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Regulation of *OsRac1* mRNA against defense-related stimuli in wild-type rice seedlings

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Abstract

A rice (*Oryza sativa* L.) homolog of mammalian Rac-GTPase, *OsRac1*, characterized only in transgenic rice, was proposed to involve in cell death/disease resistance. However, its role in wild-type rice remains uncharacterized and unclear. We examined expression of the *OsRac1* mRNA in response to stress signalling components, jasmonic acid (JA), hydrogen peroxide (H_2O_2), and two protein phosphatase inhibitors, cantharidin (CN) and endothall (EN), using rice (cv. Nipponbare) seedlings. The *OsRac1* transcript, whose accumulation required certain *de novo* synthesized protein factor(s), increased in the leaves upon CN/EN and H_2O_2 treatment, but not by JA. Using two pathogenesis-related (PR) protein gene markers, *OsPR5* and *OsPR10*, our results also reveal *OsRac1* mRNA accumulates later than that of these two important defense/stress-related *OsPR* genes.

Additional key words: *Oryza sativa*, oxidative burst, pathogenesis-related protein genes, Rac-GTPase, stress.

Introduction

Rice (*Oryza sativa* L.) is an important crop and model species for highlighting the components (and their interactions) involved in the self-defense response pathway(s) (Agrawal *et al.* 2001b). Our recent investigations aimed at the identification of *OsPR* (pathogenesis-related) genes (important markers), and the components of the signaling pathways involved in mediating the defense/stress response(s), have facilitated a primary understanding of the complexity that underlines the self-defense mechanism(s) in rice (Agrawal *et al.* 2000, 2001a,b, Rakwal *et al.* 2001a,b,c). However, there is still a considerable lack of information on the rice self-defense mechanisms, compared with the well-studied dicots, which is another reason for our continued focus on rice.

Here we look at one of the diverse group of proteins with an evolutionary history dating back to the first unicellular eukaryotic cells, the Rho family of GTPases, of which the Rac proteins are an important component (Winge *et al.* 2000). Among a variety of cellular responses affected by the Rac proteins, an important function lies in their ability to regulate the activation of a multicomponent plasma membrane-bound NADPH-dependent oxidase that triggers the oxidative burst (Abo *et al.* 1991, Winge *et al.* 2000). This transient burst of reactive oxygen species (ROS) production ("oxidative burst") has a direct microbial effect and is involved in inducing many other defense responses (Grant and Loake 2000, Park *et al.* 2000). Previously, it was shown that overexpression of the rice Rac GTPase, *OsRac1*, in transgenic rice suspension cultures and plants results in accumulation of the H_2O_2 and cell death (Kawasaki *et al.* 1999). The authors suggested that Rac is the component of a disease resistance pathway (Ono *et al.* 2001). However, authors failed to provide any evidence on *OsRac1* expression in wild-type rice, and whether its induction precedes the accumulation of classical *OsPR* marker genes.

Therefore, we conducted the present study to address the above questions in order to know the *OsRac1* mRNA regulation by defense/stress-related stimuli in wild-type rice seedlings.

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Abbreviations: CHX - cycloheximide; CN - cantharidin; EN - endothall; JA - jasmonic acid; PP - protein phosphatase; PR - pathogenesis-related; ROS - reactive oxygen species; ST - staurosporine; TET - tetracycline.
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Materials and methods

Plants: Rice (*Oryza sativa* L. cv. Nipponbare) seedlings were grown for 2 weeks in a growth chamber under white fluorescent lamps (irradiance 150 μmol m^{-2} s^{-1}, temperature 25 °C, relative humidity 70 %, 12-h photoperiod) (for details see Agrawal *et al.* 2000, 2001b). Approximately 2 cm long leaf/leaf sheath (shoot) segments and roots were cut from the seedlings, and treated with various compounds (as mentioned in Chemicals) under continuous light or complete darkness as described previously (Agrawal *et al.* 2000, 2001a,b, Rakwal *et al.* 2001a,b,c). Leaf segments floated on *Milli Q* (MQ) pure water served as an appropriate control for other treatments, and labeled as CON in figures. Sampling was done at the times indicated, and samples were stored at -80 °C until total RNA was extracted.

Chemicals: Cantharidin (CN), jasmonic acid (JA), staurosporine (ST), cycloheximide (CHX) and tetracycline (TET) were purchased from *Sigma* (St. Louis, MO, USA). EN was from *BIOMOL Research Laboratories* (Plymouth, PA, USA). Stock solutions were prepared as reported previously (Agrawal *et al.* 2000, 2001a,b). H_2O_2 was from *Wako Pure Chemicals* (Tokyo, Japan). Since the concentration of solutions used to dissolve various chemicals was well below 0.1 % (v/v), the MQ water was used as a control.

Northern analysis: Total RNA was isolated from rice seedling tissues (*RNeasy Plant Kit, Qiagen*, Hilden, Germany). Gene specific primer pairs [*OsRac1* (accession AB09508, Kawasaki *et al.* 1999): OsRac1-F (5′-GCTACACCTGCAACAAGTTC-3′) and OsRac1-R (5′-AGAAGTTTCCTCCTAGCTGC-3′); *OsPR10* (accession D38170, Midoh and Iwata 1996): OsPR10-F (5′-GCTACAGGCATCAGTGGTCA-3′) and OsPR10-R (5′-GACTCAAACGCCACGAGAAT-3′)] were designed based on the nucleotide sequence. A part of the *OsPR5* gene (accession X68197, Reimann and Dudler 1993) fragment was also PCR amplified using the pPIR2 plasmid (a kind gift from Prof. Robert Dudler, University of Zurich, Switzerland) as a template, and a specific primer pair [OsPR5-F (5′-ACCTCTTCCGCTGTCCTC-3′) and OsPR5-R (5′-GAAGACGACTTGGTAGTTGC-3′)]. The size and nucleotide sequence of the amplified fragment for *OsRac1* (693 bp), *OsPR5* (510 bp), and *OsPR10* (651 bp) were confirmed. Northern analyses were carried out as described previously (Agrawal *et al.* 2000, 2001b). Hybridization with the [α-^{32}P]dCTP-labeled *OsRac1* cDNA probe was carried out for 18 h at 65 °C, the membrane washed with 2 × SSC and 0.1 % SDS at 65 °C for 1 h, and exposed to an X-ray film (*Kodak*, Tokyo, Japan) using two intensifying screens for 4 d. To make an accurate comparison with the results obtained for *OsRac1* the same membrane was stripped, and reprobed with cDNA probes for *OsPR5* and *OsPR10*; the membrane was exposed for 18 h at -80 °C. As a representative of the equal loading (20 μg total RNA), a part of the ribosomal RNA (rRNA) from one membrane, stained with methylene blue, is shown in the figures. These results are representative of three independent experiments.

Results and discussion

Accumulation of *OsRac1* mRNA in response to PP inhibitors: The *OsRac1* transcript was identified in transgenic rice suspension cultured cells overexpressing the *OsRac1* gene (Kawasaki *et al.* 1999, Ono *et al.* 2001), but its response to wounding or stress was not determined in whole plants (which serve as much better model systems than suspension cells), and in wild-type rice. Using Northern analyses, we investigated the expression profile of the *OsRac1* mRNA accumulation in rice seedlings (Fig. 1). Under light, *OsRac1* did not respond to wounding by cut (CON) at all, and its transcript could not be detected in healthy leaves (see Fig. 2*A*). Surprisingly, the global signal, JA (Reymond and Farmer 1998), which was convincingly shown to involve in rice self-defense response (Agrawal *et al.* 2001b), did not induce the *OsRac1* transcript, indicating that JA does not influence the *OsRac1* mRNA. This forced us to ask what other stress agents can cause expression of the *OsRac1* mRNA.

In previous studies we have found that protein phosphatase (PP, Millward *et al.* 1999) inhibitors such as CN and EN are highly effective in up-regulating a variety of defense/stress-related genes (Agrawal *et al.* 2001b, and references therein). Therefore, CN and EN were used in the present study, and it was found that they are indeed effective in up-regulating the *OsRac1* expression, which provided a first evidence for *OsRac1* mRNA accumulation in wild-type rice. On the contrary under dark, with CN and EN, there was a significant down-regulation of the *OsRac1*mRNA level over the high amount of transcript accumulation under light, suggesting involvement of light signal(s) in modulating the *OsRac1* expression. A dose-dependent experiment revealed that *OsRac1* induction is related to applied doses of CN and EN (Fig. 1B). Moreover, using 1 μM EN (Fig. 1*C*), *OsRac1* mRNA was found to accumulate only in the leaves, but not in the leaf sheaths (shoot) or roots. To further investigate whether the induced *OsRac1* expression requires *de novo* synthesized protein factor(s),

the protein synthesis inhibitor CHX or TET were applied simultaneously with these chemicals (Fig. 1*D*). CHX, but not TET, completely blocked the *OsRac1* transcript accumulation by CN and EN, suggesting that *de novo* protein synthesis may be required for its expression.

Overexpression of *OsRac1* caused browning and necrosis, and was accompanied by increase in content of H_2O_2 (Kawasaki *et al.* 1999, Ono *et al.* 2001). As PP inhibitors can also elicit an increased production of the ROS (Tenhaken *et al.* 1995, Lamb and Dixon 1997), the possibility that the *OsRac1* mRNA might accumulate as a result of ROS generation after treatment with CN/EN,

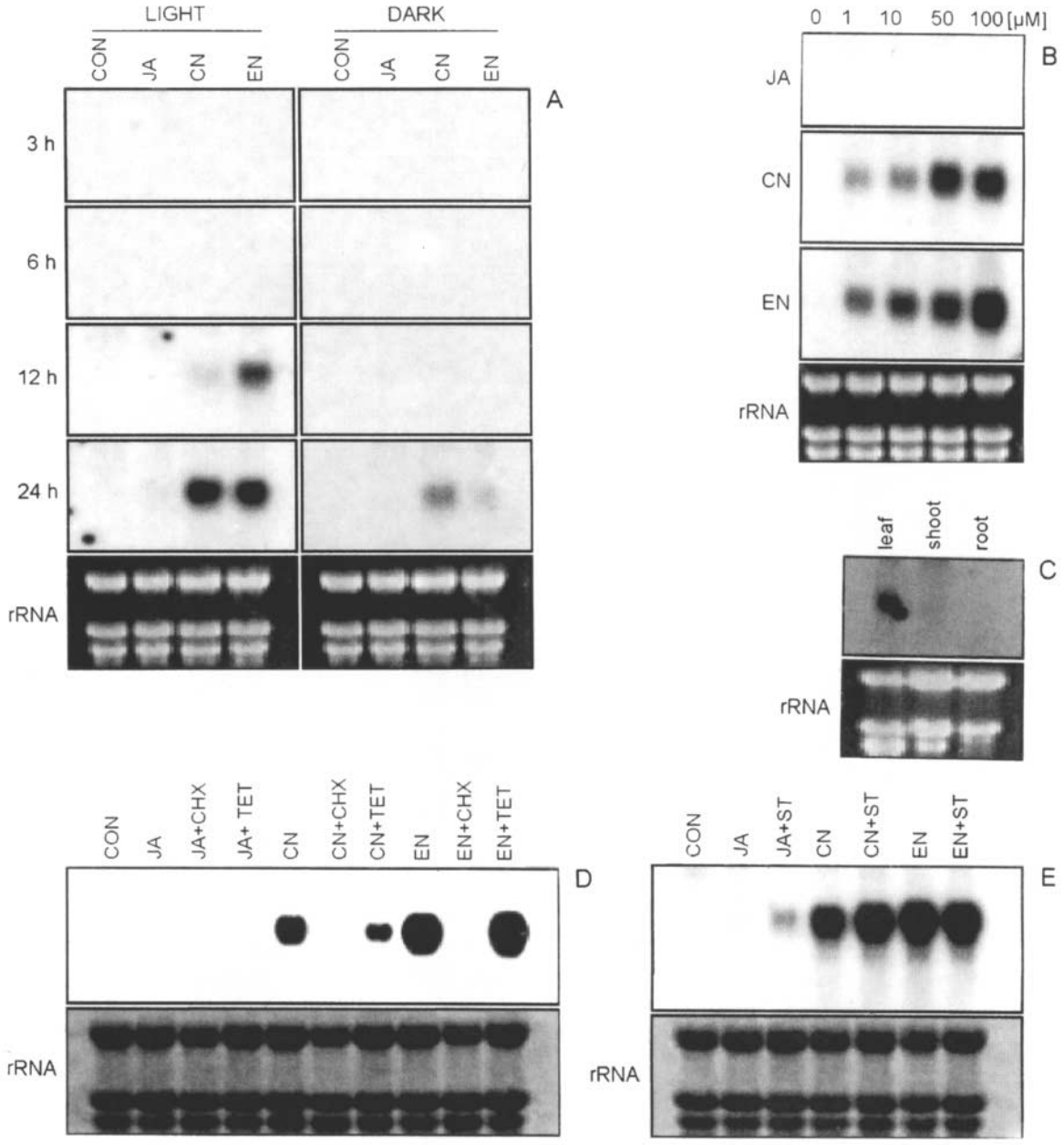

Fig. 1. *A* - Induction of the *OsRac1* mRNA by CN and EN. Northern analysis of total RNA isolated from leaves treated with 100 μM JA, CN, and EN, and cut control (CON) in a time-dependent manner, under continuous light (LIGHT) and dark (DARK). *B* - Dose dependent effect of JA, and CN or EN on *OsRac1* transcript. *C* - Tissue specific induction of *OsRac1*, as determined by treating the seedling tissues with 1 μM EN. *D* - Effect of 10 μM CHX and TET on JA (100 μM) and CN or EN (1 μM) induced *OsRac1* expression. *E* - Effect of ST (10 μM), in combination with JA (100 μM), CN and EN (1 μM) on *OsRac1* expression. In *B*, *C*, *D* and *E*, the treatments were done for 24 h under light (leaf and shoot) or dark (root). *Lane 1* is the control (CON) for the same time period (24 h) in *D* and *E*. Equal loading (20 μg) was confirmed for all experiments by ethidium bromide staining (*A*, *B* and *C*) or staining the blots with methylene blue (*D* and *E*), and as a representative a part of the rRNA from one membrane is shown in the lowermost panels. The blots were hybridized to the [α-^{32}P]dCTP-labeled *OsRac1* cDNA probe.

cannot be ruled out. CN and EN have been shown to cause phytoalexins, sakuranetin and momilactone A production in rice, accompanied by necrotic lesion formation on leaves (Rakwal *et al.* 2001b), and rapid up-regulation of *OsPR* marker genes (Agrawal *et al.* 2000, 2001a,b, Rakwal *et al.* 2001a,b,c). Therefore in light of the above findings, a possible involvement of phosphorylation/dephosphorylation events and ROS in regulation of *OsRac1* mRNA expression can be suggested.

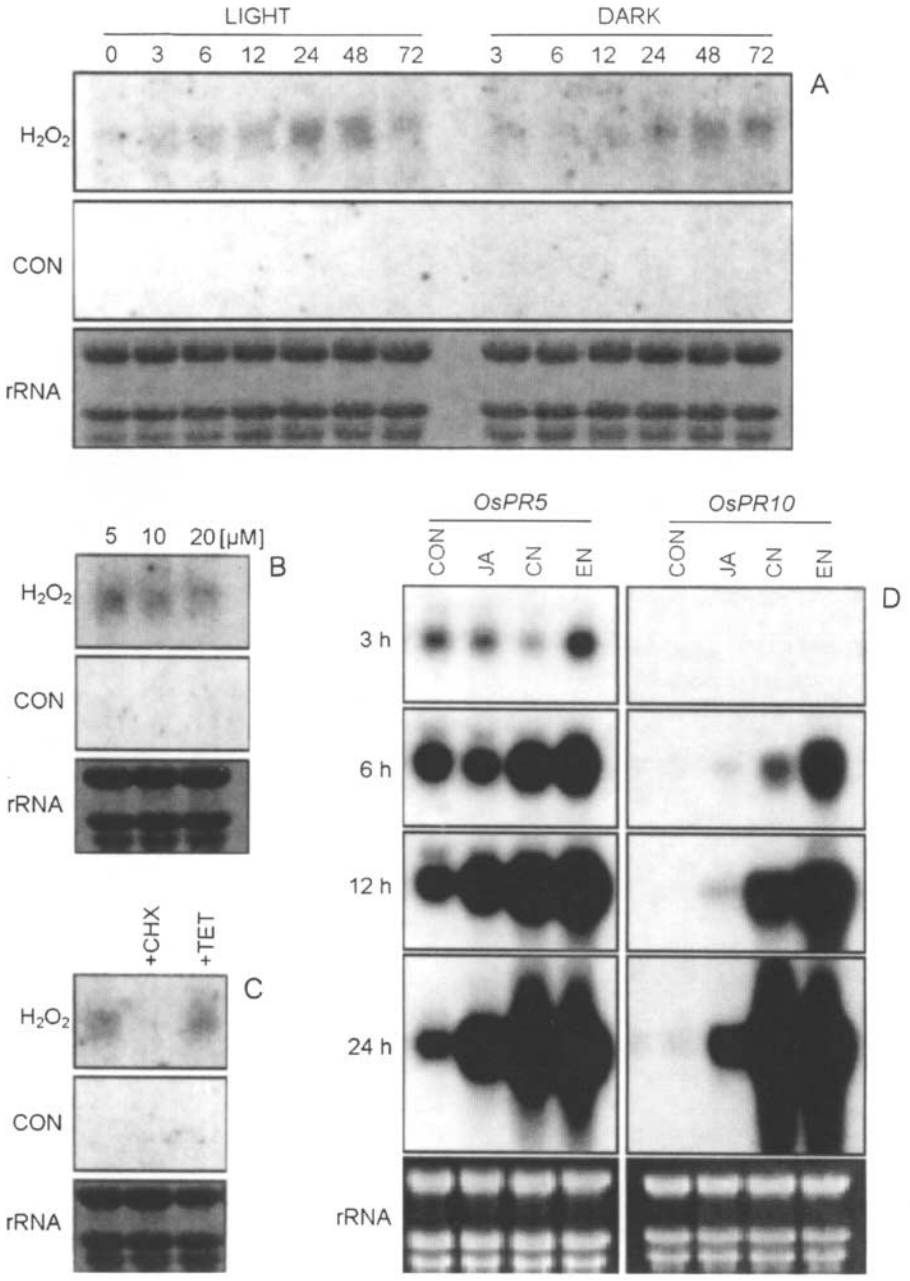

Fig. 2. Applied H_2O_2 induces *OsRac1*, and induction of two rice PR genes, the *OsPR5* and *OsPR10*, by JA, CN and EN. *A* - Leaves treated with 10 mM H_2O_2, under continuous light and dark, in a time-dependent manner, as given above each lane in h. The lower panels show the control (CON). Zero (0) indicates sampling at the start of the experiment. *B* - Dose-dependent effect of H_2O_2 at 24 h after treatment under light. *C* - Effect of 10 μM CHX and TET on 10 mM H_2O_2 induced *OsRac1* expression, measured at 24 h after treatment under light. Equal loading (20 μg) was confirmed by staining the blots with methylene blue, and hybridization ([α-^{32}P]dCTP-labeled *OsRac1* cDNA probe) was carried out as described Fig. 1. *D* - Northern analysis of total RNA isolated from leaf treated with 100 μM JA, CN, and EN, and control (CON) in a time-dependent manner, under continuous light. Total RNA was extracted at the times indicated at the left hand side. Blots were hybridized to the [α-^{32}P]dCTP-labeled *OsPR5* and *OsPR10* cDNA probes, respectively. Equal loading (20 μg) was confirmed as described in Fig. 1.

Subtle effect of ST on inducible *OsRac1* mRNA accumulation: *OsRac1* mRNA accumulation by CN/EN suggested that hyperphosphorylation of certain target proteins might modulate its expression. So we examined the effect of a potent serine/threonine protein kinase inhibitor, ST, applied simultaneously with 100 μM JA, or 1 μM CN and EN), on *OsRac1* expression (Fig. 1*E*). *OsRac1* transcript was clearly detected with ST plus JA treatment, as compared to undetectable *OsRac1* transcript with JA alone. A plausible explanation is that, a certain serine/threonine protein kinase might be negatively regulating the *OsRac1* expression. Interestingly, the *OsRac1* transcript level was also slightly enhanced by CN. It might also be possible that a serine/threonine protein kinase is associated with one or more PP in modulating *OsRac1* expression. These results imply that phosphorylation and/or hyperphosphorylation state (or contents) of a serine/threonine residue(s) of one or more unidentified phosphoproteins might be responsible for regulating *OsRac1*, as was suggested for the *OsPR1* genes in rice (Agrawal *et al.* 2000, 2001b).

***OsRac1* responds to applied H_2O_2:** One of the most rapid defense responses engaged following pathogen recognition is the oxidative burst, which constitutes the production of ROS, primarily superoxide and H_2O_2, at the site of infection (Tenhaken *et al.* 1995, Lamb and Dixon 1997). Previously it was suggested that *OsRac1* overexpression leads to ROS production (Kawasaki *et al.* 1999, Ono *et al.* 2001), however, no evidence was presented for *OsRac1* responsiveness to H_2O_2. Accumulation of *OsRac1* mRNA by H_2O_2 was detectable at 6 h, which slowly increased till 48 h, followed by a decline thereafter (Fig. 2*A*). Although accumulation of the *OsRac1* transcript was also observed under darkness, it was delayed in comparison to that seen under light. Again, the *OsRac1* transcript was not detected in healthy leaves (0) and after wounding by cut (CON). Moreover, only a subtle effect of different doses of H_2O_2 on *OsRac1* expression was seen (Fig. 2*B*). Complete inhibition of the induced *OsRac1* transcript by CHX again suggests a requirement for *de novo* synthesized protein factor(s). These results show responsiveness of *OsRac1* to H_2O_2 providing additional evidence for *OsRac1* involvement in rice defense/stress response(s).

Expression of *OsPR* marker genes precedes accumulation of *OsRac1* mRNA: As the *OsRac1* mRNA accumulates in wild-type seedling leaves in response to certain defense/stress stimuli, the question arises where does *OsRac1* stand in the self-defense pathway(s) in rice? For this the expression profiles of two important rice PR marker genes, *OsPR5* (Reimann and Dudler 1993, Datta *et al.* 1999, Agrawal *et al.* 2001a, Rakwal *et al.* 2001a) and *OsPR10* (Midoh and Iwata 1996, Rakwal *et al.* 2001c) were examined reprobing the same membranes used for determining the *OsRac1* expression (Fig. 2*D*). The transcripts of these two *OsPR* genes were detected as early as 6 h after treatment with JA, CN and EN, which was stronger and earlier than that observed for *OsRac1* (Fig. 1*A*), clearly demonstrating that *OsPR* gene induction occurs earlier than the *OsRac1* mRNA accumulation. It should be noted that in repeated experiments we observed the potent and rapid up-regulation of the *OsPR5/OsPR10* mRNAs, as compared to the late induction of the *OsRac1* transcript, indicating the consistency of the relationship. These findings indicate that, at least at the mRNA level, the *OsRac1* expression does not precede the *OsPR* gene expression. Therefore, the argument (and model therein) that *OsRac1* precedes the expression of defense genes in rice (Kawasaki *et al.* 1999, Ono *et al.* 2001) needs more thorough investigation(s).

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