ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(6): 393–402 **CN** 31-1940/Q

Rice GTPase *OsRacB*: Potential Accessory Factor in Plant Salt-stress Signaling

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Abstract As the sole ubiquitous signal small guanosine triphosphate-binding protein in plants, Rop gene plays an important role in plant growth and development. In this study, we focus on the relationship between the novel rice Rop gene *OsRacB* and plant salt tolerance. Results show that *OsRacB* transcription is highly accumulated in roots after treatment with salinity, but only slightly accumulated in stems and leaves under the same treatment. Promoter analysis showed that *OsRacB* promoter is induced by salinity and exogenous salicylic acid, not abscisic acid. To elucidate its physiological function, we generated *OsRacB* sense and antisense transgenic tobacco and rice. Under proper salinity treatment, sense transgenic plants grew much better than the control. This suggests that overexpression of *OsRacB* in tobacco and rice can improve plant salt tolerance. But under the same treatment, no difference could be observed between *OsRacB* antisense plants and the control. The results indicated that *OsRacB* is only an accessory factor in plant salt tolerance.

Key words *OsRacB*; rice; salt tolerance; promoter; transgenic analysis

Accumulation of salts in irrigated soil is a primary factor in depressing yield in crop production. Previous studies showed that products of some stress-inducible functional genes could directly counteract this detrimental condition. Transfer of these genes into plants confirmed their protective roles in stress adaptation. But the effectiveness of an individual gene is normally rather small [1,2]. It was generally thought that salt stress was regulated by a complex signaling network, and modulation of signaling regulators would be a promising method for improving plant salt stress tolerance. So it is thrilling to study the molecular mechanisms of salt tolerance underlying signal transduction, although little has been known on the topic until now [1].

Small guanosine triphosphate (GTP)-binding proteins are pivotal molecular switches in signal transduction. They are normally divided into five families, including Ras, Rho, Rab, Ran and Arf/Sar [3,4]. Among these proteins, only Ras and Rho are generally considered as signaling proteins to transmit extracellular signals in yeast and animals. Although Ras plays a crucial role in cellular signaling in animals and various lower eukaryotes, *Arabidopsis* genome sequencing reveals no Ras homologs [4]. In mammals, the Rho family contains three subgroups of proteins, including Rac, cell division cycle protein 42 (Cdc42) and Rho. In plants, however, only one specific subgroup, slightly more similar to Rac than to Rho, exists and is designated as Rop (Rho of plant) [3,5–7]. Rop genes are ubiquitous and various in plants. Based on the distributive difference between the two signal small GTP-binding proteins in plants, Rops were popularly regarded as the sole family of signaling small GTPase in plants [4]. Hence, Rops have attracted a great deal of attention over the last 10 years, since their initial identification in pea in 1993 [8–11].

Previous studies showed that Rops participate in signaling to a series of physiological processes including actin cytoskeleton remodeling, secondary wall formation, intracellular Ca^{2+} gradients establishment, regulation of polar cell growth, production of reactive oxygen intermediates, and modulation of hormone signaling and gene expression. During these studies, the function of Rop in biotic stress response was noted. The importance of Rops in defence

Received: January 15, 2006 Accepted: March 26, 2006
This work was supported by the grants from the National Plant Transgenic Research and Industrialization Special Foundation of China (No. J00-A-005), the National High Technology Research and Development Program of China (No. 2002A224061) and the National Basic Sciences Initiative (No. 2001CB1088)
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DOI: 10.1111/j.1745-7270.2006.00172.x

mechanisms has been shown in at least three plant micro-interactions, such as OsRac1 function in the rice-rice blast fungus and rice-rice bacterial leaf blight systems [12–14], and HvRacB action in the barley-barley powdery mildew fungus system [5,14,15]. But little is known about its function in abiotic stress response, except for one study of Rop action in transient flooding endurance [16]. So it is significant to study how Rop acts in abiotic stress response such as salinity resistance.

During our previous work, a novel rice Rop gene *OsRacB* was isolated from a young ear cDNA library [17]. Structural analysis and *in vitro* GTP binding test identified *OsRacB* as a new member of the Rop family [17,18]. *OsRacB* encoded a putative protein of 197 amino acids. Its transcription unit was 2930 bp in length, consisting of seven exons and six introns. Genomic Southern blotting revealed that *OsRacB* was a low abundance gene. Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that *OsRacB* was ubiquitously expressed in various tissues, but the expression level in stems and young ears was much higher than that in other organs. *OsRacB* showed very high amino acid homology to maize ZmRacB (99.5%), which can induce superoxide production when expressed in a mammalian system (NIH 3T3 cells) [19], and barley HvRacB (99.0%), which was involved in processes supporting parasitic entry into epidermal host cells [5,14,15].

In this paper, we analyzed *OsRacB*'s expression pattern, promoter regulation and transgenic plant phenotype under salinity stress. Results suggest that *OsRacB* might play an accessory role in plant salt tolerance.

Materials and Methods

Plant growth and stress treatment

Seeds of late ripening japonica rice cultivar (cv.) Nongken 58 were obtained from Prof. Tong-Min MOU (Huazhong Agricultural University, Wuhan, China). Plants were grown in soil in a growth chamber (14 h light/10 h darkness, 28 ºC). When they were grown to the five-leaf-old stage, plants were subjected to short-day conditions (9 h light/15 h darkness), which was required for this cultivar to enter into the reproductive stage, until differentiation of the primordial of secondary branches and florets (stage III).

In addition, seeds were germinated for 3 d and grown in nutrient solution comprising 0.1% (*V*/*V*) Hyponex (water) (Hyponex Co., Copley, USA) for 20 d under normal conditions at 28 ºC and 14 h light/10 h darkness. For osmotic stress treatment, 20-day-old (three-leaf-old) rice seedlings were grown in nutrient solution containing 200 mM NaCl, and incubated for 0–48 h under a normal light/dark cycle at 28 ºC.

Semiquantitative RT-PCR analysis

Following the procedure provided with the SuperScript II kit (Gibco BRL), reverse transcription of total RNA was carried out using Oligo(dT)$_{12–18}$ (Invitrogen). A set of primers specific for the rice actin gene *actin 1* (X15865), 5′-CATGCTATCCCTCGTCTCGACCT-3′ and 5′-CGCACTTCATGATGGAGTTGTAT-3′, were synthesized and used in RT-PCR as internal control. Amplification was carried out under the following conditions: 94 ºC for 5 min; 94 ºC for 1 min, 54 ºC for 0.5 min, 72 ºC for 0.5 min, 25 cycles; 72 ºC for 7 min. *OsRacB* was amplified by RT-PCR, based on its specific primer pair 5′-TTGCTTTGCTCCTCCTTCAACCTT-3′ and 5′-GCCACGACTTGTCAGTCACACG-3′. PCR thermocycling profile was 94 ºC, 60 ºC and 72 ºC, 1 min each step, for a total of 26–28 cycles. PCR products were analyzed through the Gel-Doc 2000 system (Bio-Rad).

5′-Deletion analysis of *OsRacB* promoter

Based on previous research [17], we isolated *OsRacB* promoter and subcloned it into pBluescriptII SK(+) vector (Stratagene). Four 5′ deletion *OsRacB* promoter fragments were constructed by PCR for promoter element analysis. They were covered from –1241 to +175, –721 to +175, –481 to +175, –281 to +175. 5′ deletion vectors p1301-BPn-β-glucuronidase (GUS) were constructed by cloning these fragments into *Xba*I/*Bgl*II sites of pCAMBIA1301 instead of its original constitutive cauliflower mosaic virus (CaMV) 35S promoter. To construct the negative control vector p1301-CK-GUS, pCAMBIA1301 was digested with *Xba*I and *Bgl*II to delete the original 35S promoter, and circularized by blunt end ligation after turning adhesive ends to blunt ends. Both p1301-BPn-GUS, pCAMBIA1301 and p1301-CK-GUS vectors were separately transformed into *Agrobacterium tumefaciens* EHA105 by electroporation and introduced into tobacco cv. NC89. Transformed tobaccos were screened by 50 mg/L hygromycin and confirmed by Southern blot and RT-PCR analysis. Excised leaves from transgenic plants were tested for GUS activity using quantitative fluorometric assay [20]. During the treatments, leaves were cut into flakes approximately 1 cm^2 and put into liquid culture 1/2 MS for 4 h with or without different treatments such as 50 M abscisic acid (ABA), 200 μM salicylic acid (SA) or 200 mM NaCl sepa-

rately at 22 ºC.

Construction of *OsRacB* binary vectors and plant transformation

Sense and antisense *OsRacB* vectors, pWMSB and pWMAB, were constructed by cloning full-length *OsRacB* cDNA into the *Kpn*I/*Bam*HI site or *Xba*I/*Bam*HI sites, respectively, of plant expression vector pWM101, in sense or antisense orientation under the control of enhanced CaMV 35S promoter. Using the *Agrobacterium*-mediated method [21,22], these *OsRacB* vectors were separately introduced into tobacco cv. NC89 and rice cv. Nongken 58 cells. In addition, blank vector pWM101 was introduced into the plants as the control. PCR and Southern hybridization were sequentially used to verify transgenic plants. Transformed calli were selected by hygromycin resistance, and regenerated. In a growth chamber (16 h light/8 h darkness, 28 ºC), transgenic tobaccos were separately grown in 16 cm diameter garden pots. Transgenic rice was grown in 30 cm diameter garden pots with three types grown under normal conditions.

Estimation of *OsRacB* transgenic plants salt tolerance

In a growth chamber (16 h light/8 h darkness, 28 ºC), sterilized T1 transgenic tobacco seeds were germinated on 1/2 MS agar medium [23] containing 0–400 mM NaCl used as salt-grade for salinity treatment. To eliminate environmental influences, transgenic tobaccos and the control were grown in the same culture dish.

At the same time, T1 transgenic rice seeds were germinated for 3 d and separately transplanted in 8 cm×8 cm garden pots. For salt-stress treatment, 20-day-old (three-leaf-old) rice seedlings were irrigated for 10 d with water containing 200 mM NaCl, then rinsed with water, and observed three days later. During the treatment, plants were all grown under the 14 h light/10 h darkness cycles at 28 ºC.

Results

OsRacB is highly accumulated in roots upon exposure to salt stress

The conserved Rho family of GTPases in fungi and mammals has emerged as a key regulator in stress-induced signaling. But in plants, almost all insights into Rop's defense function were focused on biotic stress [4,24,25]. In order to investigate the *OsRacB* transcription pattern under salinity stress, we analyzed various tissues of 20-day-old (three-leaf-old) rice that had been treated with 200 mM NaCl. Because of low abundance of *OsRacB*, which could be detected only poorly by Northern blot analysis [17], an RT-PCR approach was adopted with special primers. **Fig. 1** shows that *OsRacB* was highly accumulated in roots after treated with salinity, but was only partly accumulated in leaves and very slightly in stems. These results suggest that *OsRacB* probably plays a role in salt tolerance, especially in root tissue.

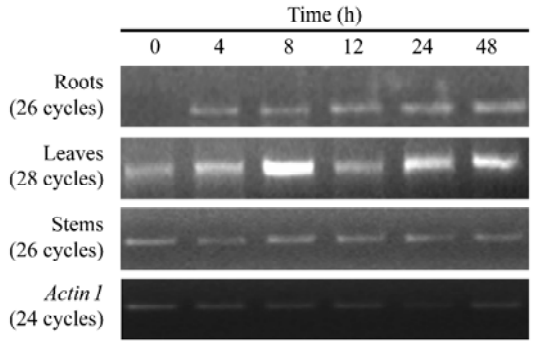

Fig. 1 Expression of novel rice Rop gene *OsRacB* in various rice tissues under salt stress

Rice actin gene *actin 1* was used as a constitutively expressed reverse transcription-polymerase chain reaction control. Twenty-day-old rice (*Oryza sativa* cv. Nongken 58) seedlings were grown in nutrient solution in a greenhouse with a 14 h light/10 h darkness cycle at 28 ºC, and 200 mM NaCl salinity treatment was conducted under the same growth conditions.

Identification of promoter sequence that regulates expression of *OsRacB*

Using *OsRacB* intron IV (nt 1957–2265) and the 5′-coding region (nt 1–327) as specific probes, we isolated *OsRacB* promoter by screening a rice genomic DNA library [17]. Analyzed by PLACE software (http://www.dna.affrc.go.jp/PLACE/), some putative regulation elements were found, such as cytoskeleton-related element (I-box), ABA responsive element (ABRE), SA response element (W box) and SEBF-specific binding sequence PyTGTCNC [26–29]. All of these elements were also found in other plant Rop promoters [30]. It was implied that *OsRacB* basic working mechanism was similar to other Rops.

To clarify its regulation pattern in plants, different 5′ deletion fragments of *OsRacB* promoter were isolated by PCR and inserted in transgenic vector pCAMBIA1301 instead of the original CaMV 35S promoter. These vectors were named p1301-BP*n*-GUS (with *n* representing A, B,

C or D). **Fig. 2** shows all deletions and their locations with respect to the structure of *OsRacB* genomic clone. To eliminate the original GUS background in transgenic plants, p1301-CK-GUS vector was constructed with original 35S promoter deleted to act as a negative control. All of these constructs and the positive control pCAMBIA1301 were introduced into tobacco cv. NC89 by means of *Agrobacterium*-mediated transformation. Transgenic plants were regenerated from hygromycin-resistant transformants. Excised leaves from transgenic plants were tested for GUS activity using quantitative fluorometric assay [20].

Functional studies showed that many plant Rop genes were regulated by ABA or entered into some defense signaling [4,12,21,24]. It was also generally thought that salt tolerance was regulated by different signaling pathways, which were ABA-dependent or ABA-independent [31]. So we selected ABA and SA, which plays a signal role in hypersensitive cell death and disease resistance [32], to treat transgenic excised leaves. Based on our previous abiotic stress studies, we also researched the effect of salinity on *OsRacB* promoter. **Fig. 3** summarizes the quantitative analysis of GUS expression in transgenic plants. For each deletion in the series, a large number of independent transformants were regenerated and assayed. **Fig. 3(A,B)** shows that ABA has a very slight effect on pA, pB and pC deletions, whereas SA has an apparent inducement effect on pA and pC deletions of *OsRacB* promoter. **Fig. 3(C)** shows that NaCl could evidently induce the activity of pC deletion, but it has no inducement effect on pB and pD deletion. Results showed that it might have some elements related to salinity response in the −281 to −481 fragment of *OsRacB* promoter, whereas some inhibitive factors were presented in the −481 to −721 area. In addition, during these experiments, pD deletion showed no response to any inducers, although there did exist an ABA responsive element. These results showed that *OsRacB* promoter can be induced by salinity and exogenous SA, but not by ABA. They implied that *OsRacB* might enter into some response to biotic and abiotic stress, based on the ABA-independent signaling pathway.

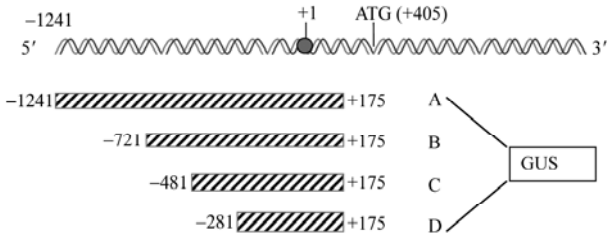

Fig. 2 Structure of the deletions used in the rice Rop gene *OsRacB* promoter study

The site of transcription initiation is indicated +1. GUS, β-glucuronidase.

Phenotypic and genetic analysis of transgenic tobacco transformed with sense and antisense *OsRacB*

To clarify the physiological role of *OsRacB* in plants, *OsRacB* cDNA was introduced into tobacco cv. NC89 cells in sense as well as antisense orientation under CaMV 35S promoter control by means of *Agrobacterium*-medi-

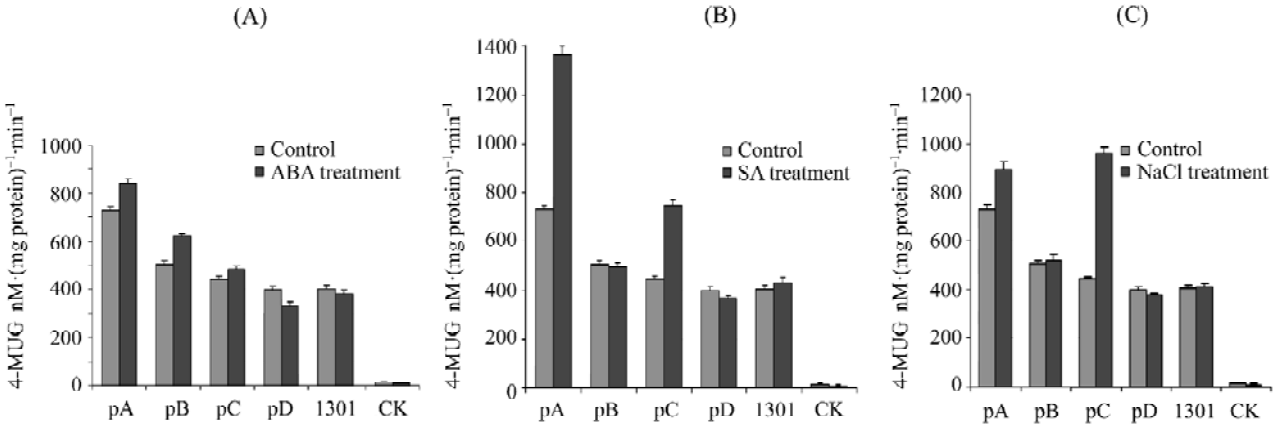

Fig. 3 β-glucuronidase assay of different rice Rop gene *OsRacB* promoter deletions in transgenic tobaccos under various treatments

All tobacco leaves were cut into 1 cm^2 pieces and put into nutrient solution comprising 1/2 MS at 22 ºC. (A) 50 μM abscisic acid (ABA) treatment. (B) 200 μM salicylic acid (SA) treatment. (C) 200 mM NaCl treatment.

ated transformation [**Fig. 4(A,B)**]. Six cDNAs encoding Rop protein homologs were isolated in tobacco [33–35], which are highly homologous to *OsRacB*. Heterologous expression of *OsRacB* in tobacco plants should influence the expression of endogenous Rop homologs. NtRac5, the highest homology tobacco Rop protein to *OsRacB* (91.4%), can regulate active oxygen species production by negative regulation of NtrbohD, an oxidase involved in active oxygen species production upon elicitation [33].

In order to simplify PCR-based molecular analysis of our transgenic plants, we designed a special 5′ primer based on the *hyp* gene sequence. The integration of hyp-35S P-OsRacB-cDNA in both types of transformants was confirmed by genomic PCR, without simultaneously amplifying endogenous Rop-related tobacco genes [**Fig. 4(C,D)**]. Twelve independent transformants with *OsRacB* in sense orientation, fourteen plants transformed with *OsRacB* in antisense orientation and six vector control plants were regenerated for further analysis. In order to minimize the interference of tissue culture, nine aseptic regenerated NC89 plants were obtained. Expression levels of these *OsRacB* transgenes were demonstrated by RT-PCR (data not shown). RT-PCR analysis confirmed that 12 transgenic tobacco plants transformed with *OsRacB* sense constructs expressed the foreign gene at mRNA level.

Several biological characters, such as shoot

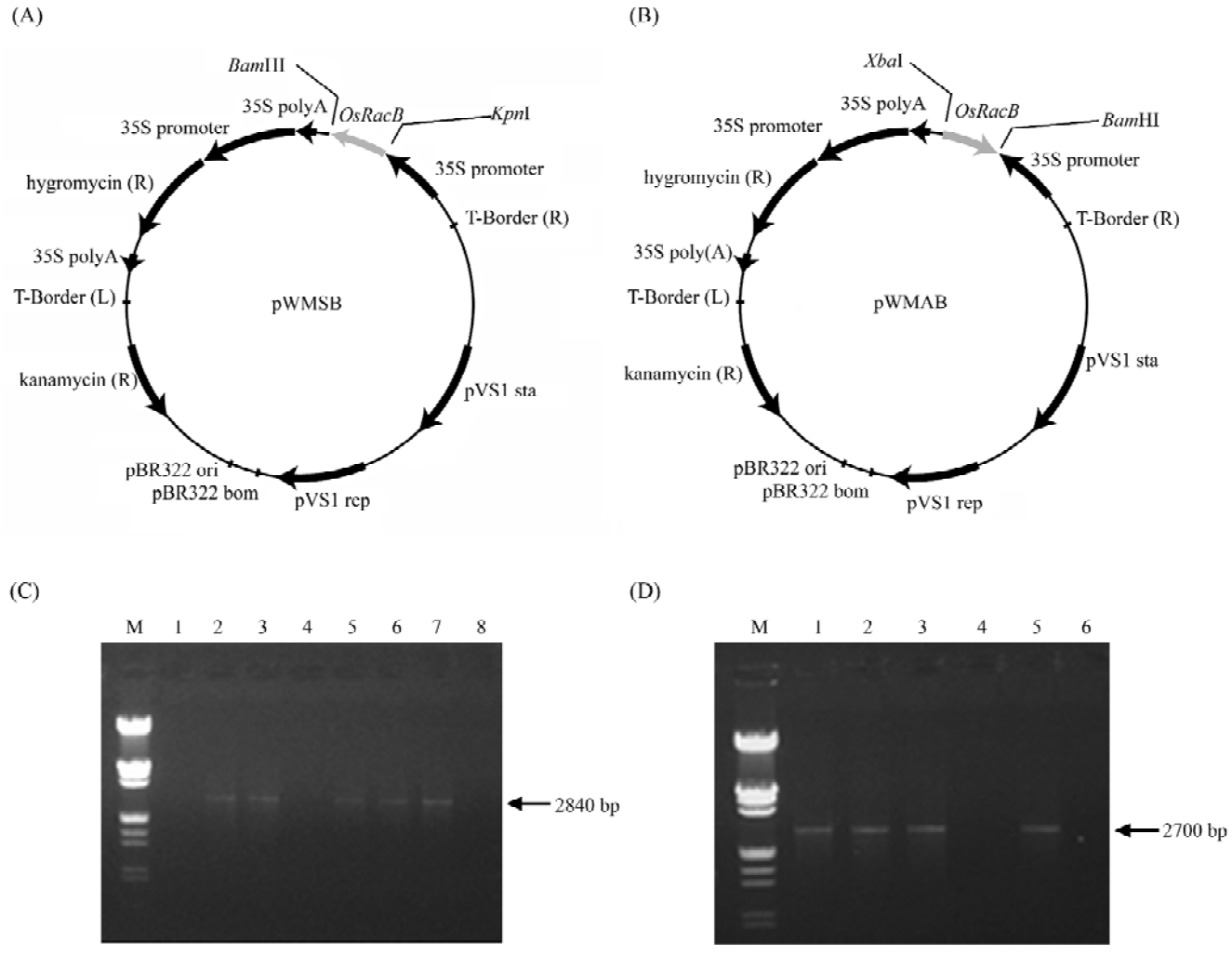

Fig. 4 Genetic analysis of tobacco cv. NC89 plants transformed with rice Rop gene *OsRacB* sense or antisense construction

(A) Sense expression vector pWMSB. (B) Antisense expression vector pWMAB. (C) Genetic analysis of tobacco *OsRacB* sense transformants by genomic polymerase chain reaction (PCR). Lanes 1–6 are test samples. Lanes 1 and 4 are negative samples, and lanes 2, 3, 5 and 6 are positive samples. At the same time, lane 7 is positive control and lane 8 is negative control. (D) Genetic analysis of tobacco *OsRacB* antisense transformants by genomic PCR. Lanes 1–4 are test samples. Lanes 1–3 are positive samples, and lane 4 is negative sample. At the same time, lane 5 is positive control and lane 6 is negative control.

Table 1 Properties of transgenic T0 and T1 tobacco progenies

Plant	*n*	Height range (cm)	Height (cm)	Stem perimeter (cm)	Seed weight (mg)
T0 *OsRacB*-sense tobacco	12	80–110	93.083±8.660 [a]	5.967±0.396 [b]	74.396±1.882 [b]
T0 *OsRacB*-antisense tobacco	14	30–80	50.643±15.184 [a]	5.529±0.343 [a]	69.942±2.305 [a]
T0 vector control	6	73–85	78.333±4.761 [b]	5.933±0.266 [b]	73.040±0.904 [b]
Wild-type control	9	74–85	77.000±4.555	5.911±0.226	74.491±2.085
T1 *OsRacB*-sense tobacco	14	79–140	101.143±21.045 [a]	5.871±0.324 [b]	75.099±1.614 [b]
T1 *OsRacB*-antisense tobacco	11	27–80	51.364±15.945 [a]	5.582±0.340 [b]	71.258±2.627 [b]
Wild-type control	9	70–85	77.889±4.676	5.756±0.207	74.589±1.580

Significant difference analysis is according to *t* distribution (α=0.05). [a] significant difference vs. corresponding wide-type control; [b] non-significant difference vs. corresponding wide-type control. For seed weight measurement, 1000 seeds for each group were measured.

development, weight of 1000 seeds, seed vitality and germination capacity, were compared between all types of T0 and T1 tobaccos (**Table 1** and **Fig. 5**). Compared with vector control plants and regenerated NC89, *OsRacB*-sense plants showed no differences except plant height. *OsRacB*-antisense plants showed distinct differences in height, stem perimeter and seed weight compared with wide-type control plants. This implied that expression changes in the *OsRacB* homolog affects tobacco shoot development, especially in plant height. Antisense expression of MS-*rac*1 cDNA in transgenic tobacco plants can also cause such growth inhibition and dwarfing [36].

To test whether *OsRacB* played a role in abiotic defence reactions, all types of tobacco plants were treated with salinity. **Fig. 6** shows that three tobaccos grew similarly in basic culture medium (1/2 MS) and low concentration salinity medium (100 mM NaCl+1/2 MS). Under proper salinity stress conditions (200 mM NaCl+1/2 MS), *OsRacB*-sense tobacco seedlings grew better than others. But in 300 mM NaCl salinity medium, the three types of transformants grew without any difference. None of the tobacco plants treated with 400 mM NaCl high concentration salinity could germinate. These results implied that overexpression of *OsRacB* in tobacco can partly improve its tolerance to proper salinity stress for plants.

Fig. 5 Morphology analysis of rice Rop gene *OsRacB* sense or antisense plants in comparison with a control plant transformed with blank vector alone

1, *OsRacB* sense plant; 2, control plant; 3, common *OsRacB* antisense plant; 4, one dwarfish *OsRacB* antisense plant.

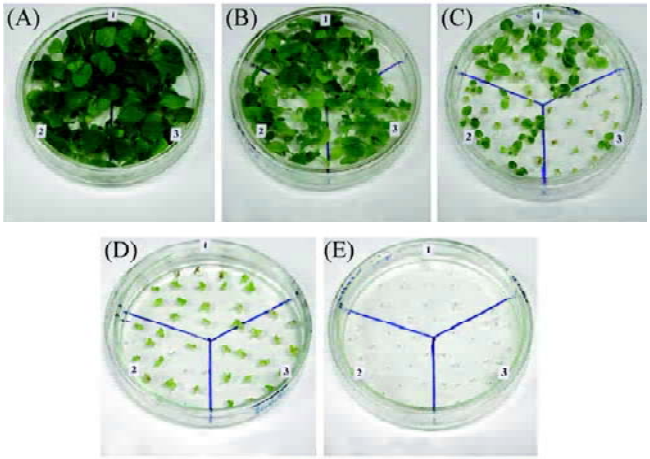

Fig. 6 Germination and growth experiment of different T1 tobacco transformant seedlings using different culture mediums

(A) 1/2 MS basic culture medium. (B) 1/2 MS+100 mM NaCl medium. (C) 1/2 MS+200 mM NaCl medium. (D) 1/2 MS+300 mM NaCl medium. (E) 1/2 MS+400 mM NaCl medium. 1, *OsRacB*-sense tobacco; 2, *OsRacB*-antisense tobacco; 3, wild-type NC89.

Overexpression of *OsRacB* cDNA in transgenic rice plants causes salt tolerance

To analyze *OsRacB* roles in rice *in vivo*, same transgenic vectors were also introduced into rice cv. Nongken 58 cells in sense as well as antisense orientations, as had been done in tobacco. Genomic PCR and Southern blot analysis confirmed the successful integration of *OsRacB* into chromosomes (data not shown). A highly sensitive RT-PCR, in which the number of amplification cycles was controlled, was developed to detect *OsRacB* expression modification in transgenic rice leaves. As shown in **Fig. 7**, we succeeded in modifying *OsRacB* expression levels in rice cv. Nongken 58 by transgenic technology.

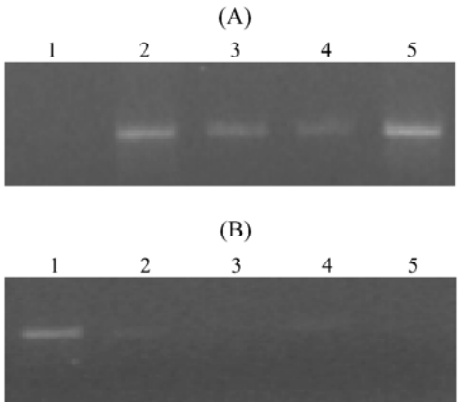

Fig. 7 Genetic analysis of rice Rop gene *OsRacB* expression levels in leaves of rice transformants by reverse transcription-polymerase chain reaction (RT-PCR)

(A) *OsRacB* transcript in *OsRacB*-sense rice. The number of PCR cycles was 25 for *OsRacB*. (B) *OsRacB* transcript in *OsRacB*-antisense rice. The number of PCR cycles was 29 for *OsRacB*. 1, cultivar Nongken 58; 2–5, rice transformants.

Based on previous studies, our insights were focused on how the altered *OsRacB* expression affected the salinity tolerance of rice plants. In **Fig. 8**, sense T1 transgenic rice treated with salinity stress grew much better than the control, suggesting that overexpression of *OsRacB* in rice can also partly improve salinity tolerance, as had been shown in tobacco. So it was inferred that *OsRacB* plays a role in the signal pathway of plant salt tolerance. There was no difference between *OsRacB* antisense plants and controls in their growth under salinity treatment, neither in transgenic tobacco nor in rice, which indicated that *OsRacB* does not play an indispensable role but is an accessory factor in plant salt tolerance. In addition, during the analysis of T0 and T1 transgenic rice, there were many anomalous growths and abortions in sense and antisense transgenic rice, which indicated that abnormal expression of *OsRacB* could induce lethality.

Discussion

As the most major crop in the world, a better understanding of stress signaling in rice has an enormous impact. In the present study, we identified a Rop protein, *OsRacB*, which acts as an accessory regulatory component in salt stress responses. Phylogenetic analyses showed that *OsRacB* belongs to Group IV of the Rop family [37]. Another rice Rop gene, *OsRacD*, and *Arabidopsis* Rops *AtRop*1 to *AtRop*6, also belong to this subgroup [4, 38]. In this group, most Rops are involved in actin dynamics

Fig. 8 Phenotypic analysis of T1 rice transformants 3 d after salt stress (200 mM NaCl for 10 d)

1 and 2, vector control plants; 3 and 9, cultivar Nongken 58; 4, 5 and 6, *OsRacB*-sense rice; 7 and 8, *OsRacB*-antisense rice.

regulation, which controls polar growth and root hair development. Other Rops were found to act in the production of active oxygen species, which can induce programmed cell death. But these similar proteins have not been attributed to salinity responses. This is the first report to suggest that Rops plays a role in salt tolerance signal pathways in plants.

Plants execute a wide range of physiological and metabolic processes to cope with adverse environmental conditions, such as high salinity or water deficit. Many genes have been found to respond to salt stress, but little is known about the signaling events involved in the mechanisms that make plants tolerant to high salt concentrations. It is popularly considered that the signaling pathway of salt tolerance is an intricate web of interconnecting signal networks rather than a collection of parallel but separate pathways. During the signal net, some phytohormones such as ABA and intracellular second messengers (phospholipids, Ca^{2+} and reactive oxygen species) regulate stress responses and contribute to the coordination of whole-plant responses to stress conditions. All of these signal molecules have proved to be linked to signal transduction and physiological functions related with Rop proteins.

One early response to salinity stress in plant cells is a transient increase in cytosolic Ca^{2+}, derived from either an influx from apoplastic space or release from internal stores [39,40]. Several lines of evidence suggested that many proteins related with Ca^{2+} release, such as calmodulin, calcineurin B-like proteins and calcium-dependent protein kinases, were involved in stress signal transduction [41]. In rice, OsCDPK7 has proved to act upstream of one small GTP-binding protein, rab16A, during the salt tolerance signaling pathway. Research into plant Rops has presented evidence that they also control tip growth through the regulation of tip-localized calcium influxes in pollen tubes and roots, such as the actions of *AtRop1* in pollen and *AtRop4* in roots [42,43].

Stimulated vesicle transport under salt stress is also an important physiological process to altered growth conditions, based on rapidly adjusted biochemistry of the lytic compartment [44]. Under salt stress, vacuolar volume and activity of vacuolar ATPase are often increased. Rab protein, the only known small GTP-binding protein involved in salt tolerance signal transduction, participated in regulating intracellular vesicular transport by activation of vesicle formation and fusion at the endoplasmatic reticulum, Golgi apparatus and plasma membrane. Studies in mammalian cells suggest that biogenesis and dynamics of endosomes and lysosomes, which share some functional and biogenetic similarities to plant vacuoles, involve Rho GTPase-dependent signaling pathways [45]. Using anti-Rop1Ps antibody and immunocytochemical techniques, Rop GTPase was also proved to mediate vacuole development in plants [46].

Salt stress also causes induction of oxidative stress. Under salt stress, stomatal closure triggered by ABA limits CO_2 supply to the leaf, leading to an over-reduction of the photosynthetic electron transport chain. This causes generation of active oxygen species such as hydrogen peroxide and superoxide anion. In addition, salt stress can also induce programmed cell death. Recent studies have revealed remarkable overlaps between the mechanisms underlying stress-, toxin- and pathogen-induced programmed cell death [47]. In the study of Rop function, it is popularly regarded that Rop proteins enter into signal transduction, which induces superoxide production and programmed cell death. *ZmRacB* and *HvRacB*, which share high homology with *OsRacB*, have proven functions in the production of active oxygen and disease defense [5,14,15,19].

In addition, research showed that G-proteins and mitogen-activated protein kinase (MAPK) cascades are also related to salt resistance. In plants, G-proteins regulate phospholipase C activity, which could modulate expression of many functional genes involved in stress signaling, such as LEA-like genes. It has been proved that the α-subunit of G protein acts upstream of Rop protein *OsRac1* in disease resistance of rice [48]. Co-immunoprecipitation showed that Rop could form a large signaling complex that includes kinase-associated protein phosphatases, CLV1 and CLV3, neighboring with *AtRop*4. It is implied that Rops might act upstream of an MAPK cascade [49].

As signaling proteins, Rho plays a very important role in the signal transduction network. It directly or indirectly participates in many signal pathways and acts as a pivotal molecular switch. In plants, research on Rop lagged behind because of its relatively late finding. Most studies have focused on polar growth, root hair development, and actions in biotic stress. In our report, we observed the phenomenon that Rop gene might enter into the signaling pathway of salt tolerance. This finding is a significant step toward a comprehensive understanding of the Rop family . Based on *OsRacB* expression patterns, the character of *OsRacB* promoter elements, and analysis of transgenic plants with other Rop research, we propose that *OsRacB* possibly participates in salt tolerance based on basic Rop gene mechanisms, such as Ca^{2+} regulation, production of active oxygen, induction of programmed cell death, and regulation of MAPK cascade. In-depth studies are expected

to shed light on the molecular mechanisms that determine how *OsRacB* acts in plant salt tolerance.

References

1 Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K. Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 2000, 23: 319–327

2 Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 1998, 10: 1391–1406

3 Winge P, Brembu T, Bones AM. Cloning and characterization of rac-like cDNAs from *Arabidopsis thaliana*. Plant Mol Biol 1997, 35: 483–495

4 Yang Z. Small GTPases: Versatile signaling switches in plants. Plant Cell 2002, 14: S375–S388

5 Schultheiss H, Dechert C, Kogel KH, Huckelhoven R. Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. Plant J 2003, 36: 589–601

6 Valster AH, Hepler PK, Chernoff J. Plant GTPases: The Rhos in bloom. Trends Cell Biol 2000, 10: 141–146

7 Zheng ZL, Yang Z. The Rop GTPase: An emerging signaling switch in plants. Plant Mol Biol 2000, 44: 1–9

8 Yang Z, Watson JC. Molecular cloning and characterization of rho, a ras-related small GTP-binding protein from the garden pea. Proc Natl Acad Sci USA 1993, 90: 8732–8736

9 Winge P, Brembu T, Kristensen R, Bones AM. Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. Genetics 2000, 156: 1959–1971

10 Vernoud V, Horton AC, Yang Z, Nielsen E. Analysis of the small GTPase gene superfamily of *Arabidopsis*. Plant Physiol 2003, 131: 1191–1208

11 Gu Y, Wang Z, Yang Z. ROP/RAC GTPase: An old new master regulator for plant signaling. Curr Opin Plant Biol 2004, 7: 527–536

12 Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H, Shimamoto K. The small GTP-binding protein Rac is a regulator of cell death in plants. Proc Natl Acad Sci USA 1999, 96: 10922–10926

13 Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K. Essential role of the small GTPase Rac in disease resistance of rice. Proc Natl Acad Sci USA 2001, 98: 759–764

14 Schultheiss H, Dechert C, Kogel KH, Huckelhoven R. A small GTP-binding host protein is required for entry of powdery mildew fungus into epidermal cells of barley. Plant Physiol 2002, 128: 1447–1454

15 Opalski KS, Schultheiss H, Kogel KH, Huckelhoven R. The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. Plant J 2005, 41: 291–303

16 Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J. RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. Science 2002, 296: 2026–2028

17 Luo M, Mi ZY, Qu H, Tang CR, Liang WH, Wu NH. Molecular cloning and structural analysis of a novel Rac gene *osRACB* in rice (*Oryza sativa* L.). Sci China Ser C 2003, 46: 481–494

18 Luo M, Tang CR, Wu NH. Prokaryotic expression and characterization of rice Rac protein osRACB. Acta Biochim Biophys Sin 2003, 35: 1149–1154

19 Hassanain HH, Sharma YK, Moldovan L, Khramtsov V, Berliner LJ, Duvick JP, Goldschmidt-Clermont PJ. Plant Rac proteins induce superoxide production in mammalian cells. Biochem Biophys Res Commun 2000, 272: 783–788

20 Jefferson RA. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 1987, 5: 387–405

21 Hiei Y, Komari T, Kubo T. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 1997, 35: 205–218

22 Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 1994, 6: 271–282

23 Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962, 15: 473–497

24 Agrawal GK, Iwahashi H, Rakwal R. Small GTPase 'Rop': Molecular switch for plant defense responses. FEBS Lett 2003, 546: 173–180

25 Luo M, Wu NH. Structure and function of Rac genes in higher plants. Progress in Natural Science 2003, 13: 721–729

26 Higo K, Ugawa Y, Iwamoto M, Korenaga T. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 1999, 27: 297–300

27 Busk PK, Pages M. Regulation of abscisic acid-induced transcription. Plant Mol Biol 1998, 37: 425–435

28 Yu D, Chen C, Chen Z. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 2001, 13: 1527–1540

29 Boyle B, Brisson N. Repression of the defense gene PR-10a by the single-stranded DNA binding protein SEBF. Plant Cell 2001, 13: 2525–2537

30 Xu YZ, You RL, Wu NH. Structural analysis and identification of cis-elements of rice osRACD gene. Acta Biochim Biophys Sin 2004, 36: 191–198

31 Ishitani M, Xiong L, Stevenson B, Zhu JK. Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: Interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell 1997, 9: 1935–1949

32 Alvarez ME. Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Mol Biol 2000, 44: 429–442

33 Morel J, Fromentin J, Blein JP, Simon-Plas F, Elmayan T. Rac regulation of NtrbohD, the oxidase responsible for the oxidative burst in elicited tobacco cell. Plant J 2004, 37: 282–293

34 Zarsky V, Cvrckova F. Small GTPases in the morphogenesis of yeast and plant cells. NATO ASI Series H: Cell Biology 1997, 101: 75–88

35 Cvrckova F, Zarsky V. Ntrop1, a tobacco (*Nicotiana tabacum*) cDNA encoding a Rho subfamily GTPase expressed in pollen. Plant Physiol 1999, 120: 634

36 Schiene K, Puhler A, Niehaus K. Transgenic tobacco plants that express an antisense construct derived from a *Medicago sativa* cDNA encoding a Rac-related small GTP-binding protein fail to develop necrotic lesions upon elicitor infiltration. Mol Gen Genet 2000, 263: 761–770

37 Christensen TM, Vejlupkova Z, Sharma YK, Arthur KM, Spatafora JW, Albright CA, Meeley RB *et al*. Conserved subgroups and developmental regulation in the monocot rop gene family. Plant Physiol 2003, 133: 1791–1808

38 Mi ZY, Wang SS, Wu NH. Isolation of osRACD gene encoding a small GTP-binding protein from rice. Chinese Sci Bull 2002, 47: 1673–1679

39 Knight H. Calcium signaling during abiotic stress in plants. Int Rev Cytol 2000, 195: 269–325

40 Xiong L, Yang Y. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 2003, 15: 745–759

41 Anil VS, Harmon AC, Rao KS. Spatio-temporal accumulation and activity of calcium-dependent protein kinases during embryogenesis, seed development, and germination in Sandalwood. Plant Physiol 2000, 12:

1035–1044

42 Li H, Lin Y, Heath RM, Zhu MX, Yang Z. Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. Plant Cell 1999, 11: 1731–1742

43 Molendijk AJ, Bischoff F, Rajendrakumar CS, Friml J, Braun M, Gilroy S, Palme K. *Arabidopsis thaliana* Rop GTPases are localized to tips of root hairs and control polar growth. EMBO J 2001, 20: 2779–2788

44 Bolte S, Schiene K, Dietz KJ. Characterization of a small GTP-binding protein of the rab 5 family in *Mesembryanthemum crystallinum* with increased level of expression during early salt stress. Plant Mol Biol 2000, 42: 923–936

45 Murphy C, Saffrich R, Grummt M, Gournier H, Rybin V, Rubino M, Auvinen P *et al*. Endosome dynamics regulated by a novel rho protein. Nature 1996, 384: 427–432

46 Lin Y, Seals DF, Randall SK, Yang Z. Dynamic localization of rop GTPases to the tonoplast during vacuole development. Plant Physiol 2001, 125: 241–251

47 Beers EP, McDowell JM. Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. Curr Opin Plant Biol 2001, 4: 561–567

48 Suharsono U, Fujisawa Y, Kawasaki T, Iwasaki Y, Satoh H, Shimamoto K. The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. Proc Natl Acad Sci USA 2002, 99: 13307–13312

49 Trotochaud AE, Hao T, Wu G, Yang Z, Clark SE. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. Plant Cell 1999, 11: 393–406

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