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ORIGINAL PAPER

OsRAF is an ethylene responsive and root abundant factor gene of rice

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Abstract ERFs, the largest group of AP2/EREBP transcription factors, are involved in diverse processes in plants. However, their importance in growth and development is not fully understood. Here, we report *OsRAF* (a Root Abundant Factor gene in *Oryza sativa*), a new member of the rice *ERF* group, expressed more abundantly in roots than in other organs of rice at the transcriptional level. Moreover, the expression could be up-regulated by ethylene or low temperature. Transient expression of OsRAF::GFP fusion in onion epidermis cells and its overexpression in Arabidopsis revealed that OsRAF was a nucleus localized protein. Down-regulation of *OsRAF*'s expression by RNAi method failed to produce aberrance in transgenic rice, which suggests existence of functionally redundant gene(s).

Keywords *OsRAF* · Root-abundant factor · ERF · *Oryza sativa*

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Introduction

AP2/ethylene responsive-element binding proteins (EREBPs) compose of a superfamily of transcription factors that exist extensively in plants and microorganisms (Okamuro et al. 1997; Magnani et al. 2004; Nakano et al. 2006). They are characterized by the presence of a highly conserved 60 ~ 70 amino acid AP2/EREBP DNA binding domain (Weigel 1995; Okamuro et al. 1997). The superfamily is divided on the basis of number of domains and function into five subfamilies namely AP2, ERF, DREB, RAV and others (Sakuma et al. 2002).

ERF is the largest subfamily. Present studies mainly from Arabidopsis and tomato indicate that ERFs are involved in diverse processes including biotic stress responses which are often associated with ethylene signaling pathway (Zhou et al. 1997; Gu et al. 2000; Gutterson and Reuber 2004; Ohme-Takagi and Shinshi 1995), plant development or metabolic regulation (Banno et al. 2001; Van der Graaff et al. 2000; Chuck et al. 2002). However, most of the ERF members have not been characterized even in Arabidopsis. In rice, only a few ERFs have been reported (Nakano et al. 2006). Further analysis on molecular and functional characteristics of novel ERFs are essential to extend our understanding of their importance in plant growth and development.

In Arabidopsis and tomato, expression of many characterized ERFs can be induced by ethylene, but a

corresponding ERF member has not been identified in rice (Gu et al. 2000; Ohme-Takagi and Shinshi 1995). Here, we report *OsRAF*, a functionally unidentified rice ERF gene with known full length cDNA sequence (AK101949) capable of encoding an AP2/EREBP domain contained protein, could be up-regulated by ethylene or coldness and expressed abundantly in roots of rice at the transcriptional level. Moreover, OsRAF accumulated predominantly in the nucleus, which suggests that it might act as a transcription factor.

Materials and methods

Plant materials and nuclear acid extraction

Rice plants (*Oryza sativa* L. ssp. *japonica* cv. Zhonghua 10) were grown under standard conditions. Leaves were collected from 2-week-old seedlings, and inflorescences were harvested at different developmental stages. Young roots and buds were gathered from seeds germinated on sterile water-soaked filter paper for 3 days.

Two-week old rice seedlings were transferred into distilled water (control) for 3 days or into a solution containing 100 μmol/l ethrel (purity, 95%), 50 μmol/l abscissic acid (ABA; Invitrogen) or 20% polyethylene glycol (PEG; –4.5 MPa), with roots immersed completely, and cultivated for 4 h at 28°C. For low-temperature treatment, seedlings were exposed to 8°C or to 28°C (as a control) for 4 h.

Total RNA was isolated by use of Trizol kit (Invitrogen) according to the manufacturer's protocol. RNA was incubated with RNase-free DNase (Takara) to remove residual genomic DNA.

Analysis of *OsRAF* mRNA accumulation

For semi-quantitative RT-PCR analysis, an amount of 5 μg of total RNA was reverse-transcribed into first-strand cDNAs by ReverTra Ace (Toyobo). PCR was performed in a 50-μl mixture containing 5 μl of first-strand cDNA, 20 pmol each of the gene-specific primers 5′-GACAGATCTGATGTGTGGAGGCGC CATC-3′ and 5′-TCAACTAGTGAAAAGGGCGC CGTCGATTG-3′, 0.4 μM dNTPs, 1× GC buffer (Takara) and 2.5 U Taq DNA polymerase (5 U/μl, Takara) for 35 cycles. Rice *Tubulin A* cDNA (*Tub A*) was amplified for 25 cycles as a constitutive control (Ding et al. 2002).

Northern blot analysis was used to confirm the RT-PCR results. An amount of 25 μg of total RNA was fractioned on a 1.2% agarose-formaldehyde gel and transferred onto Hybond-N+ nylon membranes before UV crosslinking. Hybridization was performed with a ^{32}P-labeled cDNA fragment of *OsRAF* amplified by the gene-specific primers. The resulting membrane was autographed at –80°C.

Construction of *OsRAF* expression vector and RNAi vector

A fragment spanning the *OsRAF* open reading frame (ORF) was PCR amplified from rice cDNA by use of the primers: 5′-GACAGATCTGATGTGTGGAGG CGCCATC-3′ and 5′-TCAACTAGTGAAAAGGG CGCCGTCGATTG-3′, which contained added *Bgl*II and S*pe*I enzyme sites, respectively. After gel purification, the amplicon was double-digested with *Bgl*II and *Spe*I, then inserted into pCAMBIA1302 (CAMBIA), which was digested with the same endonucleases to generate an OsRAF::GFP fusion construct under the control of CaMV35S promoter. The resulting *OsRAF* construct 35S::OsRAF::GFP was first introduced into *Agrobacterium tumefaciens* strain EHA105 and then into Arabidopsis Col. plants by a floral dip method (http://www.nlh.no/research/narc/protocols/floral_dip.htm).

A construct for RNAi analysis of *OsRAF* was derived by inserting a nonconserved region (nt 2–534) in the cDNA of *OsRAF* (AK101949) into a RNAi tool vector pWTC605 (Zhang et al. 2006). The construct was introduced in rice as described previously (Zhang et al. 2006).

Subcellular localization of OsRAF protein

35S::OsRAF::GFP was transformed into *Allium cepa* (onion) epidermis by *A. tumefaciens* transfection (Yang et al. 2000). GFP fluorescence signals were detected on microscopy with use of an FITC filter (Zeiss) after incubation of the transformed cells on a MS medium in light at 25°C for 2.5 days. Subcellular localization of OsRAF was further examined in T1 seedlings of transgenic Arabidopsis lines expressing OsRAF::GFP.

Identification of RNAi lines in rice and examination of endogenous *OsRAF* transcript in the RNAi lines

Positive lines of *OsRAF* RNAi rice were first screened by use of hygromycin followed by PCR confirmation with the primer pair 5′-AATGAGCTC TGAACGTGCCGAGCCAGAC-3′ and 5′-CGGGAA CTACAAGACACGTGC-3′. Endogenous *OsRAF* transcript in *OsRAF* RNAi lines was checked by semi-quantitative RT-PCR with the gene specific primer pair. Rice *Tubulin A* (*Tub A*) cDNA was amplified as a constitutive control (Ding et al. 2002).

Results

OsRAF is an *ERF*-like gene

Genomic sequence prediction revealed a putative rice ERF gene, designated *OsRAF* (Os03g08470), with a 1575-bp cDNA and a 1005-bp ORF capable of encoding 334 amino acids (AK101949). The predicted protein has a calculated molecular mass of 35.7 kD and a pI of 6.07, with a relatively hydrophilic feature. A BLASTN search with DNA sequence of *OsRAF* retrieved only one hit with 100% identity in the TIGR rice genomic database, which suggests that *OsRAF* exists as a single-copy gene. The genomic sequence of *OsRAF* localizes in chromosome 3 (AC079633) (Map Viewer, http://www.ncbi.nlm.nih.gov/mapview) and contains two introns.

Phylogenetic analysis based on alignment of full amino acid sequences of OsRAF and other characterized rice AP2/EREBP proteins revealed that OsRAF grouped with known ERFs but not DREBs (Fig. 1a). In addition, the amino acids 15 and 20 in the AP2/EREBP domain of OsRAF are A and D, which are conserved in the known ERF proteins from different species and essential for binding to GCC box (Sakuma et al. 2002; Sessa et al. 1995), so OsRAF is suggested to be an ERF-like protein.

OsRAF contains a single AP2/EREBP DNA binding domain (amino acids 110–166) (Fig. 1b) and an aspartic acid-rich region of 25 amino acids (amino acids 53–77) proposed as a transcriptional activation domain (Mitchell and Tjian 1989; Jofuku et al. 1994; Elliott et al. 1996). Moreover, the protein has a putative nuclear-targeting motif KKKR (amino acids 104–107). All these features imply that *OsRAF* is a potential transcription factor.

OsRAF is a nucleus localized protein

ERF proteins are identified as transcription factors that regulate target gene expression in nuclei (Fujimoto et al. 2000). To explore whether *OsRAF* possesses this characteristic, we first checked its transient expression in onion epidermis cells, fluorescence observation revealed the OsRAF::GFP fusion protein accumulated predominantly in the nucleus, which was supported by the expression of OsRAF::GFP fusion protein in root tips of *OsRAF* transgenic Arabidopsis (Fig. 2). The nuclear localization in combination with the domain/motif features of OsRAF further suggested that the protein is a transcription factor.

OsRAF is predominantly expressed in roots of rice

To elucidate the function of *OsRAF*, we analyzed its expression profile by semi-quantitative RT-PCR (data not shown), which was confirmed by Northern blot analysis (Fig. 3a). *OsRAF* was expressed more abundantly in roots but less so in leaves, inflorescences, buds and embryos (Fig. 3a), a pattern of expression very much like that of *RAF*, a root-abundant ERF-type gene of barley (*Hordeum vulgare*) (Jung et al. 2007). OsRAF and RAF share 49.14% amino acid identity, which implies they may have the same or similar function(s).

OsRAF responds to ethylene and low temperature

Considering that most of the reported ERF/DREB proteins from rice or other plant species play important roles in response to biotic and/or abiotic stresses (Nakano et al. 2006), we investigated *OsRAF* expression under stress-associated stimuli, including ethrel, ABA, cold, and water deficit mimicked by PEG solution. Both RT-PCR (data

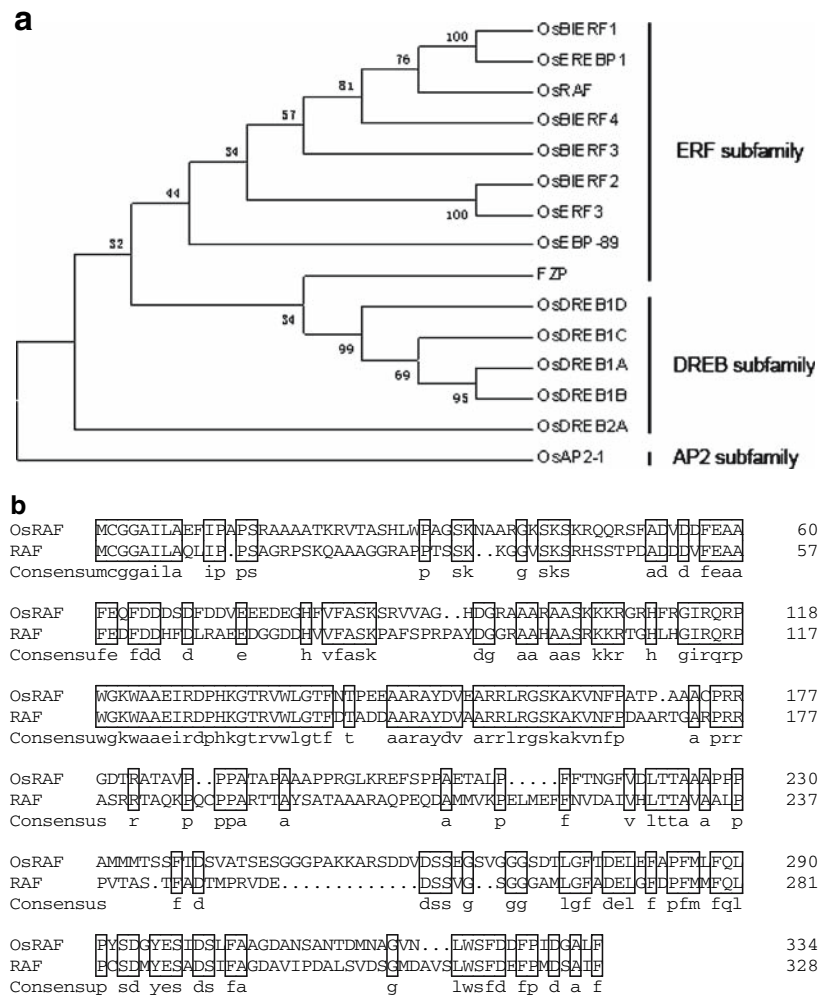

Fig. 1 *OsRAF encodes an ERF-like protein*. (**a**) Phylogenetic tree (ME) constructed by use of MEGA version 3.1 (Kumar et al. 2004) based on amino acid sequence alignment of OsRAF and other characterized AP2/EREBP proteins in rice by ClustalX (version 1.81). Bootstrap values, evaluated for 500 bootstrap trails, are shown at branch points. The sequences are OsRAF, *Oryza Sativa*, this study; OsBIERF3, CAC39058; OsBIERF1, AAV98700; OsBIERF4, NP_001049180; OsEBP-89, AAK92636; OsEREBP1, AAF23899; OsBIERF2, AAV98701; OsDREB1A, AAQ06658; OsAP2-1, BAE78578; OsDREB1B, AAP83888; OsDREB1C, NP_001056661; OsDREB1D, NP_001056909; OsDREB2, NP_001042107; FZP, AAX83561; OsERF3, BAB03248). (**b**) Alignment of OsRAF and RAF (from *Hordeum vulgare*; AAZ14086) amino acid sequences

not shown) and Northern blot results showed *OsRAF*'s expression was up-regulated prominently by ethylene and also induced by low temperature (Fig. 3b), so *OsRAF* could respond to both environmental factor and stress-associated plant hormone inducement.

We further explored the detailed response of *OsRAF* to ethylene. RT-PCR results (Fig. 3c) revealed that the mRNA level of *OsRAF* accumulated shortly after 1-h treatment with 1 mM ethrel and waned subsequently. Quantitative analysis showed that *OsRAF* expression at 1, 2 and 4 h of the treatment were 2.11, 2.07 and 1.27 folds of the control (Fig. 3c), which indicated that *OsRAF* was a fast-response ERF member to ethylene inducement.

OsRAF may have functionally redundant counterpart(s) in rice

To explore *OsRAF* function, we introduced an RNAi construct of *OsRAF* into rice. Surprisingly, both T0 and T1 RNAi rice did not show an aberrant phenotype. However, *OsRAF* expression was decreased at the transcriptional level in many RNAi transgenic lines (including L1, L2, L5, L11 and L16), as detected by RT-PCR (Fig. 4), which implies that the RNAi construct was effective in transgenic rice. Knock down of *OsRAF*'s expression failed to produce an aberrant phenotype, which suggests the existence of functionally redundant gene(s). In fact, we retrieved three functionally unidentified AP2/EREBP

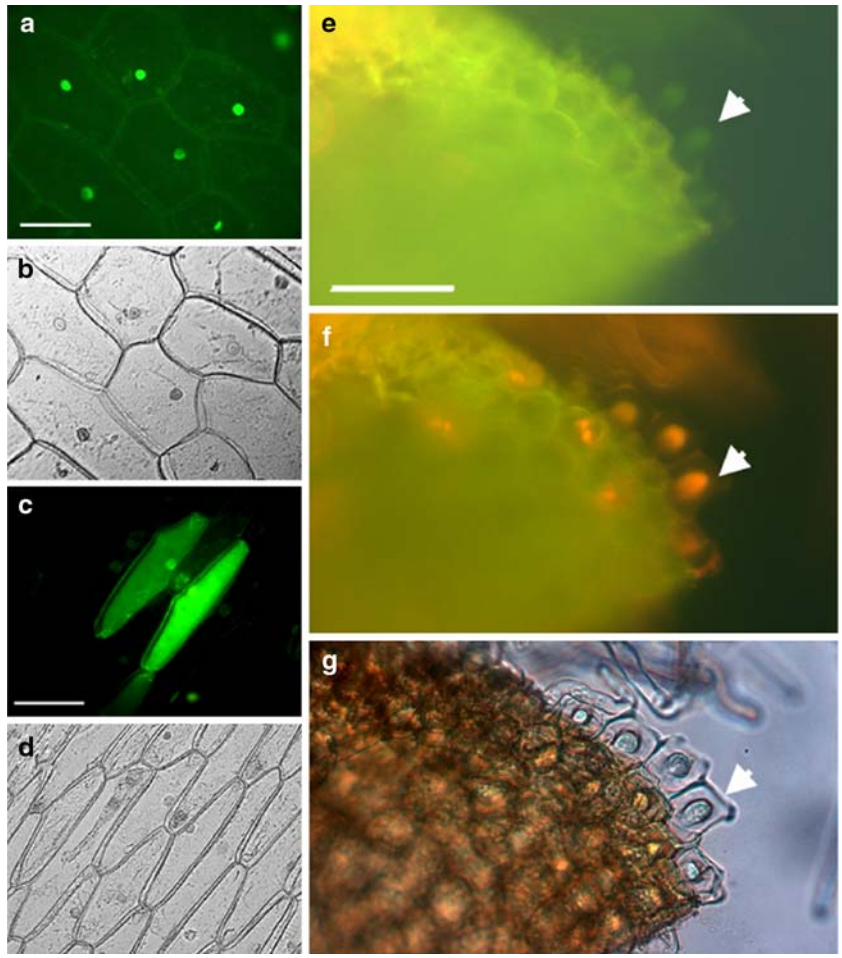

Fig. 2 *OsRAF is a nucleus-localized protein*. (**a**–**d**) Onion epidermis transformed with 35S::OsRAF::GFP construct (**a**, **b**) or pCAMBIA1302 (control) (**c**, **d**) observed under microscopy with an FITC filter (Zeiss) (**a**, **c**) or under bright field (**b**, **d**). (**e**–**g**) OsRAF::GFP signals in root tip cells from transgenic Arabidopsis plants observed under microscopy (**e**) or stained with propidium iodide (**f**) with an FITC filter, **g** is a bright-field graph. Arrowhead indicates endopleural cell. Scale bar = 50 μm in **a** for **a** to **b**, in **c** for **c** to **d** and in **e** for **e**, **f**, **g**

domain-containing proteins in the rice database (gene locus: Os09g0434500; Os03g0182800; Os09g0286600). Sequence analyses revealed that they share more than 40% amino acid identity with OsRAF, which implies that they may have similar functions.

Discussion

On the basis of phylogenetic analysis and the characteristics of ERF conserved amino acids 15 and 20 in the AP2/EREBP domain, *OsRAF* encodes an ERF-like protein. Nuclear localization of OsRAF::GFP fusion suggested that it was a potential transcription factor. At the transcriptional level, *OsRAF* accumulated predominantly in roots of rice and could be up-regulated by ethylene or low temperature, suggests that like *RAF* from barley which confers enhanced pathogen resistance and salt tolerance in Arabidopsis (Jung et al. 2007), it is a biotic and abiotic stress-responsive factor. Moreover, the *OsRAF* response to ethylene was very fast as compared with that of other *ERF* genes such as *AtERF1, AtERF2, AtERF4*, and *AtERF5* from Arabidopsis (Fujimoto et al. 2000). The significance of the expression pattern probably lies in the fact that animals can avoid adverse surroundings by moving away, but plants must rely on regulating the expression of stress-associated genes such as ERFs to adapt. The fast response of *OsRAF* to ethylene inducement suggests that it may have important function(s) in the adaptation process.

In Arabidopsis and rice, only a small part of *ERFs* contain introns (Nakano et al. 2006). Interestingly, *OsRAF* possesses two introns in its DNA sequence, the first between the two exons (AC079633: nt 94653–94761) and the second within the 3′

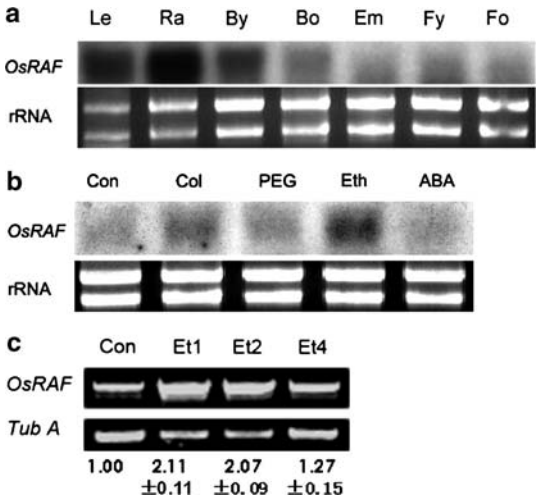

Fig. 3 Expression of *OsRAF* detected by Northern blot analysis (**a**, **b**) or RT-PCR (**c**). In **a** and **b**, rRNAs used as markers to indicate that equal amount of RNAs were loaded in each lane. In **c**, expressions of *Tubulin A* (*Tub A*) were used as constitutive controls. Expression intensity was quantitatively analyzed by Scion Image (Scion Corporation). (**a**) OsRAF expression in leaf (Le), radicle (Rad), young bud (By), old bud (Bo), embryo (Em), young flowe r (Fy), old flower (Fo). (**b**) OsRAF expression after 4-h treatment with plant hormones or environmental factors. Control (Con), cold (Col), PEG (20%), ethrel (Eth), ABA. (**c**) *OsRAF* expression at 1, 2 and 4 h of 1 mM ethrel treatment were 2.11, 2.07 and 1.27 folds of control respectively. Water was used as a control (Con) of the treatment. Results shown below the image represent quantified values of *OsRAF* mRNA levels (ratios of normalized data by use of the tubulin signals for control or ethrel-treated samples), and the data are mean ± SD of triplicates

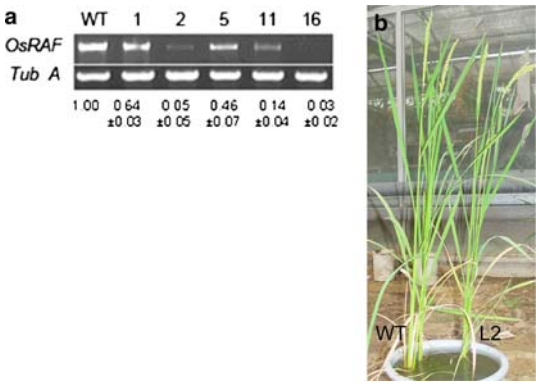

Fig. 4 *RT-PCR analysis and phenotypes of OsRAF RNAi T1 rice*. (**a**) RT-PCR result of *OsRAF* expression in different lines of RNAi T1-positive rice: line 1, line 2, line 5, line 11, line 16. WT=wild type. Quantitative analysis was performed according to the method described in Fig. 3c. (**b**) Comparison of WT and T1 *OsRAF* RNAi line 2

noncoding region (AC079633: nt 95540–95650). Generally, the 3′ noncoding region is essential for the stability of mRNA and efficiency of translation (Russell and Liebhaber 1996; Decker and Parker 1995). In *OsRAF*, removal of the second intron in the 3′ noncoding region after transcription suggests that the spliced 3′ noncoding sequence might play an important role in post-transcriptional regulation.

The inability of RNAi to identify *OsRAF* function implies the existence of functionally redundant gene(s) in rice genome. The repression of one gene's function may be remedied by that of other(s), a situation often encountered in the functional analysis of AP2/EREBP family members by RNAi method (Nakano et al. 2006; Zhang 2003). Perhaps overexpression is the best way to address a gene's function under the circumstances.

In summary, our experiment identified that *OsRAF* was an ethylene responsive gene. As mentioned in the introduction, no such genes in rice had been reported previously although ethylene responsive genes were extensively investigated in Arabidopsis and in tobacco. Moreover, our data from rice together with research from other species showed that these ERF genes were similar in function (Nakano et al. 2006). In contrast, functions of AP2 subfamily members varied obviously. For example, *APETALA2* and its homologues in maize and in petunia play different roles in plant development (Moose and Sisco 1996; Maes et al. 2001). An intriguing question is why stress responsive ERF members are more conserved in function, whereas development related AP2 members are more versatile? Further investigation will help us gain new insight into the function concerning this superfamily.

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