RESEARCH PAPER

The rice ERF transcription factor *OsERF922* negatively regulates resistance to *Magnaporthe oryzae* and salt tolerance

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Abstract

Rice *OsERF922*, encoding an APETELA2/ethylene response factor (AP2/ERF) type transcription factor, is rapidly and strongly induced by abscisic acid (ABA) and salt treatments, as well as by both virulent and avirulent pathovars of *Magnaporthe oryzae*, the causal agent of rice blast disease. OsERF922 is localized to the nucleus, binds specifically to the GCC box sequence, and acts as a transcriptional activator in plant cells. Knockdown of *OsERF922* by means of RNAi enhanced resistance against *M. oryzae*. The elevated disease resistance of the RNAi plants was associated with increased expression of *PR*, *PAL*, and the other genes encoding phytoalexin biosynthetic enzymes and without *M. oryzae* infection. In contrast, *OsERF922*-overexpressing plants showed reduced expression of these defence-related genes and enhanced susceptibility to *M. oryzae*. In addition, the *OsERF922*-overexpressing lines exhibited decreased tolerance to salt stress with an increased Na^+/K^+ ratio in the shoots. The ABA levels were found increased in the overexpressing lines and decreased in the RNAi plants. Expression of the ABA biosynthesis-related genes, 9-*cis*-epoxycarotenoid dioxygenase (NCED) *3* and *4*, was upregulated in the *OsERF922*-overexpressing plants, and *NCED4* was downregulated in the RNAi lines. These results suggest that OsERF922 is integrated into the cross-talk between biotic and abiotic stress-signalling networks perhaps through modulation of the ABA levels.

Key words: Abscisic acid, *Magnaporthe oryzae*, ERF, *Oryza sativa*, salt tolerance.

Introduction

Plants have evolved efficient resistance mechanisms to protect themselves against pathogen infection through a combination of preformed physical and chemical barriers and induced defence systems. In plant cells, perception of conserved pathogen-associated molecular patterns (PAMPs) leads to activation of a battery of defence mechanisms, including the production of reactive oxygen species, the synthesis of antibiotic phytoalexins, deposition of callose, and expression of defence-related genes (Zhou and Chai, 2008; Clay *et al.*, 2009). These inducible defences are regulated by complex signal transduction networks. Plant hormones have been demonstrated to play important roles in orchestrating defence responses. Salicylic acid (SA) is mostly associated with resistance to biotrophic phytopathogens, whereas jasmonic acid (JA) and ethylene (ET) are usually related to defence against necrotrophic pathogens and herbivorous insects (Rojo *et al.*, 2003; De Vos *et al.*, 2005; Glazebrook, 2005). However, interactions between the SA- and JA/ET-signalling pathways can be either mutually antagonistic or synergistic (Beckers and Spoel, 2006; Mur *et al.*, 2006; Adie *at al.*, 2007).

Recently, investigations have highlighted that abscisic acid (ABA), a global regulator of abiotic stresses, is also a key determinant in the induction of plant defence responses (Asselbergh *et al.*, 2008a; Ton *et al.*, 2009). Studies of mutants with ABA deficiency showed increased resistance to the necrotrophic fungus *Botrytis cinerea* (Audenaert *et al.*, 2002), the bacterial pathogen *Erwinia*

chrysanthemi (Asselbergh *et al.*, 2008b) in tomato, and the oomycete *Hyaloperonospora parasitica* in *Arabidopsis* (Mohr and Cahill, 2003). In contrast, exogenous application of ABA enhances the susceptibility of various plants species to phytopathogens (Mohr and Cahill, 2003; Asselbergh *et al.*, 2008b). Treatment of *Arabidopsis* with a virulent isolate of *Pseudmonas syringae* pv. *tomato* or ectopic expression the bacterial effector AvrPtoB induces ABA biosynthesis and modulates regulators of basal defence and callose deposition, suggesting that antagonistic interaction of ABA on SA-dependent defence responses underlies the impact of ABA as a susceptibility factor for this bacterium (de Torres-Zabala *et al.*, 2007). Similarly, the activation-tagged *Arabidopsis cds2-1D* mutant with increased endogenous ABA levels accumulates JA and exhibits a complex antagonistic relationship with SA. These mutants have suppressed resistance to *P. syringae* and the biotrophic oomycete *Hyaloperonospora arabidopsis*, while the resistance to the necrotrophic fungus *Alternaria brassicicola* is enhanced (Fan *et al.*, 2009). Thus, ABA has profound roles in defences mediated by cross talk with the JA- and SA-signalling pathways, depending on the specific pathosystems and infection stages (Mauch-Mani and Mauch, 2005; Asselbergh *et al.*, 2008a; Ton *et al.*, 2009).

Ethylene responsive factors (ERFs), composing a superfamily of transcription factors unique to plants, have been demonstrated to modulate multiple responses to abiotic and biotic stresses. For example, overexpression of *OsDREB1A* (Ito *et al.*, 2006), *AP37*, and *AP59* (Oh *et al.*, 2009) in rice increases tolerance to drought and high salinity. *Arabidopsis* ERF1 (Solano *et al.*, 1998), tomato Pti4 (Gu *et al.*, 2002), and tobacco OPBP1 (Guo *et al.*, 2004) bind to GCC-box-containing DNA fragments and activate expression of defence-related genes. Overexpression of tomato *TSER1* in tobacco has been shown to confer resistance against *Ralstonia solanacearum* as a result of modulation in ABA and ET levels (Zhou *et al.*, 2008). Similarly, transgenic tobacco plants expressing *SodERF3*, an ABA inducible *ERF* of sugarcane, display enhanced tolerance to drought and osmotic stress (Trujillo *et al.*, 2008). *Arabidopsis* ORA59 is implicated the in JA- and ET-mediated defence signal pathways and has a positive impact on resistance against *B. cinerea* (Pré *et al.*, 2008). Ectopic expression of *TiERF1*, a gene from a wild wheat relative *Thinopyrum intermedium*, enhances disease resistance in both wheat and tobacco through the ET-dependent pathway (Chen *et al.*, 2008; Liang *et al.*, 2008). Other AP2/ERF members are also reported to take part in regulation of metabolism, developmental processes, and/or defence responses in plants (Chuck *et al.*, 1998; Fukao *et al.*, 2006; Andriankaja *et al.*, 2007; Oñate-Sánchez *et al.*, 2007; Hattori *et al.*, 2009).

Rice is not only one of the most important cereal crops in the world, but also a model monocot plant for biological research (Devos and Gale, 2000). Pathogens, salinity, and drought are among the major factors that affect the growth and productivity of the crop. Transcription factors play important roles in adaptation to these adverse environmental conditions by regulating expression of the specific responsive genes. Overexpression of transcription factor genes, such as *OsWRKY45* and *OsWRKY89*, enhances disease resistance against *Magnaporthe oryzae*, the causal agent of rice blast disease, through the SA-mediated signal pathway (Shimono *et al.*, 2007; Wang *et al.*, 2007). On the other hand, application of exogenous ABA enhances rice susceptibility to *M. oryzae*, implying a negative role of ABA in defence against the pathogen (Koga *et al.*, 2004; Bailey *et al.*, 2009). Previous studies found that transgenic plants overexpressing *OPBP1* enhanced disease resistance and salt tolerance in both tobacco and rice (Guo *et al.*, 2004; Chen and Guo, 2008). These results promoted the study of the function of OsERF922, a close homologue of OPBP1. Expression of the *OsERF922* gene was induced rapidly and strongly by inoculation with the rice blast fungus as well as by treatments with ABA and salt in rice seedlings. Knockdown of the expression of *OsERF922* by means of RNA interference (RNAi) reduced the accumulation of ABA and enhanced resistance against *M. oryzae*.

Materials and methods

Construction of plasmids and plant transformation

The coding sequence of *OsERF922* (Os01g54890) was amplified with primers E922F (5′-ACAGGATCCCCGCATGTCTCTCT-CCTTG-3′) and E922R (5′-TTACTCGAGTATCACGCACT-CACCGTCGCT-3′) from a rice IR72 cDNA library and ligated into a T-vector to give pUCmT-OsERF922. To construct plasmids for overexpression, the encoding region of OsERF922 was fused at its N-terminal with the sequence encoding the Flag-tag and put under the control of a maize ubiquitin promoter, generating *pCo-Ubi:Flag-OsERF922*. To knock down OsERF922 transcription, a fragment of *OsERF922* gene (from 1 to 203 bp) was constructed in a form to generate a self-complementary hairpin RNA structure, which was driven by the cauliflower mosaic virus (CaMV35S) promoter (pCaMV35S:dsOsERF922).

For analysis of OsERF922 localization, the full-length coding sequence of *OsERF922* was amplified with primers E922F and E922R and was fused in frame to the N-terminus of the green fluorescent protein (GFP) gene to generate the *CaMV35S: OsERF922-GFP* construct. All of the vectors constructed were verified by sequencing and introduced into rice calli of cultivar Zhonghua 17 (japonica, Zh17) by the *Agrobacterium*-mediated transformation method. More than 10 independent transgenic lines were obtained for each construct. The overexpressing plasmid *pCo-Ubi:Flag-OsERF922* was also transformed into tobacco (*Nicotiana tabacum* cv. Xanthin nc) using the similar *Agrobacterium*-mediated method. The transgenic lines at the T_2 generation were screened by planting seeds on 1/2 MS medium (containing 0.5% agar) supplemented with 40 mg l^{-1} hygromycin and the antibiotic resistant seedlings were used in the following experiments unless otherwise indicated.

Plant growth and treatments

Seeds of *Oryza sativa* Zh17 were surface sterilized, imbibed in water at 37 °C for 2 d before germination and seedlings were grown in vermiculite supplemented with mineral nutrient solution in a greenhouse around 28 °C under natural sunlight. Three-week-old rice seedlings were used for abiotic stress treatments, including spraying with 100 µM ABA (dissolved in 10 mM 4-morpholinee-thanosulphonic acid (MES) buffer, pH 6.0), or the submerging roots in the nutrient solution containing 150 mM NaCl.

For analysis of salt tolerance, the transgenic and wild-type (WT) plants were grown on 1/2 MS medium with 150 mM NaCl (containing 0.5% agar) under a 16/8 light/dark cycle at 28 °C for 3 weeks. These plants were then transferred to paddy pots for recovery at normal growth conditions. The plants were evaluated 8 d later.

Pathogen inoculation

Inoculation of rice blast fungi, *M. oryzae*, was conducted as described by Wang *et al.* (2007). Briefly, spores were harvested and suspended in 0.1% (w/v) gelatin to 10^5 conidia ml^{-1}. Three-week-old rice plants were inoculated with the spore solution by spraying. The inoculated plants were maintained in the growth chamber at 24 °C in darkness for 24 h, then under growth conditions of a 16/8 light/dark cycle with 95% humidity. For analysis of gene expression, rice plants were inoculated with an avirulent *M. oryzae* strain P131 or a virulent strain P140. Leaves were sampled at designated times and used for RNA isolation.

To examine the capability of disease resistance, 3-week-old transgenic and the WT plants were inoculated with the virulent strain P140 as mentioned above. Some leaves from each line were sampled for analysis of gene expression 6 h post inoculation and disease severity was evaluated by a method of image analysis described by Fukuoka *et al.* (2009). The area of lesions formed 7 d after the infection was counted on the third leaves (15 cm in length) of 20 plants for each line.

Subcellular localization of OsERF922

Subcellular localization of OsERF922 in rice was examined by using the *CaMV35S:OsERF922-GFP* transgenic seedlings and the *CaMV35S:GFP* transgenic plants were used as the control. 4′-6-Diamidino-2-phenylindole (DAPI) staining was used as a nuclear marker. Fluorescence signals were detected using a confocal microscope (Eclipse TE2000, Nikon).

Western blot analysis

Total proteins were extracted from leaves of 7-d-old seedlings by grinding with small plastic pestles in the extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na$_3$VO$_4$, 10 mM NaF, 50 mM β-glycerophosphate, 10 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 µg ml^{-1} leupeptin, 5 µg ml^{-1} aprotinin, 5% glycerol). After centrifugation at 13,000 *g* for 15 min, the supernatants were transferred into clean tubes, quickly frozen in liquid nitrogen, and stored at –80 °C until use. Protein concentration was determined by using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Protein samples (2 µg) were separated on a 10% SDS-PAGE and analysed by protein gel blot. Mouse anti-Flag antibody (Sigma-Aldrich) with 1:4000 dilution was used as the primary antibody and goat anti-mouse peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) with 1:8000 dilution was used as the secondary antibody. The membranes were visualized using a Super-Signal West Femto Trial Kit (Themor) following the manufacturer's instructions.

Transcriptional activation assays

A reporter plasmid was constructed by replacing the full CaMV35S promoter in pBI121 using a fragment harbouring four repeats of the GCC-box and the CaMV35S minimal promoter (from –46 to +10), kindly provided by Prof. Huang (Zhang *et al.*, 2004). *Agrobacterium* cells harbouring the reporter plasmid were harvested by centrifugation and resuspended in 10 mM MES, pH 5.6, 10 mM MgCl$_2$, and 0.2 mM acetosyringone to an optical density (600 nm) of 0.7. After 4 h incubation at 28 °C, the bacteria were infiltrated into the leaves of 10-d-old *pCo-Ubi:Flag-*

OsERF922 transgenic and WT tobacco plants using a needle-free syringe. The tissues around the infiltration sites were punched off into microcentrifuge tubes 48 h post treatment. GUS activity was determined as described using 4-methylumbelliferone as the standard (Jefferson, 1987).

Real-time quantitative RT-PCR

DNase-treated RNA (5 µg) was reverse transcribed in a total volume of 50 µl using reverse transcriptase (Takara). Real-time quantitative PCR (qPCR) was performed using 20 ng of total RNA in a 20 µl reaction volume using SYBR Green PCR MasterMix (Takara) on a StepOne Quantitative PCR system (Applied Biosystems) following the manufacturer's protocols. Primer sequences used for the qPCR analysis are shown in Supplementary Table S1 (available at *JXB* online).

Determination of endogenous ABA

For estimation of endogenous ABA levels of rice seedling, samples were snap-frozen with liquid nitrogen and lyophilized. About 30 mg of dried sample was ground to powder with liquid nitrogen and extracted at 4 °C at 12 h with 1 ml of solution A (10% MeOH, 1% acetic acid) containing 50 ng of each D6-ABA (ICON Isotopes) as internal standards. The total extract was centrifuged (13,000 *g*, 10 min) and the pellet was re-extracted once with 1 ml of solution A. The combined supernatant was dried with N$_2$ gas, and resultant residue was taken up in 50 µl solution A for LC-MS analysis (Fan *et al.*, 2009).

Samples (10 µl) were then analysed by HPLC-electrospray ionization/MS-MS using an Agilent 1100 HPLC coupled to an Applied Biosystems Esquire 6000 (Bruker). Chromatographic separation was carried out on a Phenomenex Luna 3 µm C18 150 × 2.0 mm column at 35 °C. The solvent gradient used was 100% solvent A (H$_2$O/CH$_3$CN/CHOOH 94.9:5:0.1) to 100% solvent B (H$_2$O/CH$_3$CN/CHOOH 5:94.9:0.1) over 20 min. Solvent B was held at 100% for 5 min then the solvent returned to 100% A for 10 min equilibration prior to the next injection. The solvent flow rate was 200 µl min^{-1}. Analysis of the compounds was based on appropriate multiple reaction monitoring of ion pairs for labelled and endogenous ABA.

Determination of Na$^+$ and K$^+$ contents

For analysis of Na$^+$ and K$^+$ contents, the transgenic and WT plants were grown on 1/2 MS medium with 100 mM NaCl (containing 0.5% agar) under a 16/8 light/dark cycle at 28 °C for 3 weeks. Leaves were sampled and used for analysis of Na$^+$ and K$^+$ contents with a flame photometer (2655-00 Digital Flame Analyzer, Cole- Parmer Instrument Company, Chicago, USA), as described by Song *et al.* (2005).

Results

Expression of OsERF922 was induced by Magnaporthe oryzae, ABA, and NaCl treatments

Previous studies demonstrated that tobacco OPBP1, an ERF transcription factor, is involved in disease resistance and salt tolerance (Guo *et al.*, 2004). Multiple database searches and DNA sequence alignment analyses showed OsERF922 is one of the genes with high similarity to OPBP1 (Supplementary Fig. S1). OsERF922 has an ERF/AP2 domain, which contains the 14th Ala and 19th Asp residues, two key amino acids contributing to GCC-box binding in

many ERFs (Sakuma *et al.*, 2002). In addition to the ERF/AP2 domain, OsERF922 protein includes a relatively acidic and Ser-rich (56–81 aa) region, which might act as a transcription activation domain.

Many ERF members have been shown to take part in hormonal signal transduction in response to biotic and abiotic stresses (Zhang *et al.*, 2004; Liang *et al.*, 2008; Pré *et al.*, 2008). To investigate whether *OsERF922* is involved in rice defence responses, the current study used qPCR to analyse the expression patterns of *OsERF922* during both the compatible and incompatible interactions between rice and rice blast fungi. Following infection with an avirulent *M. oryzae* strain, P131, transcription of the *OsERF922* gene was induced as early as 1 h post inoculation (hpi), and its level peaked at 3 hpi with a 14-fold increase in mRNA accumulation compared with that at 0 h and decreased sharply to near basal level at 12 h (Fig. 1A). Expression of *OsERF922* was also induced by inoculation with a virulent P140 strain, but the induction was slower and weaker than during the incompatible interaction (Fig. 1A). The results suggested that *OsERF922* might be involved in early defence responses against pathogens. Further, this study examined the expression levels of *OsERF922* in 3-week-old rice seedlings under abiotic stresses and hormone treatments. The mRNA accumulation of *OsERF922* was increased remarkably after treatment with ABA (100 μM) for 1 h, reached to a maximal level after 6 h, and was maintained at a relatively high level throughout the rest of the experimental period (Fig. 1C). Similarly, expression of *OsERF922* was induced about 5-fold by the NaCl treatment (Fig. 1C). Induction of *PR1a* (Fig. 1B) and *Rab16a* (Fig. 1D) revealed the efficacious pathogen and chemical treatments, respectively. However, no significant changes of OsERF922 expression were observed during methyl jasmonate or ET treatments (data not shown). The regulation of *OsERF922* expression by various stresses suggests that it might play multiple roles in response to environmental stimuli.

OsERF922 protein interacts specifically with the GCC box in planta

To examine the interaction of OsERF922 with the GCC box *in vivo*, a transient expression experiment was performed. A DNA fragment harbouring four GCC-element repeats was fused to the minimal CaMV35S promoter (–46 bp) and the GUS gene and used as a reporter plasmid (Zhang *et al.*, 2004) (Fig. 2A). Tobacco plants overexpressing *OsERF922* under the control of a maize ubiquitin promoter were used as effectors. The 4 × GCC-GUS reporter construct was introduced into the leaves of the homozygous transgenics of T$_3$ progenies by means of *Agrobacterium*-mediated infiltration method (Yang *et al.*, 2000). The GUS activity was increased about 5–10-fold in the six transgenic lines, when compared with the WT control, demonstrating that OsERF922 could bind to the GCC-box *in planta* and act as a transcriptional activator (Fig. 2B).
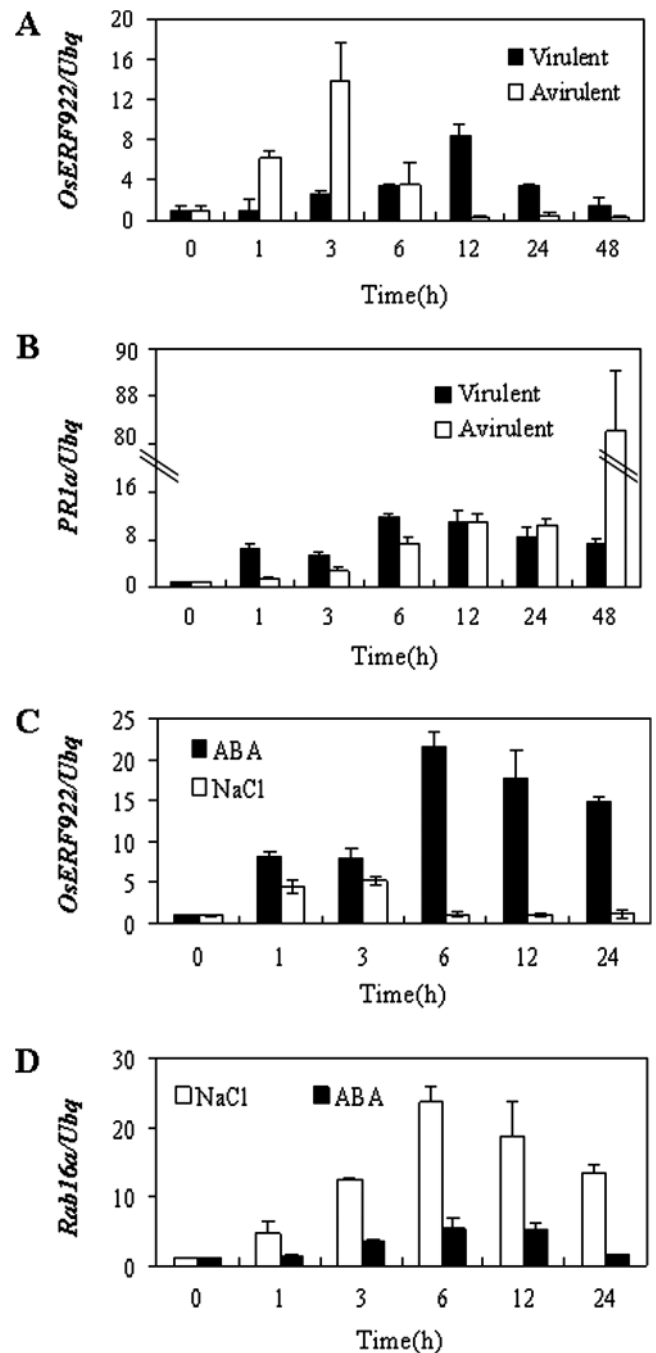

Fig. 1. Expression of the *OsERF922* gene in response to biotic and abiotic stresses. RNA was extracted from leaves of 3-week-old seedlings treated with 100 μM abscisic acid (ABA), 100 mM NaCl, or inoculation with an avirulent *Magnaporthe oryzae* strain, P131, or a virulent strain, P140, for the indicated hours: (A) relative *OsERF922* expression with infection; (B) relative *PR1a* expression with infection; (C) relative *OsERF922* expression with ABA and salt treatments; (D) relative *Rab16a* expression with ABA and salt treatments. Transcription levels were quantified by quantitative reverse-transcription PCR and the data were normalized using the rice ubiquitin gene (Ubq) as standard. Values are mean ± SD of three separate analyses for each RNA template. The experiments were performed in duplicate using different RNA samples for the template; similar results were obtained from each duplicate.
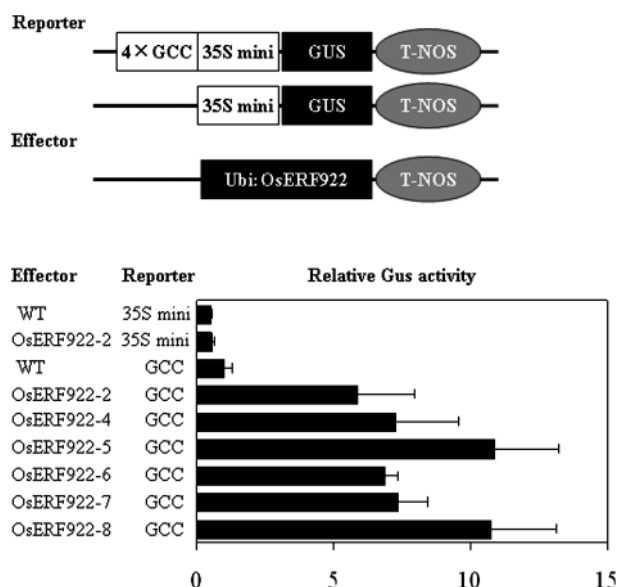
Fig. 2. Transcriptional activation of OsERF922: the constructs of the reporter and effector genes (upper panel) and induction of GUS activities (lower panel). The *GUS*-reporter gene is driven by a synthetic promoter consisting of the –46 minimal CaMV 35S promoter and four copies of the GCC box, and the construct without the GCC box (35S mini) was used as the control. The effector gene was driven by a maize ubiquitin promoter and the resultant construct was transformed into tobacco. *Agrobacterium* cells harbouring the reporter plasmid were infiltrated into the leaves of transgenic (OsERF922) and wild-type (WT) tobacco plants using a needle-free syringe. The ratios of GUS activities were calculated from the GUS activities determined in the leaves harvested 48 h after the infiltration over those determined in the WT control. Values are means and errors calculated from triplicate samples and three independent experiments.

OsERF922 is a nuclear targeted protein

The deduced OsERF922 protein has no obvious nuclear localization signal, as predicted by PSORT analysis (Nakai and Kanehisa, 1992). To determine the subcellular localization of OsERF922 protein in rice, the construct of a CaMV35S-controlled OsERF922-GFP chimeric gene (*CaMV35S:OsERF922-GFP*) and the control *CaMV35S:GFP* vector were stably transformed into rice. Inner epidermal cells of the transgenic T_1 progenies were observed by microscopy technique for fluorescence signal. Localization of the OsERF922-GFP fusion protein was visualized mainly in the nuclei (Fig. 3). These results suggest that OsERF922 could target to the nucleus.

OsERF922 negatively regulates disease resistance in rice

To reveal the biological function of *OsERF922* in plants, full-length cDNA of *OsERF922* was fused downstream of the maize ubiquitin promoter, generating a *Ubi:OsERF922* construct with a Flag-tag at the N-terminus for rice trans-

formation. For *OsERF922* knockdown, a hairpin structure (*CaMV35S:dsOsERF922*) was used for RNA interference. More than 15 independent transgenic lines were obtained for each construct, none of which showed a significant difference in plant morphological features (such as root length, plant height, and numbers of spikelets) compared with the WT plants under normal growth conditions (data not shown). To determine the level of Flag-OsERF922 in the transgenic lines, total proteins were isolated from six randomly selected transgenic plants and analysed by Western blotting using the anti-Flag antibody. The results showed high levels of the fusion proteins in all lines tested, although the levels differ between lines (Fig. 4A).

To examine whether the *OsERF922* gene is involved in disease resistance, the transgenic plants were inoculated with the virulent fungal pathogen *M. oryzae* P140. As illustrated in Fig. 4I, lesions formed by pathogen infection were remarkably increased in the *OsERF922*-overexpressing plants of the T_2 progeny compared to the WT plants, whereas the *OsERF922* RNAi plants displayed a significant decrease of lesion formation. The differences were further evaluated by quantification of the lesion areas (Fig. 4J), which again indicates that knockdown of *OsERF922* expression enhanced disease resistance against the rice blast pathogen.

OsERF922 downregulates the expression of defence-related genes

To examine whether the altered disease resistance phenotypes of the overexpressing lines and the RNAi lines are associated with altered defence gene expression, this study analysed transcript levels of several defence-related genes with or without pathogen inoculation using the qPCR method. The level of the *OsERF922* transcript was increased more than 18-fold in the overexpressing plants and decreased 4-fold or more in the RNAi plants compared with the WT plants (Fig. 4B). As expected, expression of *OsERF922* was induced in WT plants 6 h post *M. oryzae* inoculation, whereas its level did not change significantly in the *OsERF922*-overexpressing linesm which is most likely due to the high level of constitutive expression of the transgene. The transcript level remained low in the knockdown plants following pathogen infection, suggesting efficient suppression of *OsERF922* in the RNAi lines. Among the defence-related genes, expression of the *PR* genes, including *PR1a*, *PR1b*, and *PR10* were reduced in the overexpressing plants but enhanced in the RNAi plants in comparison with these in WT plants (Fig. 4C–E). Increases in expression of these genes were observed in all of the plants after inoculation with the rice blast pathogen. However, the transcript levels of the *PR1a*, *PR1b*, and *PR10* genes were increased much more in the knockdown lines than those in the control plants. In contrast, the induction of these genes by the pathogen infection was compromised in the *OsERF922*-overexpressing lines.

Phenylalanine ammonia lyase (PAL) catalyses the first committed step of phenolic biosynthesis in plants, including
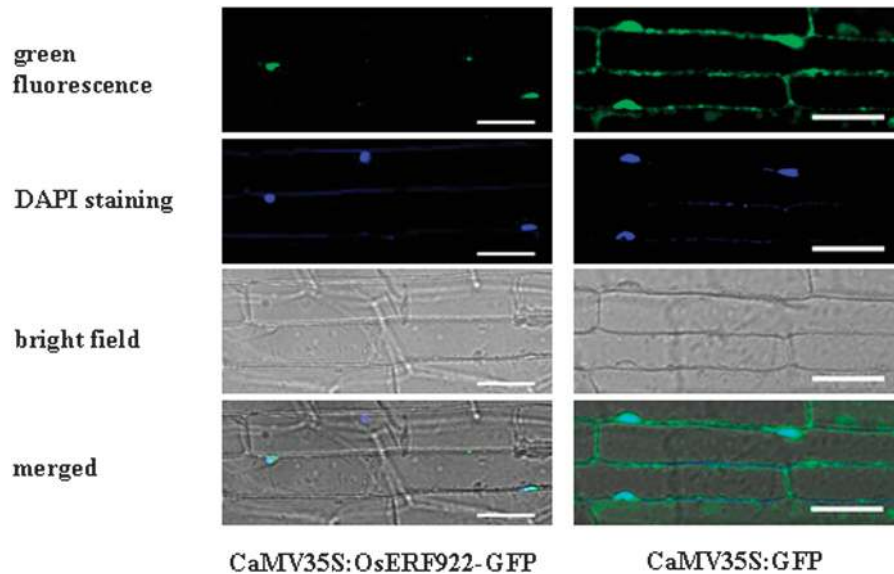
Fig. 3. Subcellular localization of OsERF922. The *OsERF922-GFP* chimeric gene was put under the control of the CaMV35S promoter and transformed into rice. The OsERF922:GFP fusion was localized exclusively to the nucleus of rice inner epidermal cells of the sheaths. GFP alone was detected in both the nucleus and the cytoplasm. Bright-field images of the inner epidermal cells are shown in the bottom half.

SA. Analysis of *PAL* expression revealed slight suppression in the overexpressing lines and an increase of 5-fold or more in the RNAi lines compared with the control plants (Fig. 4F). Stronger induction of *PAL* expression was observed in the RNAi plants during blast fungal infection, whereas its transcript level was not increased significantly in the over-expressing plants. Diterpene cyclases, *ent*-copalyl diphosphate (*ent*-CDP) synthase (OsCPS2/OsCyc2), and *syn*-CDP synthase (OsCPS4/OsCyc1) catalyse the conversion of geranylgeranyl diphosphate (GGDP) to *ent*-CDP and *syn*-CDP, respectively, and *ent*-CDP leads to synthesis of phytocassanes A to E and oryzalexins A to F and *syn*-CDP leads to synthesis momilactones A and B and oryzalexin S (Kanno *et al.*, 2006). These antimicrobial compounds, known as phytoalexins or allelochemicals, are synthesized in the rice leaf in response to infection with the blast pathogen or exposure to UV irradiation (VanEtten *et al.*, 1994; Kato-Noguchi and Ino, 2003). Similarly to the expression pattern of the *PAL* gene, accumulation of *OsCPS2* mRNA was increased in the RNAi lines and enhanced with the inoculation with blast fungi (Fig. 4G). However, expression of the *OsCPS4* gene was suppressed more than 20-fold in the overexpressing progenies and the level was much lower than the control plants even upon the infection with *M. oryzae* (Fig. 4H). These results suggest that OsERF922 is involved in disease resistance as a negative regulator by suppressing defence-related genes.

OsERF922 negatively regulates salt tolerance in rice

Induction of *OsERF922* expression by NaCl and ABA treatments prompted this study to test the tolerance of the transgenic rice plants to NaCl. The transgenic seeds of T_2 progeny and WT plants were germinated on MS medium and the seedlings were then transplanted on MS medium containing 150 mM NaCl. After growing on the NaCl medium for 3 weeks, leaves of the *OsERF922*-overexpressing lines became scorched particularly at the top of the leaves (Fig. 5A). The symptom was less severe in the RNAi and WT plants. After these plants were transferred to paddy pots for recovery (Fig. 5B), nearly half of the plants overexpressing *OsERF922* died during the 8-d recovery period (Fig. 5E). Those plants that survived also showed significant retardation of growth and less biomass than that of the WT plants (Fig. 5C and D).

Salt tolerance in plants is the consequence of several physiological processes, such Na^+ uptake, Na^+/K^+ balance, and ion compartmentalization. Damage of leaves caused by salinity is attributed to accumulation of Na^+ in the shoot by transport of Na^+ from the roots experiencing high external salt concentration (Lin *et al.*, 2004). Thus, the current study measured the accumulation of Na^+ and K^+ in the rice shoots. The levels of K^+ were lower in *OsERF922*-overexpressing lines than those in the WT and RNAi plants on 1/2 MS media, whereas the difference of Na^+ accumulation was not obvious among the plants tested (Fig. 6A). During NaCl treatment, an increase of Na^+ content was observed, with significantly higher accumulation in *OsERF922*-overexpressing lines than the WT and RNAi plants. On the other hand, the content of K^+ was suppressed during the salinity treatment (Fig. 6B), in agreement with competition between Na^+ and K^+ uptake in the shoots (Lin *et al.*, 2004). However, the level of K^+ in the overexpressing plants was even lower than those in the WT and *OsERF922* RNAi lines (Fig. 6B). The results indicate that the enhanced salt sensitivity is possibly the consequence of inhibition in K^+ accumulation in *OsERF922*-overexpressing plants.

OsERF922 regulates ABA accumulation

In addition to its well-established role in response to abiotic stress, ABA is also involved in defence responses. Therefore,
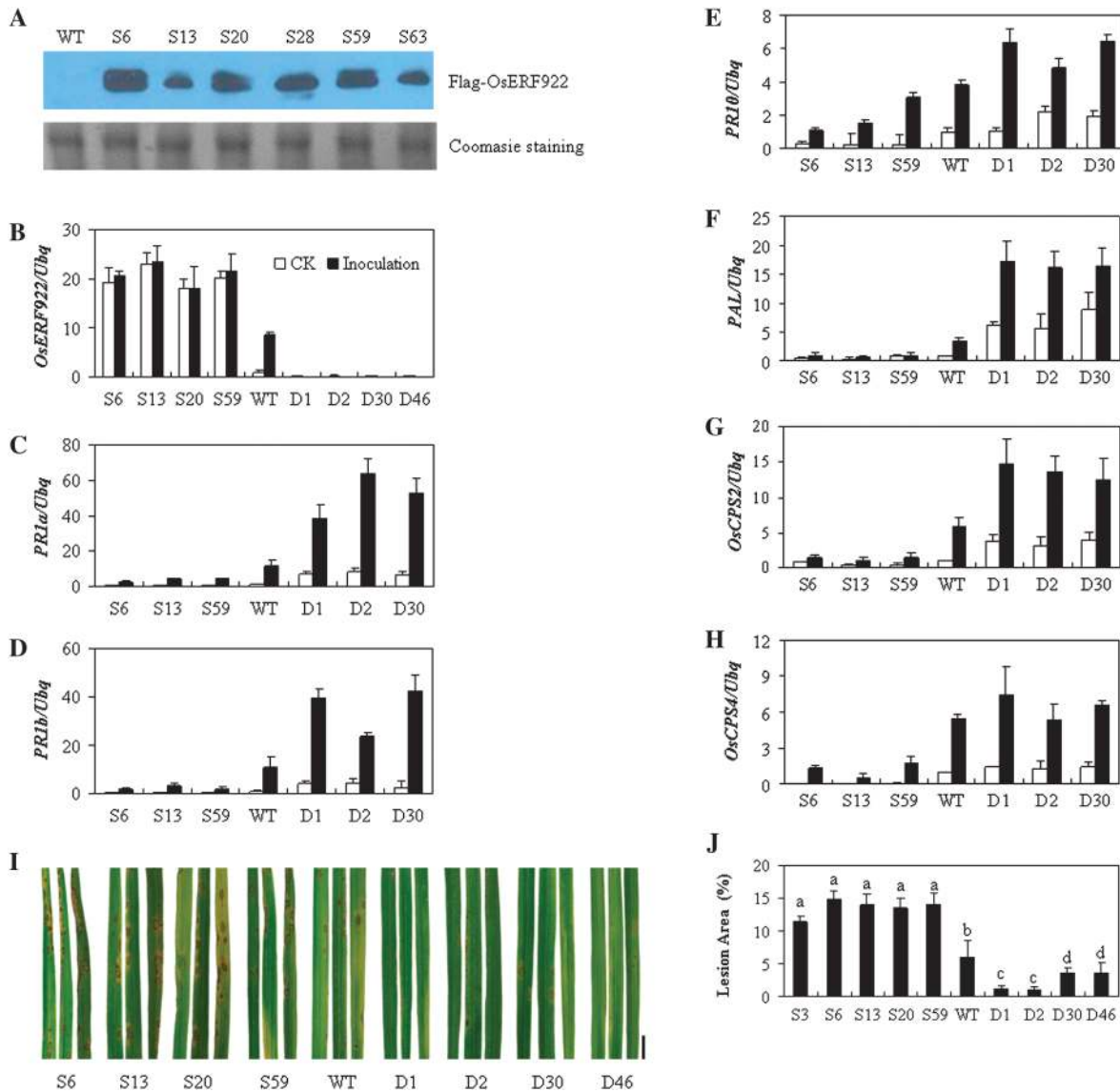
Fig. 4. Overexpression and knockdown of *OsERF922* alter resistance to *Magnaporthe oryzae* and the expression of defence-related genes. (A–H) Analysis of *OsERF922* expression in *OsERF922* overexpression (S6, S13, S20, S28, S59, and S63) and knockdown (D1, D2, D30, D46) rice lines by Western blot (A) and quantitative reverse-transcription PCR (B, *OsERF922*; C, *PR1a*; D, *PR1b*; E, *PR10*; F, *PAL*; G, *OsCPS2*; H, *OsCPS4*), as described in Fig. 1. Total proteins were extracted from leaves of 7-d-old seedlings of the transgenic and wild-type plants, separated on a 10% SDS-PAGE, and detected by the mouse anti-Flag antibody. Three-week-old transgenic and the wild-type plants were inoculated with the virulent *M. oryzae* P140 isolate and several leaves from each line were sampled for analysis of gene expression 6 h post inoculation. Data are representative of two independent biological replicates. (I) Transgenic plants of *OsERF922* were tested for resistance to *M. oryzae*. Leaves of 3-week-old rice plants were inoculated with conidial suspensions of *M. oryzae* (10^5 conidia ml^{-1}) isolate P140. Leaves were detached from inoculated plants 7 d post the infection and photographed. Experiments were repeated three times with similar results. Bar, 1 cm. (J) Histograms showing the area of lesions formed on the third leaves (15 cm in length) of 20 plants for each line. Values marked with different letters are significantly different ($P < 0.01$, Fieldman Rank Sums test).

this study measured ABA levels and expression of ABA biosynthesis-related genes. The amount of ABA was about 2-fold more in *OsERF922*-overexpressing line S6 and 1.4-fold in line S59 than that in the WT plant (Fig. 7A). A decrease in ABA accumulation was observed in the *OsERF922* RNAi lines: in the D1 line, the ABA level in the D1 and D30 lines was about one-sixth and one-third of that in WT, respectively (Fig. 7A). 9-*cis*-Epoxycarotenoid dioxygenase (*NCED*)

catalyses the conversion of 9-*cis*-epoxycartenoids to xanthoxin, a rate-limiting step in ABA biosynthesis (Schwartz *et al.*, 2003). The expression of *OsNCED* genes was examined in the transgenic and control plants by qPCR. *OsNCED3* and *4* genes were both upregulated in the *OsERF922*-overexpressing plants and *OsNCED4* was suppressed in the RNAi plants, in comparison with the WT plants (Fig. 7B and C). Further, this study examined the expression of
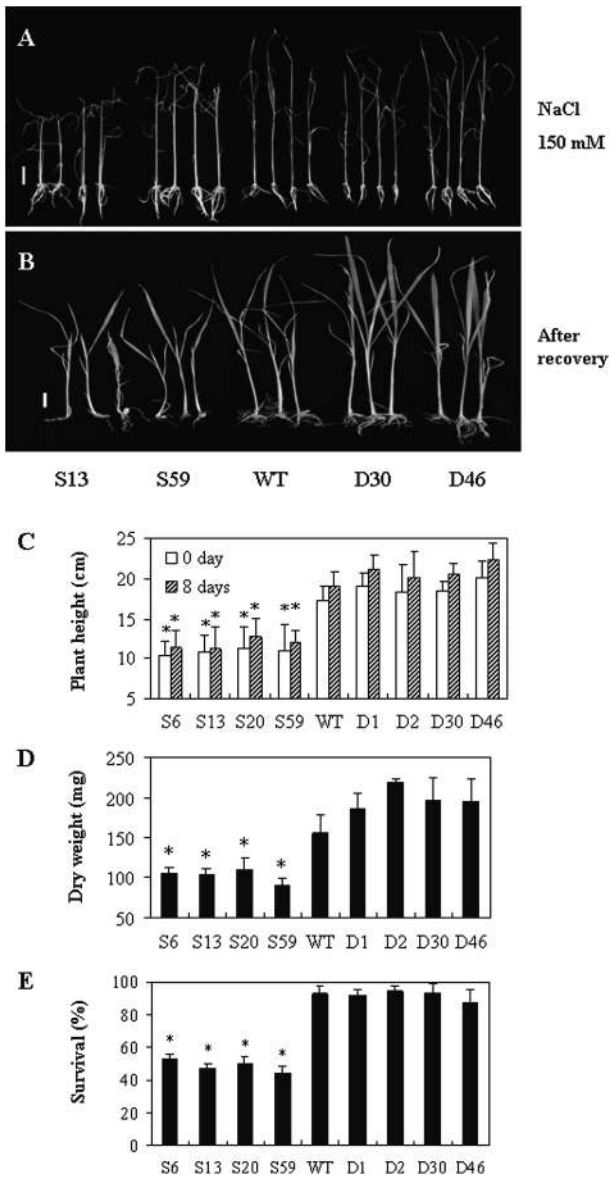
Fig. 5. Overexpression of *OsERF922* enhances salt sensitivity. (A) Transgenic and wild-type plants grown on 1/2 MS medium containing 150 mM NaCl under a 16/8 light/dark cycle at 28 °C for 3 weeks. (B) Plants after 8 d recovery in paddy pots at normal growth conditions. (C–E) Histograms showing: plant heights on the MS medium supplemented with 150 mM NaCl and after 8 d recovery (C), dry weight (D), and the percentage of survived plants (E) post the recovery. Values are means ± SE of three replicates with 10 seedlings per replicate. Asterisks indicate significant differences between the control and the overexpressing lines (*P* < 0.05, Fieldman Rank Sums test). Bars, 1 cm.

Rab16a, an ABA-responsive marker gene (Yamaguchi-Shinozaki *et al.*, 1990). A much higher level of *Rab16a* transcripts was observed in the *OsERF922*-overexpressing plants as compared to the WT, whereas the expression of *Rab16a* was decreased in the RNAi lines (Fig. 7D). The increase of ABA accumulation was consistent with the enhanced expression of the ABA-biosynthesis and -responsive genes in the overexpressing plants.
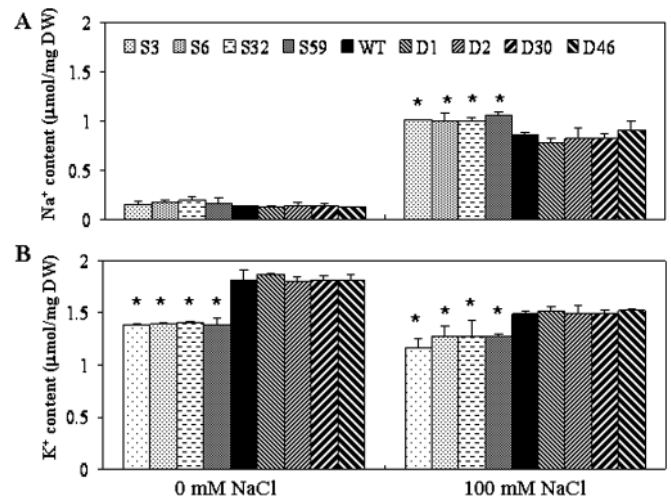

Fig. 6. Na⁺ (A) and K⁺ (B) contents in the shoots of WT and the transgenic plants under non-salt-stress and 100 mM NaCl treatment for 21 d (*n* = 3). Asterisks indicate significant differences between the control and the overexpressing lines (*P* < 0.05, Fieldman Rank Sums test).

Discussion

This study reports characterization of OsERF922, which was classified into phylogenetic group IX of AP2/ERF superfamily by Nakano *et al.* (2006). Genes in this group have been shown to play crucial roles in biotic and abiotic stress responses. For example, overexpression of *AtERF1*, tomato *Pti4/5*, or tobacco *OPBP1* enhances disease resistance (Berrocal-Lobo *et al.*, 2002; Gu *et al.*, 2002; Guo *et al.*, 2004). Further, ectopic expression of *OsBIERF3*, a member of rice ERFs in group IX, increases disease resistance against infection by tomato mosaic virus and the bacterial wild fire pathogen, *P. syringae* pv. *tabaci*, as well as tolerance to salt stress in tobacco (Cao *et al.*, 2006). The data indicate that these ERFs are positive regulators of defence- and/or stress-related transcription. *OsERF922* is induced rapidly and strongly in response to salt, ABA, and *M. oryzae* pathogen (Fig. 1). The *OsERF922* RNAi plants showed increased resistance against *M. oryzea* (Fig. 4). OsERF922 also regulates Na⁺/K⁺ homeostasis, as revealed from the increased Na⁺ and decreased K⁺ accumulation in the *OsERF922*-overexpressing plants. DNA binding and reporter gene activation assays, suggest that OsERF922 is a transcriptional activator with a high affinity for DNA molecules containing the GCC-box sequences (Fig. 2). In combination with the result of nuclear localization (Fig. 3), the data suggest that OsERF922 directly regulates gene expression and plays a significant role in mediating plant responses to environmental stimuli.

Through analysis of the responses to pathogen infection, *OsERF922*-overexpressing lines were more susceptible to rice blast fungus than that of the knockdown plants (Fig. 3), indicating OsERF922 function as a negative regulator of plant disease resistance. The question is why plants promote expression of a negative regulator in response to pathogen
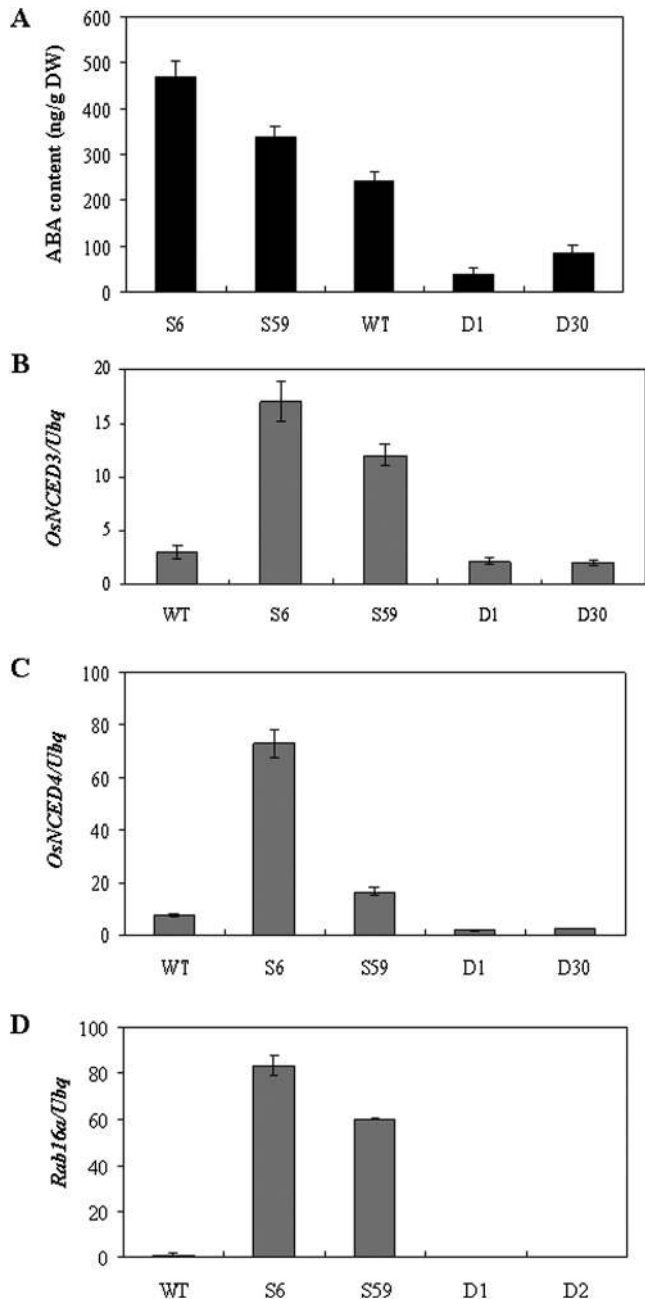
Fig. 7. OsERF922 alters the expression of ABA biosynthesis genes as well as ABA accumulation. (A) Endogenous levels of ABA in 3-week-old leaves were quantified by LC-MS/MS. Values are mean and SE of three independent samples. (B–D) RNA was isolated from 3-week-old leaves of the *OsERF922* transgenic and the wild-type plants for the *OsNCED3* (B), *OsNCED4* (C) and *Rab16a* (D) genes and quantified as described in Fig. 1. Experiments were repeated in triplicate and similar results were obtained.

modifies the host ABA-signalling pathway to suppress defence responses (de Torres-Zabala *et al.*, 2007). AvrPto functions as a kinase inhibitor, which directly interacts with receptor kinases, such as *Arabidopsis* and tomato FLS2, to block PAMP-triggered immunity and leads to host susceptibility (Xiang *et al.*, 2007). Transcription activator-like effectors of *Xanthomonas oryzae* pv. *oryzae*, a causal agent of bacterial blight of rice, strongly induced rice *Os8N3*, *OsTFX1*, and *OsTFIIAγ1* genes to compromise the defence mechanism and promote a state of disease susceptibility (Yang *et al.*, 2006; Sugio *et al.*, 2007). Further, *Arabidopsis* WRKY48 has also been characterized as a transcriptional activator that represses plant basal resistance (Xing *et al.*, 2008). Induction of *WRKY48* expression is not enhanced during infection by the *PstDC3000hrcC* mutant strain defective in the type III secretion system, suggesting that certain virulent factors from the pathogen are required to promote the expression of the negative regulator. Further experiments will be necessary to determine whether any effectors or secreted proteins from rice blast fungal pathogen are involved in expression of negative regulatory genes such as *OsERF922* to attenuate host defence responses.

A large number of genes related to plant defence are induced in compatible and incompatible interactions, although the kinetics and amplitude of response are attenuated during compatible interactions (Tao *et al.*, 2003; Vergne *et al.*, 2007). *OsERF922* expression also followed predicted patterns, showing induction by avirulent *M. oryzae* P131 strain was as early as 1 hpi and reached a peak expression level in 3 hpi, but it was elevated at 3 hpi and reached to the peak at 12 hpi during the compatible interaction (Fig. 1A). In the period of 3 hpi, spores attached on the surface of rice leaf are in the stages of germination and appressorium development (Talbot, 2003; Ribot *et al.*, 2008). The results suggest that rice quickly recognizes the invading pathogen through spores, germ tubes, contaminated hypha, and/or other unknown secreted factors and elicits innate defence responses or enhances host susceptibility. Likely, OsERF922 is an important component of the signalling pathway. MAL10, a barley resistance protein against the powdery mildew fungus, has been shown to directly associate with HvWRKY1/2 transcription factors upon recognition of the fungal avirulence A10 effector (Shen *et al.*, 2007). Importantly, the identified WRKY proteins are repressors of PAMP-activated basal defence, revealing MAL10 functions as a de-repressor of the immune response. *Arabidopsis* AtERF104, also an ERF member of group IX, is characterized as a substrate of MAPK 6 (Bethke *et al.*, 2009), which is a positive regulator of the flg22-mediated basal defence. Interestingly, both the over-expressor and the mutant of *erf104* showed enhanced susceptibility to *B. cinerea* and a non-adapted bacterial pathogen, *P. syringae* pv. *phaseolicola*. It has been demonstrated that ERF104 must be maintained at an optimal level and any alterations of this crucial threshold can tip the 'signalling balance' (Bethke *et al.*, 2009). OsMPK5, a MAPK protein in rice, is inducible by infection of rice blast fungi as well as ABA and abiotic stresses and its role is

attack that would cause them more susceptible to the invading pathogen. One possibility is that OsEFR922 might be activated by pathogens to suppress host defence as a pathogen's counter-defence mechanism. In the case of bacterial pathogens, a number of studies have demonstrated that plant-pathogenic effectors modulate diverse host processes to enhance virulence. AvrPtoB of *P. syringae* effector

well documented as negatively modulating broad-spectrum disease resistance and positively regulating abiotic tolerance (Xiong and Yang, 2003). It will be interesting to reveal whether OsMPK5 and OsERF922 are involved in the same pathways during biotic and abiotic stress responses.

This study indicates that OsERF922 functions as a transcriptional activator in plants (Fig. 2), although it suppresses expression of defence genes and resistance against rice blast fungal pathogen (Fig. 4). It is plausible that OsERF922 first activates some unknown negative regulators, instead of suppressing defence genes directly. In turn, the negative regulators repress defence-related genes and compromise disease resistance. OsERF922 could also play a role in preventing overactivation of defence reactions that may have an overall fitness cost. Nevertheless, identification of the available targets of OsERF922 will provide useful tools to dissect the poorly understood mechanism of transactivators acting negatively on plant immune pathways.

The phytohormone ABA is a key regulator of responses to diverse abiotic stresses as well as in plant growth and development. A high ABA content is often associated with stresses such as drought, cold, and salt. The transgenic lines of overexpressing *OsERF922* showed more sensitivity to salty conditions in comparison with the WT plants. An explanation is probably due to the disturbance of Na^+/K^+ homeostasis (Fig. 6). Similarly, the transgenic plants of *OsAP2-39*, another rice *ERF* gene are more susceptible to drought comparing with the WT plants, although the transgenic plants contain higher amount of ABA (Yaish *et al.*, 2010). Increasing reports have shown that ABA may also participate directly or indirectly in responses to a range of pathogens. Using ABA-deficient mutants, inhibition of ABA biosynthesis or exogenous application of ABA, several studies have shown a positive correlation between endogenous ABA levels and resistance to pathogens (Adie *et al.*, 2007; Fan *et al.*, 2009), while others have documented a negative role for this hormone in plant defence reactions (Audenaert *et al.*, 2002; Mohr and Cahill, 2003; de Torres-Zabala *et al.*, 2007, 2009; Asselbergh *et al.*, 2008b; Jensen *et al.*, 2008). In rice plants, ABA has been shown as an activator of resistance against rice brown spot-causing *Cochliobolus miyabeanus* (De Vleesschauwer *et al.*, 2010). However, ABA appears to play a negative role in response to rice blast fungus, since leaves treated with ABA become slightly more susceptible to *M. oryzae* (Koga *et al.*, 2004). The current data also indicate that ABA might enhance host susceptibility. Expression of ABA biosynthetic genes and accumulation of ABA were decreased in the *OsERF922* RNAi plants, in line with enhanced resistance against *M. oryzae*, whereas overexpressing plants were more susceptible to the pathogen (Fig. 4). Recently, the blast fungus *M. oryzae* was found to produce and secrete ABA, which antagonizes the SA-signalling pathway to attenuate host defence responses (Jiang *et al.*, 2010). These results imply that fungus- and host-derived ABA may play a critical role in the interactions between rice plant and the hemi-biotrophic fungus.

PAL is a key enzyme catalysing the first committed step of the phenylpropanoid biosynthetic pathway, leading to production of hormone SA and antimicrobial phytoalexins. ABA suppression of PAL activity is a possible mechanistic explanation for its negative impact on disease resistance. Application of ABA reduced the accumulation of SA and *PAL* expression in *Arabidopsis*, consistent with enhanced susceptibility to an avirulent stain of *P. syringae* pv. *tomato* (Mohr and Cahill, 2007) and the production of the phytoalexin glyceollin is reduced in an ABA-dependent manner in soybean upon infection with *Phytophthora sojae* (Mohr and Cahill, 2003). In agreement with this, the current study also found that *PAL* expression was elevated and further increased by inoculation with *M. oryzea* in *OsERF922* RNAi plants coincident with the low level of ABA accumulation. In contrast, the pathogen-inducible *PAL* expression was suppressed in the overexpression lines (Fig. 4F). The large majority of phytoalexins isolated in rice are diterpenoids with the exception of the flavanone sakuranetin (Kodama *et al.*, 1992). The diterpenoids have been classified into phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S, according to their structures of diterpene hydrocarbon precursors (Kanno *et al.*, 2006). Expression of *OsCPS2* and *OsCPS4*, two cyclase genes leading to the synthesis of the diterpenoid phytoalexins, was suppressed significantly at the basal levels as well as under the conditions of pathogen inoculation (Figs. 4G and H). In mutants deficient in ABA biosynthesis, the synthesis of capsidiol, a sesquiterpene phytoalexin in tobacco is induced higher than WT plants when infected with *B. cinerea* or elicited with elicitors, while the application of exogenous ABA reversed the elevated effect (Mialoundama *et al.*, 2009). Collectively, the data indicate that ABA is implicated in the biosynthesis of phytoalexins in challenged plants, which affects host susceptibility.

Recently, Bailey and associates (2009) have found that exogenous application of ABA drastically reduces endogenous ET levels in rice and enhances susceptibility to *M. oryzea*. Antisense suppression of *OsEIN2*, a key component of ET signal transduction, confers ET insensitivity and ABA supersensitivity and enhances rice blast susceptibility, while the resistance against *C. miyabeanus* is increased (De Vleesschauwer *et al.*, 2008, 2010). Further, the OsEIN2 antisense plants are more tolerant to drought, salinity, and cold. Interestingly, rice ETHYLENE INSESITIVE3-LIKE 1, an orthologue of *Arabidopsis* EIN3 has been found to interact with OsMPK5, which increases ET level and resistance against *M. oryzae* and decreases the ABA level and tolerance to abiotic stresses in the RNAi-mediated OsMPK5 suppression plants (Xiong and Yang, 2003; Bailey *et al.*, 2009). Based on the model of ET signal transduction in *Arabidopsis* (Yoo *et al.*, 2009), EIN3 and EIN3-LIKE (EIL) proteins transduce the signal from the upstream component EIN2 to downstream components including the ERF transcription factors. These results indicate that ABA antagonistically interacts with ET-signalling networks and consequently influences disease resistance as well as abiotic stress tolerance, and also implies that OsERF922 is possibly

such a candidate downstream of OsEIL1 signal transduction. Overlap between abiotic and biotic stress signal transduction pathways is well known, including some similar mechanisms, such as the generation of reactive oxygen species and nitric oxide (Pastori and Foyer 2002), and the involvement of MAPK-signalling cascades (Xiong and Yang, 2003; Fujita *et al.*, 2006) and various transcription factors. Increased expression of transcription factors, such as a barley ERF and rice OsMyb4, lead to enhanced resistance to pathogen attack and tolerance to abiotic stresses (Vannini *et al.*, 2006; Jung *et al.*, 2007). This study's data suggest that the negative regulation of OsERF922 on basal resistance and salt tolerance is mediated through the ABA-signalling pathway, suggesting that OsERF922 is integrated into the complicated cross-talk between biotic and abiotic stress-signalling networks.

Supplementary material

Supplementary data are available at *JXB* online.
 Supplementary Table S1. Primers used for qRT-PCR.
 Supplementary Fig. S1. *OsERF922* sequences and phylogenetic analysis.

Acknowledgements

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