang et al., 2006; Cho et al., 2008; Lee et al., 2008; Xu and Meier, 2008). If this is the case, it is suggested that Ran is involved in abiotic response signalling by regulating the transfer of specific proteins or RNA into or out of the nuclear envelope under different abiotic stresses. Moreover, it was found that co-expression of OsRAN2 with maize Lc, which is a reporter gene for the nuclear import (Shieh et al., 1993) that locates extensively in the nucleus (Fig. 11A), resulted in a low level of maize Lc being localized in the cytoplasm as well as mainly in the nucleus (Fig. 11B). This result illustrated that OsRAN2 overexpression did indeed alter the nuclear import and suggests that OsRAN2 overexpression results in hypersensitivity to salinity, osmotic stresses or exogenous ABA through disturbing the traffic of key regulatory proteins.

Plants transformed with RAN2-dsRNA display pleiotropic developmental abnormalities

To study OsRAN2’s function in abiotic stresses further, RNAi plants were generated in which OsRAN2 expression was efficiently reduced (Fig. 7). The RNAi plants were male sterile (Fig. 9) and they displayed pleiotropic developmental abnormalities such as small size, abnormal tillering, and inflorescence development (Fig. 8). OsRANI was expressed at a reduced level (Fig. 7C, D). It was presumed that all the phenotypic defects of RNAi plants were likely to result from reduced expression of OsRAN1 and OsRAN2. Evidence was provided that the abnormality during the Ran GTPase cycle might lead to male sterility (Wu et al., 2007). RAN3, a member of the Ran GTPase subfamily, was highly up-regulated in the sterile plants of the homozygous DGMS (dominant genic male sterility) two-type line, Rs1046AB. The higher expression of RAN3 in the sterile plants may cause the abnormal assembly of the spindle and the abnormal nuclear trafficking, which may be the reasons of male sterility. Importin z, another gene involved in the Ran GTPase cycle, could not be detected in sterile anthers, while it was only expressed in early buds of normal anthers. Thus, reduced Ran (RAN1 and RAN2) transcriptional expression may cause an abnormal Ran GTPase cycle, including abnormal nuclear trafficking and abnormal mitotic spindle or nuclear envelope assembly, which finally may result in male sterility and other developmental defects in RANi plants.

**Supplementary data**

Supplementary data are available at JXB online.

**Supplementary Table S1.** Gene-specific primers used in qPCR experiments.

**Supplementary Table S2.** Primers used in plasmid constructions.

**Supplementary Table S3.** Primers used in RT-PCR in detecting gene expression level in transgenic plants.

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