

Expression of a gene encoding a rice RING zinc-finger protein, OsRZFP34, enhances stomata opening

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Abstract By oligo microarray expression profiling, we identified a rice RING zinc-finger protein (RZFP), *OsRZFP34*, whose gene expression increased with high temperature or abscisic acid (ABA) treatment. As compared with the wild type, rice and *Arabidopsis* with *OsRZFP34* overexpression showed increased relative stomata opening even with ABA treatment. Furthermore, loss-of-function mutation of *OsRZFP34* and *AtRZFP34* (*At5g22920*), an *OsRZFP34* homolog in *Arabidopsis*, decreased relative stomata aperture under nonstress control conditions. Expressing *OsRZFP34* in *atrzfp34* reverted the mutant phenotype to normal, which indicates a conserved molecular function between *OsRZFP34* and *AtRZFP34*. Analysis of water loss and leaf temperature under stress conditions revealed a higher evaporation rate and cooling effect in *OsRZFP34*-overexpressing *Arabidopsis* and rice than the wild type,

atrzfp34 and *osrzfp34*. Thus, stomata opening, enhanced leaf cooling, and ABA insensitivity was conserved with *OsRZFP34* expression. Transcription profiling of transgenic rice overexpressing *OsRZFP34* revealed many genes involved in *OsRZFP34*-mediated stomatal movement. Several genes upregulated or downregulated in *OsRZFP34*-overexpressing plants were previously implicated in Ca^{2+} sensing, K^+ regulator, and ABA response. We suggest that *OsRZFP34* may modulate these genes to control stomata opening.

Keywords ABA · Heat stress · Rice · RING zinc finger protein · Stomata · Transpiration

Abbreviations

ABA	Abscisic acid
Col	<i>Arabidopsis thaliana</i> ecotype Columbia
HS	Heat shock
HSPs	Heat shock proteins
OS	Opening solution
ROS	Reactive oxygen species
RZFP	RING zinc-finger protein
sHSPs	Small heat shock proteins
TRIM	Taiwan rice insertion mutant
WT	Wild type

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Introduction

Plants, being sessile organisms, cannot escape from their growing environments. Extremes in environmental factors can result in stressful conditions that inhibit plant growth and development. These detrimental effects inevitably limit crop productivity. Understanding how the plant responds to environmental stress is critical for increasing stress

tolerance in crops through genetic engineering (Shinozaki and Yamaguchi-Shinozaki 2007).

Heat stress due to elevated temperature is the major environmental limitation of plant growth and development. High temperatures can create fluid membranes, reduce photosynthetic electron transport, and denature cellular proteins (Sun et al. 2002; Suzuki and Mittler 2006; Hüve et al. 2011). Plants have evolved a complex set of molecular responses to adapt to stressful high temperatures. For example, high temperature invokes the synthesis of heat shock proteins (HSPs), associated with acquired thermotolerance (Yeh et al. 1997; Kotak et al. 2007). Other pathways mediate thermotolerance through an HSP-independent mechanism. In *Arabidopsis*, mutation of *MBF1c* or *Siz1*, involved in the salicylic-acid signaling pathway, causes thermosensitivity but no alteration in expression of HSPs or heat shock (HS) factors (Yoo et al. 2006; Suzuki et al. 2008).

In addition to synthesizing protective molecules, plants may incorporate other physiological responses to cope with heat stress. For example, after exposure to high temperature, plant leaves can respond by actively regulating stomata opening to evaporate water, which results in heat dissipation from the leaves (Lewis and Nobel 1977). However, transpirational cooling involves water usage, which often forces plants to face water deficits under field conditions (Liu et al. 2005). Levels of the phytohormone abscisic acid (ABA) are triggered by water deficit to regulate necessary changes in gene expression and physiological adaptation (Chaves et al. 2002). One of the major adaptive responses mediated by ABA is stomata closure (Schroeder et al. 2001). Factors that function in ABA-mediated stomata closure include protein phosphatase 2C proteins such as ABI1 and ABI2, protein kinases such as SnRK2.6/OST1 and GHR1, transmembrane ATP binding cassette proteins such as ABCG22 and MRP5, and anion channels such as SLAC1 (Mustilli et al. 2002; Umezawa et al. 2009; Kuro-mori et al. 2011; Hua et al. 2012). ABA can mediate stomata closure by changing the cytosolic $[Ca^{2+}]$ and $[H^+]$ levels in guard cells, or enhancing the Ca^{2+} sensitivity for activation of *SLAC1* and downregulation of inward-rectifying K^+ channels *KAT1* (Lebaudy et al. 2007; Siegel et al. 2009). In addition, stress can induce stomata closure through ABA-independent reactive oxygen species (ROS) homeostasis (Mustilli et al. 2002; Huang et al. 2009). These measures, although effective in preventing water loss, can ultimately hamper transpirational cooling. Plants must have mechanisms to balance these interconnected yet counteractive field-survival strategies, but the genetic determinants of the mechanisms are largely unclear.

Rice is a staple food crop consumed by more than half of the world's population. Rice plants often face more than one stress during their growth in paddy fields. For instance, stomata close in rice plants grown under water deficit in

arid regions, which leads to limited carbon uptake by leaves and increased leaf temperature under high irradiance (Hüve et al. 2011). Thus, discovery of genes involved in multiple environmental stress responses provides new insights to study the responses to multiple stresses and improve multiple stress tolerance in rice.

This study aimed to identify the genes involved in HS and ABA responses. We used oligo microarray expression profiling of leaves of rice (*Oryza sativa* L. cv. Tainung No. 67) to screen for an HS- and ABA-responsive novel gene and identified *OsRZFP34* (cDNA accession no. AK068005). *OsRZFP34* encodes a RING zinc-finger protein (RZFP, Freemont et al. 1991) (Supplementary Fig. S1). *OsRZFP34* transcripts specifically accumulated in rice leaves at germination and vegetative stages after HS or ABA treatment. Gain- and loss-of-function analyses revealed that *OsRZFP34* could have a critical role in mediating stomatal aperture and function in leaf cooling with exposure to high temperature and drought in rice.

Materials and methods

Plant materials

Seeds of rice (*Oryza sativa* L. cv. Tainung No. 67) were soaked in running water at 28 °C and when germinated, were transferred to soil and grown under a 12-h-light (28 °C)/12-h-dark (26 °C) cycle in a growth chamber with 70 % relative humidity. For HS treatment, 3-week-old rice plants were incubated in shaking buffer [1 % (w/v) sucrose in 5 mM potassium phosphate buffer, pH 6.8] at 41 °C for the indicated times. For ABA treatment, 3-week-old rice plants were incubated in shaking buffer with or without 100 μ M ABA at 28 °C for the indicated times.

The *Arabidopsis thaliana* ecotype Columbia (Col) was used as the wild type. *Arabidopsis* plants were grown at 23 °C in a 16-h-light/8-h-dark cycle in a growth chamber with 60 % relative humidity. For HS and ABA treatment, 3-week-old *Arabidopsis* plants were used.

RNA isolation and first-strand cDNA synthesis

Total RNA was extracted as described (Guan et al. 2010). To remove genomic DNA contamination, total RNA for cDNA synthesis was first treated with DNase I (Promega, Madison, WI, USA) for 30 min at 37 °C. The first-strand cDNA was synthesized from 2 μ g total RNA in a 20- μ l reaction volume with use of the SuperScriptIII First-Strand Synthesis system (Invitrogen, Carlsbad, CA, USA). RNA and cDNA were quantified by optical density measurement with use of a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA).

Analysis of *OsRZFP34* transcripts by RT-PCR

For analysis of *OsRZFP34* in rice plants, leaves collected from seed germination, seedling, tillering, booting, heading, and ripening stage rice plants were incubated in shaking buffer at 41 °C and with or 100 μM ABA at 28 °C for the indicated times, respectively. RT-PCR was used to analyze transcript levels of *OsRZFP34*. PCR amplification conditions were 24 cycles of 30 s at 95 °C, 30 s at 48 °C and 30 s at 72 °C, then 5 min at 72 °C. Primer sequences for *OsRZFP34* were forward (5'-GCATGTGTTGAAGGAGCG-3') and reverse (5'-CTGGCACTTGTGCGCAAT-3'). DNA from 15 μl of each PCR reaction was fractionated by 1.2 % (w/v) agarose gel electrophoresis. ImageJ for Windows (<http://rsbweb.nih.gov/ij/>) was used to quantify the intensity of the ethidium-bromide-stained DNA bands. The expression of *18S rRNA* was an internal control.

Microarray analysis

To analyze the genes involved in the response to HS and ABA, Agilent 60-mer rice oligonucleotide microarrays (Agilent Technologies, CA, USA) were used to study the transcriptome profiles of ABA- and HS-treated 3-week-old rice leaves. The hybridizations were conducted as suggested by the manufacturer (Agilent Technologies, CA, USA). We performed two independent biological replicates of hybridizations.

For microarray analysis of *OsRZFP34*-overexpressing rice plants, we used the Rice Whole Genome OneArray v1.1 (Phalanx Biotech Group, Taiwan), which contains 22,003 DNA oligonucleotide probes, and each probe is a 60-mer designed in the sense direction. Among the probes, 21,179 probes correspond to the annotated genes in RGAP v.6.1 and BGI (2008) database. In addition, we included 824 control probes. The detailed descriptions of the gene array list are available from http://www.phalanx.com.tw/products/RiOA_Probe.php. Fluorescent antisense RNA (aRNA) targets were prepared from 1 μg total RNA samples with the OneArray Amino Alkyl aRNA Amplification Kit (Phalanx Biotech Group, Taiwan) and Cy5 dyes (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Human Whole Genome OneArray with Phalanx hybridization buffer by the Phalanx Hybridization System. The hybridizations were conducted as suggested by the manufacturer (Phalanx Biotech Group, Taiwan). The slides were dried by centrifugation and scanned by use of an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensities of each spot were analyzed by use of GenePix 4.1 (Molecular Devices). Normalized spot intensities were transformed to gene expression \log_2 ratios between control and treatment groups. The spots with \log_2 ratio ≥ 1 or ≤ -1

and P value < 0.001 were tested for further analysis. Two independent biological replicates of hybridizations were performed.

Quantitative real-time RT-PCR (qRT-PCR)

To analyze transcript levels of *OsRZFP34*-upregulated genes, qRT-PCR involved use of the iCycler- iQ5 Multicolor Real time PCR Detection System and iQ SYBR Green Hot-start Supermix (Bio-Rad, Hercules, CA, USA) with the primer sequences in Supplementary Table S1. Real-time PCR reaction involved 60 ng cDNA with each set of primers and the iQ™ SYBR® Green Supermix (Bio-Rad). PCR cycling included an initial step at 95 °C for 5 min, then 40 cycles of 10 s at 95 °C, 30 s at 56 °C, and 20 s at 72 °C. The comparative C_T method was used to determine the relative amount of each sample, with the expression of *18S rRNA* as an internal control.

Preparation of DNA constructs and transformation

To generate the construct for overexpressing *OsRZFP34*, we synthesized the coding region of *OsRZFP34* by PCR amplification of the first-strand HS-induced rice cDNAs with the primer sequences 5'-ACTAGTATTCAAGATGGGCGCCAT-3' and 5'-GCGGCCGCTTCGCCACTTCAGATCTG-3' with built-in *SpeI* and *NotI* restriction sequences, respectively. The PCR product was subcloned into the *SpeI* and *NotI* sites of the *pCYH8* vector (Guo and Ho 2008) in the sense orientation downstream of the ubiquitin 1 promoter (*Ubi* promoter). The resulting *Ubi::OsRZFP34* construct was cloned into the *pPZP/35H* Ti-based vector by inserting 35S-hph-tml as a *HindIII* fragment into the *HindIII* site of *pPZP200* (Hajdukiewicz et al. 1994), which was introduced into *Agrobacterium tumefaciens* (strain *EHA105*). Transformation of *Arabidopsis* involved the floral-dip method (Weigel and Glazebrook 2002). Rice calli (*Oryza sativa* L. cv. Tainung No. 67) were transformed by *Agrobacterium*-mediated transformation as described (Hiei et al. 1994). Transformed rice or *Arabidopsis* seedlings were selected on medium that contained 50 μg/ml hygromycin. The hygromycin-resistant T1 seedlings were transferred to soil and grown to maturity. Homozygous T2 plants were selected by growth of their T3 generations on medium that contained hygromycin. T3 seeds derived from homozygous T2 plants were used for subsequent tests.

Stomata analysis

For imaging rice stomata, leaves of 3-week-old plants were fixed as described (Hurng et al. 1988). Stomata were observed under a Hitachi S-4700 scanning electron microscope. For ABA treatment, detached rice leaves were

incubated in stomata-opening solution (OS) containing 10 mM KCl, 100 μ M CaCl₂, and 10 mM MES, pH 6.1, for 2 h and then transferred to OS supplemented with 5 μ M ABA for 30 min. To measure rice stomatal aperture under high temperature, 3-week-old rice seedlings were subjected to 38 °C for the indicated times and leaves were immediately cut and fixed. More than 30 guard cells from each sample were used to measure stomatal aperture. For imaging *Arabidopsis* stomatal aperture, mature leaves of 3-week-old *Arabidopsis* plants grown at 23 °C for 16 h of light were used. 3 M clear tape was applied to the abaxial surface of each leaf to peel off the epidermal layer. Epidermal strips were immediately mounted on glass slides and observed under a Nikon SMZ-1500 stereoscopic zoom microscope. For ABA treatment, detached leaves were treated as described above. More than 100 guard cells from each sample were used to measure stomatal aperture.

Thermal imaging

Thermal imaging of stressed plantlets was as described (Mustilli et al. 2002). *Arabidopsis* and rice plants were grown at 23 or 28 °C for 16 h of light for 14 or 21 days, respectively. Drought stress was induced by withholding watering as described above and transferring the pots to a drier atmosphere (24 °C, 50 % relative humidity, 16-h photoperiod). Thermal images were obtained by use of an Avio TVS-200EX infrared camera (Nippon Avionics, Japan).

Stress treatment of transgenic *Arabidopsis*

For water loss measurement, the aerial parts of 4-week-old plants grown in soil were separated from roots and allowed to dry (25 °C, 60 % relative humidity). The fresh weight of each sample was recorded at 30-min regular intervals.

Statistical analysis

Data are shown as mean \pm SE from 3 independent experiments. Statistical differences were analyzed by Student's *t* test or Duncan's multiple range test. A *P* < 0.05 was considered statistically significant.

Results

Cloning and expression analysis of *OsRZFP34*

Among the genes with >twofold difference in expression between nonstress control and HS- and ABA-treated rice leaves (CY Hong, unpublished results), we selected and further characterized *OsRZFP34* because it showed the highest HS responsiveness among the ABA responsive

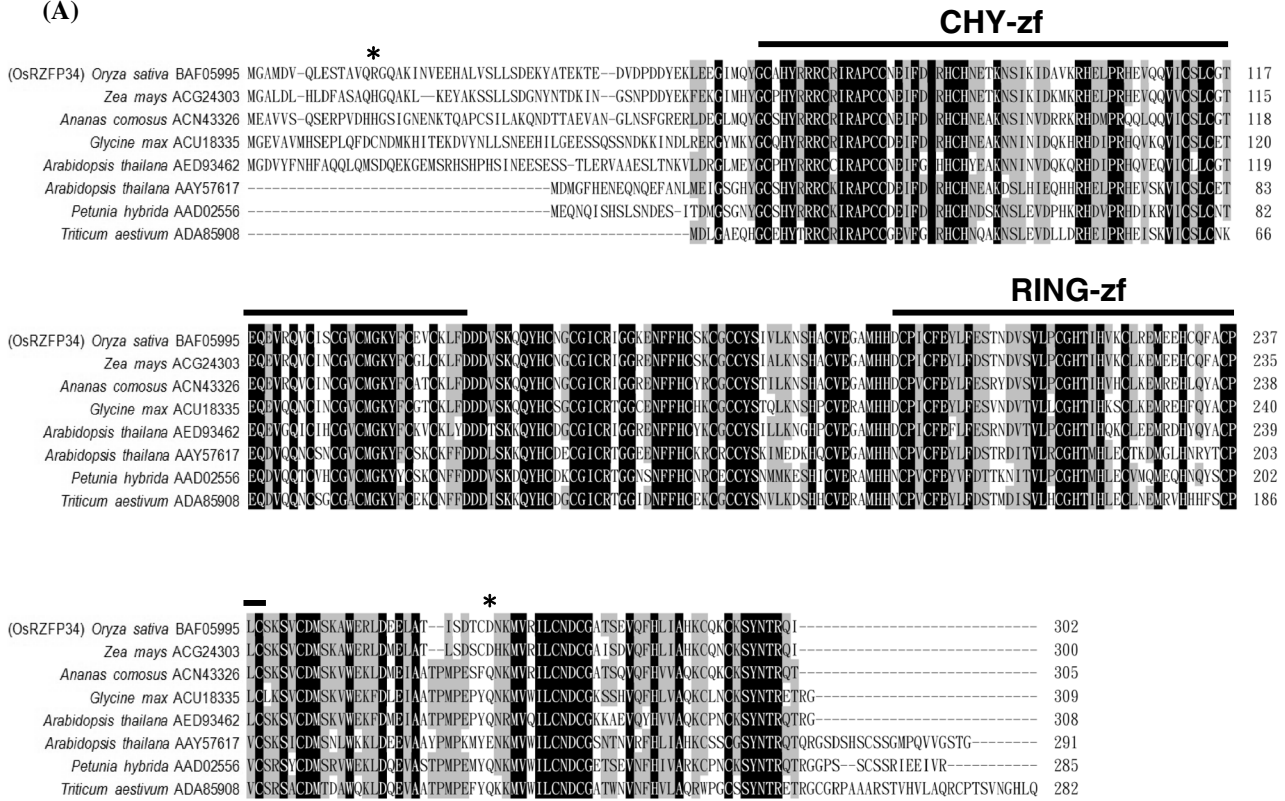
Fig. 1 a Alignment of *OsRZFP34* and other plant RZFPs based on deduced amino acid sequences. ClustalW analysis showed that they all contain conserved CHY zinc finger and RING-finger. The conserved CHY zinc finger domain (CHY-zf) and the RING zinc finger domain (RING-zf) were *underlined*, respectively. The different amino acids between *OsRZFP34* and *OsRFP1* were indicated (*). **b** RT-PCR analysis of *OsRZFP34* in rice. mRNA expression of *OsRZFP34* in the shoot and root of rice seedlings under HS and ABA stresses. Expression of *OsRZFP34* in leaves from seed germination, seedling, tillering, booting, heading, and ripening stage of rice plants treated with **c** 41 °C and **d** 100 μ M ABA for the indicated times. Bars are mean expression relative to that of *18S* rRNA from 3 independent experiments

genes. The predicted protein of *OsRZFP34* consists of 302 amino acids of 34 kDa. Multiple sequence alignment was performed by ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) (Fig. 1a). The deduced amino acid sequence is mostly identical to that of *OsRFP1* (*OsRFP1*; GenBank accession no. AY574990) but differs in amino acids 15 (R in *OsRZFP34* and H in *OsRFP1*) and 265 (D in *OsRZFP34* and G in *OsRFP1*) (Zhou et al. 2008) (Fig. 1a). A search of rice DNA sequences revealed that these 2 proteins were derived from the same gene, so the differences may be due to polymorphism between different cultivars. To understand the evolutionary relationship among plant RZFPs, we constructed a phylogenetic tree with the full-length amino acid sequences using the UPGMA method of Mega5 (<http://megasoftware.net/>). The members of the *OsRZFP34* clade exist in *Ananas comosus*, *Arabidopsis thaliana*, *Glycine max*, *Oryza sativa*, *Petunia hybrida*, *Populus trichocarpa*, *Ricinus communis*, *Selaginella moellendorffii*, *Sorghum bicolor*, *Triticum aestivum*, *Vitis vinifera*, and *Zea mays* (Supplementary Fig. S2), which suggests that they may have conserved molecular functions in these plants, although many of their physiological roles are not identical.

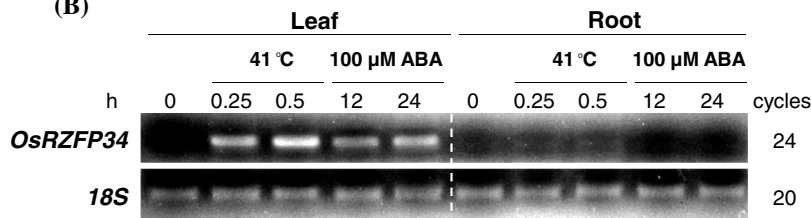
To further confirm the responsiveness of *OsRZFP34* to heat and ABA, we analyzed its transcript levels in various rice tissues exposed to HS and ABA treatments. *OsRZFP34* mRNA level in leaves of rice seedlings was markedly higher with HS and ABA than control treatment (Fig. 1b), which suggests that *OsRZFP34* is implicated in the rice leaf response to thermal and ABA conditions. In contrast, *OsRZFP34* mRNA was not detectable in seedling roots, even under HS and ABA treatments.

Under HS, transcripts of *OsRZFP34* were detected in leaves from germination, seedling, tillering, and booting but not heading or ripening stages (Fig. 1c). *OsRZFP34* transcripts were induced by ABA in leaves from germination, seedling, and tillering stages but were low or not detected in booting, heading, and ripening stages (Fig. 1d). Therefore, the expression of *OsRZFP34* depends on developmental stage and is tissue specific.

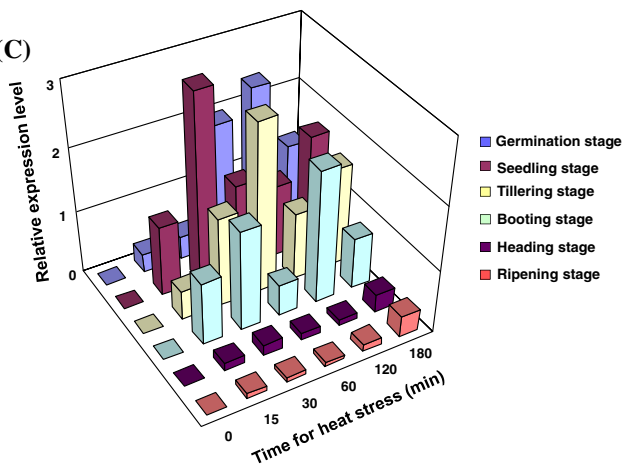
(A)



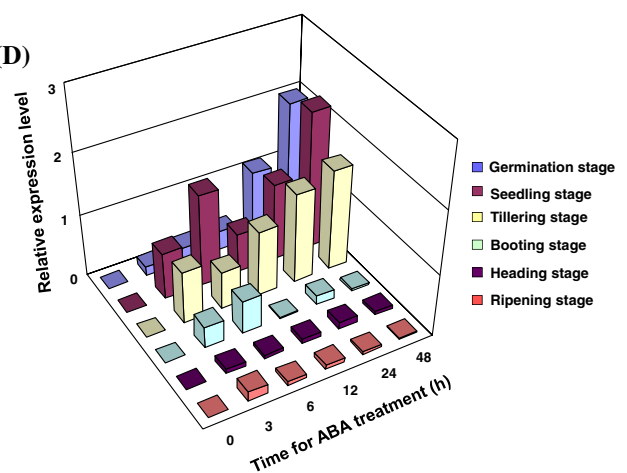
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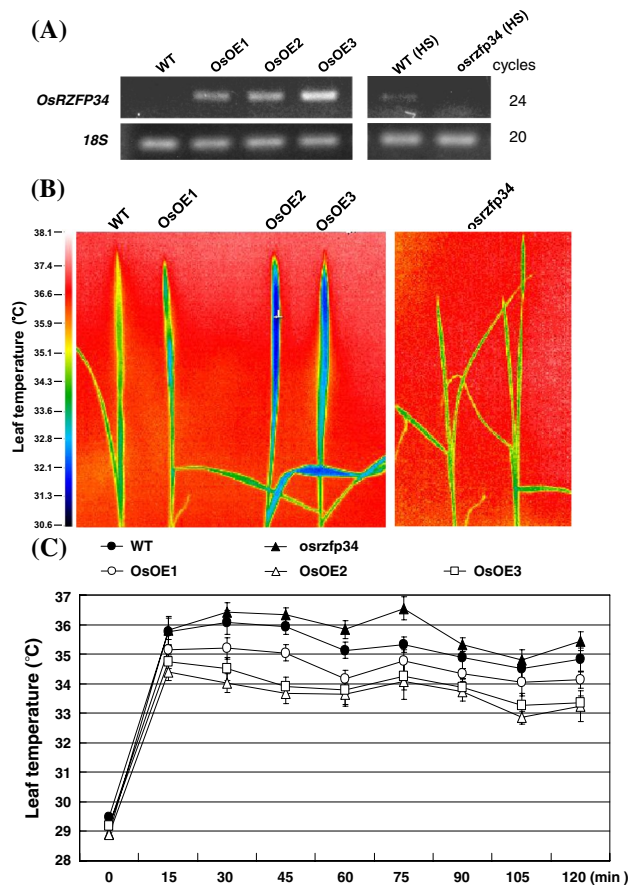


Fig. 2 Analysis of cooling effect in *OsRZFP34*-overexpressing rice plants. **a** Expression of *OsRZFP34* in *OsRZFP34*-overexpression and-knockout rice plants. **b** Leaf temperature of heat-stressed rice plants. Thermal images of the 4th leaf of 3-week-old soil-grown seedlings of wild-type (WT), OsOE1, OsOE2, OsOE3, and *osrzfp34* plants exposed to 38 °C for 60 min. **c** Data are mean \pm SE temperature of the 4th leaf from 3 independent experiments (5 plants in each test)

Constitutive expression of *OsRZFP34* increases rice stomata opening and leaf cooling

We used both loss- and gain-of-function strategies to examine the biological function of *OsRZFP34*. We obtained an *OsRZFP34* rice mutant, *M0028121* (*osrzfp34*), with a transfer DNA (T-DNA) insertion in the last intron, from the Taiwan Rice Insertion Mutant (TRIM) pool; *OsRZFP34* was not expressed in *osrzfp34* even under HS stress (Fig. 2a). Also, we constructed 3 independent T_3 homozygous transgenic rice lines constitutively expressing *OsRZFP34*:OsOE1, OsOE2, and OsOE3 (Fig. 2a). We compared agronomic traits between the wild-type (WT) and the transgenic rice plants grown in a growth chamber under nonstress control conditions. Among the traits, plant height of the OsOE1, OsOE2, and OsOE3 was significantly lower than that of the WT (16–20 % reduction)

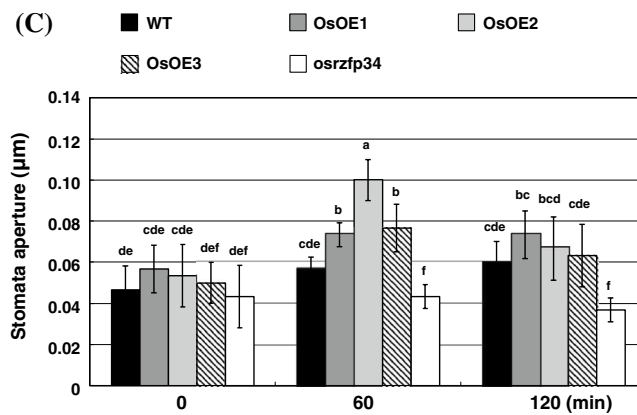
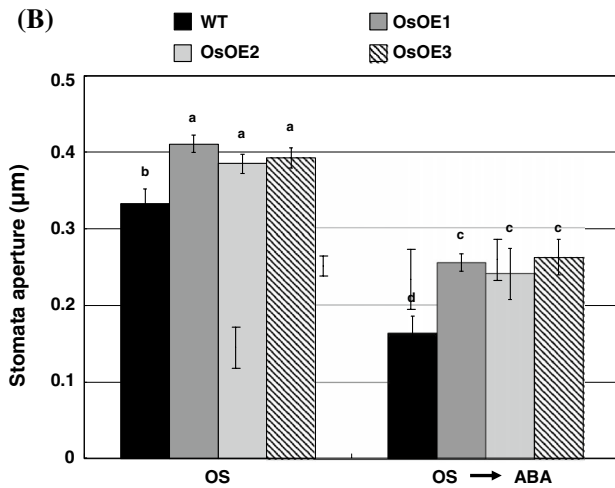
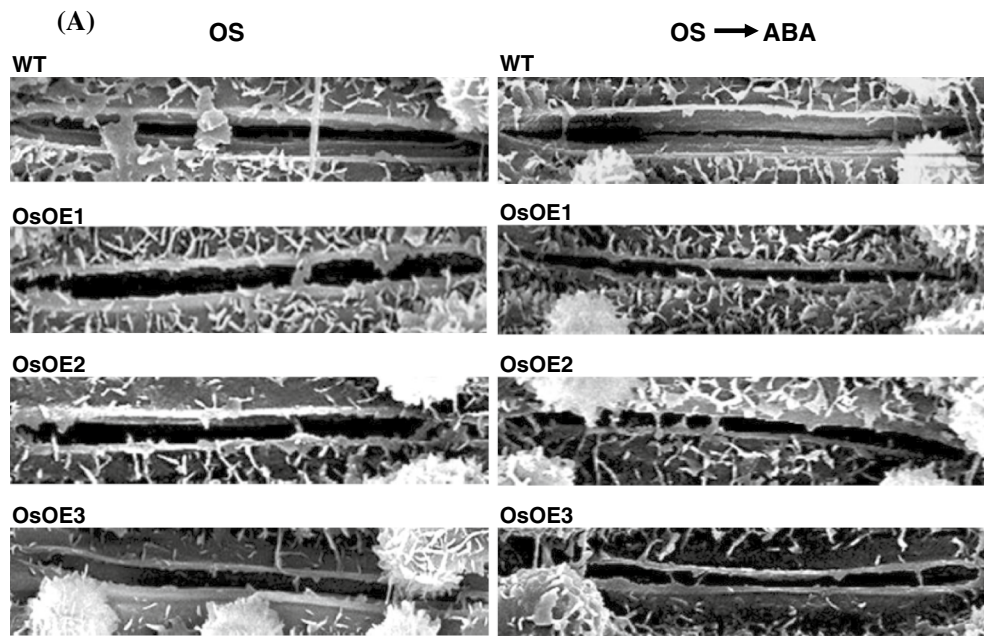
Fig. 3 Analysis of stomatal aperture in *OsRZFP34*-overexpressed rice plants. **a** The 4th leaf of 3-week-old soil-grown seedlings of WT, OsOE1, OsOE2, and OsOE3 was detached and incubated under light in stomata-opening solution (OS) for 2 h and then treated with 5 μ M ABA for 30 min (OS \rightarrow ABA). Scanning electron microscopy of stomatal aperture. Scale bar = 1 μ m. **b** The stomata aperture of each plant incubated in OS for 150 min. **c** 3-week-old soil-grown seedlings of WT, OsOE1, OsOE2, OsOE3, and *osrzfp34* were subjected to 38 °C heat stress. Data are mean \pm SE from 3 independent experiments (30 stomata in each test). Bars with the same letter are not significantly different at $P < 0.05$

at the heading stage (data not shown). We did not find significant difference in panicle length, number of tillers per plant, numbers of panicles, spikelets per panicles, filling rate, and total grain weight of 1,000 grains among the WT, OsOE1, OsOE2, OsOE3, and *osrzfp34* plants (data not shown). Since *OsRZFP34* was induced by heat and ABA, we tested whether *OsRZFP34* is involved in transpirational cooling of rice leaves, which is an important aspect to stressful high temperatures and ABA-dependent regulation. We used infrared thermal imaging to measure leaf surface temperature of the transgenic rice plants with high temperature treatment (Mustilli et al. 2002). The results revealed that leaf temperature was significantly lower in OsOE1, OsOE2, and OsOE3 than WT plants, and the difference was up to 1–2 °C within 60 min (Fig. 2b, c). Conversely, leaf temperature was higher in *osrzfp34* than in the WT under high temperature (Fig. 2b, c). *OsRZFP34* may play an important role in regulating leaf temperature under heat stress.

Stomatal movement in response to high temperature is critical for leaf cooling. Thus, we used scanning electron microscopy to observe stomata. Under nonstress control conditions, stomatal aperture was significantly greater in rice plants constitutively expressing *OsRZFP34* than in WT plants (Fig. 3a, b, c). Of note, in response to ABA, the mean stomata opening was significantly greater in *OsRZFP34* transgenic plants than the WT (Fig. 3a, b), which suggests that *OsRZFP34* may mediate stomata opening even with ABA treatment in rice. Furthermore, during heat stress, the stomatal aperture was larger in *OsRZFP34*-overexpressing rice than the WT, and elevated temperature did not enhance stomata opening in *osrzfp34* (Fig. 3c). This finding correlates well with the increased cooling effect in *OsRZFP34* transgenic plants and increased leaf temperature in *osrzfp34*.

OsRZFP34 and *AtRZFP34* are functional orthologs

To gain better insight into the function of *OsRZFP34*, we overexpressed *OsRZFP34* in *Arabidopsis thaliana* ecotype Col plants. We selected three independent T_3 homozygous transgenic lines AtOE2, AtOE3, and AtOE5, which



constitutively expressed *OsRZFP34* transcripts (Supplementary Fig. S3), to examine stomatal movement phenotypes. Stomatal aperture was measured in the focal planes

of the outer edges of guard cells in epidermal strips of 3-week-old leaves. As compared with Col WT leaves, *AtOE2*, *AtOE3* and *AtOE5* leaves showed a 21–37 %

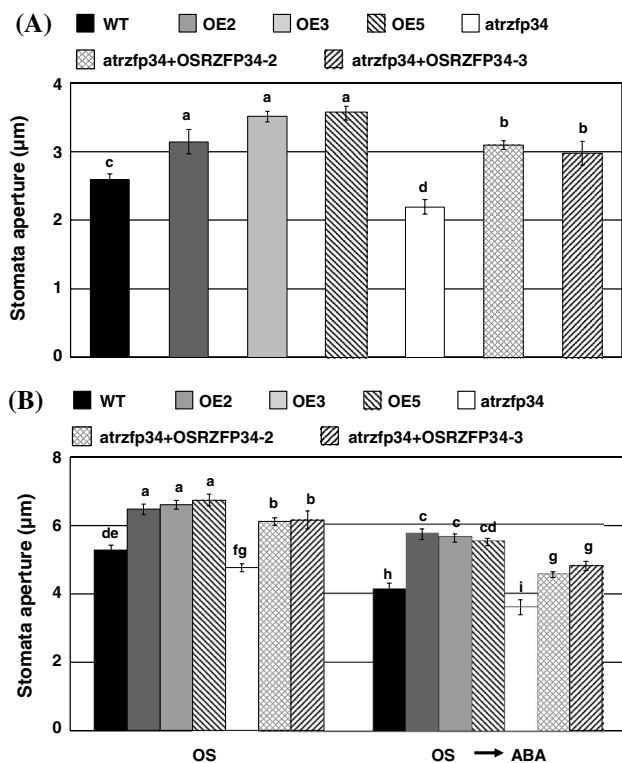


Fig. 4 *OsRZFP34* transgenic *Arabidopsis* show increased stomata opening. **a** Col WT; *OsRZFP34* transgenic plants AtOE2, AtOE3, and AtOE5; *atrzfp34*; and the complemented *atrzfp34* lines *atrzfp34* + *OsRZFP34-2* and *atrzfp34* + *OsRZFP34-3* were grown on soil for 3 weeks, and abaxial epidermal imprints were observed by light microscopy. **b** Detached leaves were incubated under light in OS for 2 h, then treated with ABA for 30 min (OS → ABA). Data are mean ± SE from 3 independent experiments. Bars with the same letter are not significantly different at $P < 0.05$

increase in stomatal aperture under nonstress control conditions (Fig. 4a), which supports the expression of *OsRZFP34* possibly related to stomata opening. We did not find significant difference in plant height, leaf morphology, and flowering time among the WT, AtOE2, AtOE3, and AtOE5 (data not shown).

To confirm that *OsRZFP34* was responsible for regulating stomatal aperture, we used the deduced amino acid sequence to search for its homologs in the *Arabidopsis* genome database. We identified an *Arabidopsis* *RZFP* gene, *At5g22920* (*AtRZFP34*), sharing 66 % amino acid identity with *OsRZFP34*, and obtained an allelic mutant, *salk_017562* (*atrzfp34*), with a T-DNA insertion in its promoter region (Supplementary Fig. S4). After confirmation of abolished *AtRZFP34* expression (Supplementary Fig. S4), homozygous mutant plants derived from a single T-DNA insertion were checked for stomatal aperture. As compared with leaves of the Col WT, those of *atrzfp34* showed significantly decreased (~15 % reduction) stomatal aperture under nonstress control conditions (Fig. 4a). To test whether

OsRZFP34 complements the mutation of *AtRZFP34*, we characterized the phenotype of 2 independent T₃ homozygous complemented mutants, *atrzfp34* + *OsRZFP34-2* and *atrzfp34* + *OsRZFP34-3* (Supplementary Fig. S5). The *OsRZFP34* transgene could restore stomata opening in the *atrzfp34* mutant (Fig. 4a). Loss-of-function and complementation results indicated that these two highly similar proteins shared similar function in mediating stomatal aperture. Furthermore, we measured the stomatal aperture of mutant plants in response to ABA. After ABA treatment, the mean stomata opening was significantly greater for *OsRZFP34* transgenic plants and *OsRZFP34*-complemented mutants than the Col WT and *atrzfp34* (Fig. 4b). The expression of *OsRZFP34* in transgenic *Arabidopsis* plants may increase stomata opening even under ABA treatment.

Ectopic expression of *OsRZFP34* can elevate water loss and reduce leaf temperatures during stress

To test the effect of increased stomatal aperture in *OsRZFP34* transgenic *Arabidopsis* plants, we determined the rate of water loss by measuring the fresh weight of shoots detached for 5 h. The fresh weight of detached shoots of AtOE2, AtOE3, and AtOE5 was reduced to <40 % of the initial weight, but that of the Col WT and *atrzfp34* was maintained at 45 and 52 %, respectively (Fig. 5a). In addition, the change in fresh shoot weight was similar between *atrzfp34* + *OsRZFP34-2* and the Col WT during drought stress (Fig. 5a). Thus, increased stomatal aperture caused by the expression of *OsRZFP34* is associated with enhanced water loss, which is an early response to high temperature stress (Chaves et al. 2002).

Using infrared thermography, we investigated the change in leaf temperature with high temperature treatment. AtOE2, AtOE3, and AtOE5 leaves maintained a significantly lower temperature within 60 min at 30 °C temperature as compared with Col WT leaves, whereas the temperature was higher for *atrzfp34* than Col WT leaves (Fig. 5b). In contrast, leaf temperatures did not significantly differ among the Col WT, AtOE3, and *atrzfp34* after 150 min of heat treatment. These results confirm the role of *OsRZFP34* in a cooling effect during heat stress. Next, we examined the leaf temperature of plants under drought. After withholding water for 5 days, the leaf temperature of *atrzfp34* was ~1 °C higher than that of the Col WT, which reflects reduced cooling from leaves of this mutant (Fig. 5c). This result is consistent with the lower water-loss rate of *atrzfp34* (Fig. 5a). However, leaf temperature of *OsRZFP34*-overexpressing *Arabidopsis* was not significantly lower than that of Col WT with drought treatment. Nevertheless, these results clearly show that under heat and drought stress, *OsRZFP34* was able to complement the *atrzfp34* mutation and the leaf cooling effect.

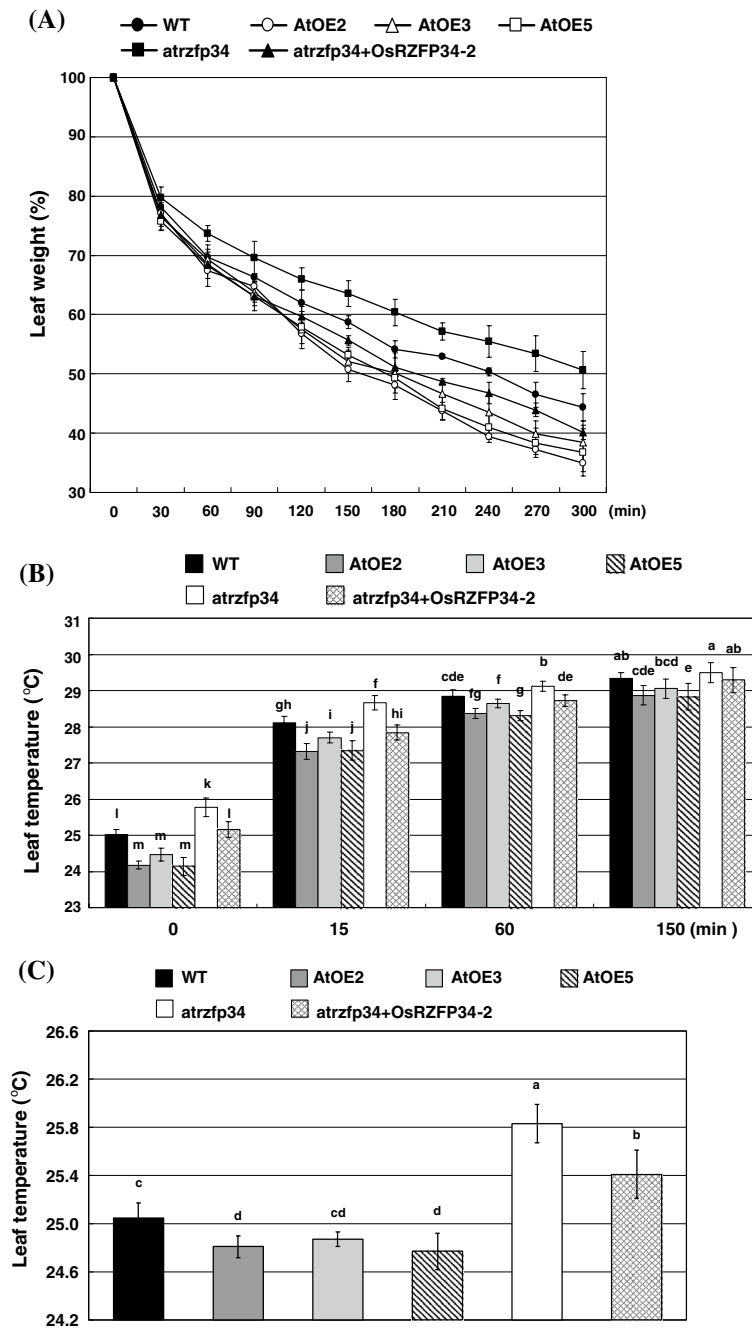


Fig. 5 Analysis of water loss and cooling effect in *OsRZFP34*-overexpressing *Arabidopsis* plants. **a** Water loss analysis. The aerial parts of 4-week-old plants were allowed to dry under the fume hood. Water loss is expressed as the percentage of initial fresh weight. Data are mean \pm SE from 3 independent experiments (10 plants in each test). **b** Leaf temperature of heat-stressed *Arabidopsis* plants. Twenty-day-old seedlings were subjected to up to 30 °C heat stress. Data are

mean \pm SE temperature of the 8th, 9th, and 10th leaf from quantification of infrared thermal images of 3 independent experiments (5 plants in each test). **c** Leaf temperature of drought-stressed *Arabidopsis* plants. Water was withheld from 12-day-old seedlings for 5 days. Data are mean \pm SE temperature from 3 independent experiments (5 plants in each test). Bars with the same letter are not significantly different at $P < 0.05$

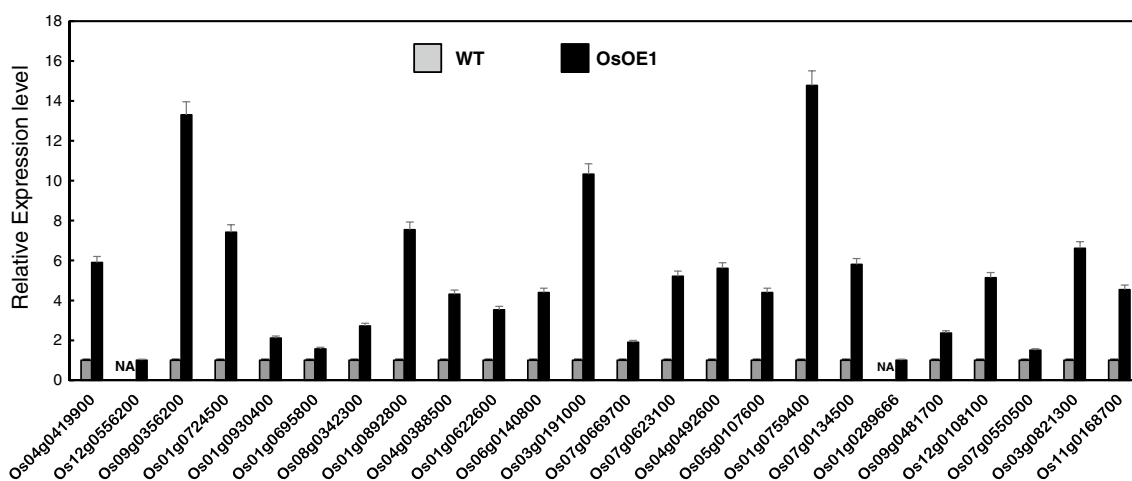
Identification of genes upregulated by overexpression of *OsRZFP34*

To further identify the target genes regulated by *OsRZFP34* and elucidate the molecular mechanism mediated

by this gene, we performed a transcriptome analysis of leaves using the Rice Whole Genome OneArray v1.1 (Phalanx Biotech Group, Taiwan) containing 22,003 DNA oligonucleotide probes. Relative change was calculated by comparing the data for *OsOE1* against those for

Table 1 Selected up-regulated genes in transgenic rice plants overexpressing OsRZFP34 by GeneChip analysis

Locus ID	Change folds (transgenic/wild-type)	P value	Annotation
Os04g0419900	4.37	0.000209	Serine/threonine-protein kinase receptor precursor, putative, expressed
Os12g0556200	3.61	0.000001	Calmodulin binding protein, putative, expressed
Os09g0356200	3.35	0.000009	Serine/threonine-protein kinase, putative, expressed
Os01g0724500	3.33	6.97E-14	Plant PDR ABC transporter associated domain containing protein, expressed
Os01g0930400	3.07	1.24E-09	Potassium transporter, putative, expressed
Os01g0695800	3.04	0.000001	ABC transporter, ATP-binding protein, putative, expressed
Os08g0342300	2.96	1.37E-09	Serine/threonine-protein kinase BRI1-like 1 precursor, putative, expressed
Os01g0892800	2.90	0.000009	Serine/threonine-protein kinase, putative, expressed
Os04g0388500	2.86	0.000055	Calmodulin-binding transcription activator 4, putative, expressed
Os01g0622600	2.82	8.68E-10	CAMK_CAMK_like.9—CAMK includes calcium/calmodulin dependent protein kinases, expressed
Os06g0140800	2.68	1.18E-09	Serine/threonine-protein kinase, putative, expressed
Os03g0191000	2.51	0	Calmodulin-binding transcription activator, putative, expressed
Os07g0669700	2.42	0.0003	Potassium transporter, putative, expressed
Os07g0623100	2.42	0.000001	Calmodulin-binding transcription activator, putative, expressed
Os04g0492600	2.41	1.53E-08	Serine/threonine-protein kinase GCN2, putative
Os05g0107600	2.34	0.00051	ABC transporter, ATP-binding protein, putative, expressed
Os01g0759400	2.32	0.000044	CAMK_KIN1/SNF1/Nim1_like.11—CAMK includes calcium/calmodulin dependent protein kinases, expressed
Os07g0134500	2.27	0.000066	Serine/threonine-protein phosphatase family protein, putative
Os01g0289666	2.25	0.000882	OsWRKY9—superfamily of TFs having WRKY and zinc finger domains, expressed
Os09g0481700	2.17	0.000136	OsWRKY80—superfamily of TFs having WRKY and zinc finger domains, expressed
Os12g0108100	2.07	0.000957	Serine/threonine-protein kinase, putative, expressed serine/threonine-protein kinase, putative, expressed
Os07g0550500	2.07	0.000015	Serine/threonine-protein kinase receptor precursor, putative, expressed
Os03g0821300	2.03	0.000211	Protein phosphatase 2C, putative, expressed
Os11g0168700	2.01	0	Serine/threonine-protein kinase BRI1-like 1 precursor, putative, expressed

**Fig. 6** qRT-PCR analysis of upregulated genes in *RZFP34*-overexpression rice. WT and *OsRZFP34* transgenic plants OsOE1 were grown in soil for 3 weeks. Leaves were harvested for extraction of total RNA. The mRNA expression of transcript levels of OsRZFP34-upregulated genes. The expression of genes in each sample is relative to that of the WT control, and the expression of *18S rRNA* was an internal control. Data are mean \pm SE from 3 independent experiments

WT plants grown at 28 °C, with relative change of two-fold or more considered significantly different. We found 549 upregulated and 478 downregulated genes in OsOE1 plants as compared with WT plants (Supplementary Tables S2, S3). Calmodulin binding proteins, CAMKs, serine/threonine-protein phosphatase, serine/threonine-protein kinases, protein phosphatase 2C, potassium transporter genes, ABC transporter genes, and OsCML27, candidate components involved in ABA, K⁺, or Ca²⁺ signaling, comprised a significant portion of the differentially expressed genes (Table 1) (Sanders et al. 2002; Zhang and Lu 2003; Nagy et al. 2009; Siegel et al. 2009; Sirichandra et al. 2009; Umezawa et al. 2009). We also found transcripts of *OsWRKYs*, suggested to function in abiotic stresses including ABA stress, significantly upregulated in OsOE1 plants (Table 1) (Ross et al. 2007). qRT-PCR was performed to confirm the expression of 24 upregulated genes, and the results suggested that all of the chosen genes showed elevated expression levels in OsOE1 plants (Fig. 6), although the absolute values of the fold changes showed some variation for some genes between microarray and qRT-PCR analysis.

Discussion

RING motifs, functioning in protein–protein interaction and ubiquitination, can be classified into 2 subgroups by presence of a cysteine or histidine in the fifth position: C3HC4 (RING-HC) and C3H2C3 (RING-H2) fingers. OsRZFP34 contains 2 types of zinc-finger domains, a Pfam:zf-CHY and a RING domain, and is one of the C3H2C3-type RZFPs (Supplementary Fig. S1). Most studies of the function of *RZFP* genes suggest a link between their expression and the stress response in plants. One RZFP, HOS1, has been shown to be a negative regulator of cold-responsive gene expression and physically interacts with ICE1 to mediate the ubiquitination of ICE1 and downregulate cold-induced genes such as *CBF1* and *COR15a* (Lee et al. 2001; Dong et al. 2006). Others, such as *SDIR1* and *XERICO*, are positive regulators of drought tolerance by altering the homeostasis of the ABA signaling pathway (Ko et al. 2006; Zhang et al. 2007). From microarray results, we identified a rice *RZFP* gene, *OsRZFP34*, that responds to ABA and heat. Phylogenetic analysis of RZFPs indicated the existence of OsRZFP34 and its homologs in higher land plants (Supplementary Fig. S2). Further analysis revealed that *OsRZFP34* was expressed before the early flowering stage and transcripts were detected mainly in leaves (Fig. 1b, c, d), which is not often found with the expression patterns of *RZFP* genes (Zhang et al. 2007). Thus, OsRZFP34 may have a specific physiological function in rice leaves for the effect induced by heat and ABA stress.

High temperature and ABA treatment have opposite effects on mediating stomatal aperture; heat stress induces stomata opening for transpirational cooling to lower leaf temperature, but an increase in ABA triggers stomata closure to reduce water loss. Under natural growth conditions of plants, heat-induced rapid and continuous evaporation or a decrease in water content of the leaf causing immediate desiccation results in accumulation of ABA and stomata closure. Overexpression of *OsRZFP34* may result in increased water loss even under drought treatment (Fig. 5a). Thus, the ABA responsiveness of *OsRZFP34* may be contrary to ABA-induced stomata closure and coordinate transpiration cooling in response to elevated ambient temperatures. We further confirmed that transgenic rice and *Arabidopsis* overexpressing *OsRZFP34* with larger stomata aperture can have a better cooling effect and reduce leaf temperature during the early stages of the heat condition (Figs 2c, 3c, 5b) or under drought (Fig. 5c). By contrast, null mutation of *OsRZFP34* and *AtRZFP34* conferred smaller stomata opening and limited evaporative cooling. Thus, OsRZFP34 and its homolog AtRZFP34 may ensure stomata opening when heat dissipation is more urgent than water preservation. Recent studies found that rice yield significantly declined for each 1 °C rise in the growing season minimum temperature in some low-latitude regions (Peng et al. 2004; Welch et al. 2010), which implied a negative impact on rice yields from increasing in night temperature during the vegetative phase. Consequently, it would be interesting to test whether OsRZFP34, which functions in lowering leaf temperature, may ameliorate the negative effects of thermal stress on the yield of irrigated rice.

During the heading stage, which in rice is the most sensitive stage to temperature, upregulation of stress-related proteins such as small HSPs (sHSPs) may protect metabolic activities and enhance heat tolerance (Jagadish et al. 2010). However, we found the survival of *OsRZFP34* transgenic plants comparable to that of WT plants after HS treatment (unpublished observations in this lab), which suggests that OsRZFP34 cannot suppress HS-induced cellular damage. In addition, HS-responsive *OsRZFP34* transcripts were less abundant in the heat-sensitive flowering stage (Fig. 1c). These results are consistent with our suggestion that OsRZFP34 functions in cooling of vegetative leaves during heat stress.

A number of signaling compounds, including ABA, ROS, nitric oxide and Ca²⁺ ions are involved in the regulation of stomatal aperture. The ABA signaling in guard cells has been reported to engage a complex network of both positive and negative regulating protein kinases and serine/threonine-protein phosphatase (Hirayama and Shinozaki 2007; Umezawa et al. 2009), and cytosolic Ca²⁺ elevation or sensitivity in *Arabidopsis* (Sanders et al. 2002; Zhang and Lu 2003; Siegel et al. 2009). However, little is known about the

HS-induced signals for stomata opening and transpiration cooling. In this study, our microarray analysis showed the changed expression of serine/threonine-protein phosphatase and kinase genes, protein phosphatase 2C genes, calmodulin binding proteins, CAMKs, and *OsCML27* in *OsRZFP34*-overexpressing rice plants as compared with WT plants (Table 1). Meanwhile, 2 potassium transporter genes, 3 ABC transporter genes, and 3 *OsWRKY* genes were upregulated in *OsRZFP34*-overexpressing rice plants. In *Arabidopsis*, a calcium-modulated serine/threonine-protein phosphatase 2C, *AtABII*, is suggested to integrate ABA and Ca^{2+} signals to regulate stomata aperture in leaves (Leung et al. 1994); an ABC transporter, *AtMRP5*, might regulate Ca^{2+} and K^{+} release into the cytosol and promote stomata opening (Suh et al. 2007; Nagy et al. 2009); a plasma membrane K^{+} inward-rectifying channel, and *AtKATI*, is extremely efficient in K^{+} uptake in the guard cells (Lebaudy et al. 2007). In addition, among the *OsRZFP34*-upregulated *OsWRKY* genes, *OsWRKY80* shows ABA responsiveness (Ricachenevsky et al. 2010). Therefore, the role of overexpression of *OsRZFP34* as a pleiotropic regulator may lead to enhanced stomatal opening by mediating the expression of these genes to result in leaf cooling (Figs. 2c, 5b, c). The direct involvement of *OsRZFP34* protein in transpiration cooling would still need to be clarified by, for instance, characterization of the roles of these specific genes in mediating stomatal movement and the interaction of *OsRZFP34* with these genes. However, the features of gain- and loss-of-function of *OsRZFP34* raise attractive possibilities regarding its putative role in mediating transpiration cooling described above.

In conclusion, we identified an RZFP, *OsRZFP34*, as an important regulator of the response of heat and ABA treatments in rice plants. *OsRZFP34* may increase stomatal opening and function in transpiration cooling when rice plants are exposed to high temperatures. Higher transpirational cooling lowers leaf temperature and thus provides an early avoidance strategy of heat stress to irrigated rice grown at supra-optimal temperatures. *OsRZFP34* may be a good candidate for understanding the signaling pathways for transpiration cooling.

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