A rice microsomal delta-12 fatty acid desaturase can enhance resistance to cold stress in yeast and *Oryza sativa*

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Abstract A full-length cDNA clone of *OsFAD2*, which encodes a Δ-12 fatty acid desaturase, the key enzyme for the conversion of oleic acid (18:1) into linoleic acid (18:2), was isolated from rice (*Oryza sativa* ssp. *japonica*) leaves. The deduced amino acid sequence of *OsFAD2* displayed three histidine boxes characteristic of all membrane-bound desaturases, and possessed a C-terminal signal for endoplasmic reticulum retention. Phylogenetic analysis showed that *OsFAD2* is grouped within plant housekeeping FAD2 sequences. Expression analysis by real-time PCR showed that the gene is expressed in all tissues of rice tested, including root, seed, stem, and leaf.

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In situ hybridization showed that *OsFAD2* mRNA accumulated in leaf mesophyll cells and in root epidermis cells when exposed to 15°C for 4 days in dark conditions. When *OsFAD2* was expressed in *Saccharomyces cerevisiae*, the cells could convert oleic acid to linoleic acid, which wild-type yeast cells cannot do, suggesting that the isolated gene encoded a functional FAD2 enzyme. Heterologous expression of *OsFAD2* enhanced the yeast cells' cold tolerance capacity compared to wild-type yeast. *OsFAD2* was also shown to be a highly active desaturase when expressed in *Xenopus* oocytes. In addition, when the *OsFAD2* gene was transferred into an *Arabidopsis thaliana fad2-1* mutant, it effectively restored wild-type fatty acid composition and growth characteristics. Stress tolerance and light regulatory elements were identified in the predicted promoter of the *OsFAD2* gene. Exogenously supplied hormone affected the level of *FAD2* mRNA accumulation, accompanied by a change of content of di-unsaturated fatty acid species in rice leaves. Furthermore, *OsFAD2* enhanced tolerance to low temperature when overexpressed in rice at the vegetative stage. More importantly, the 35S:: OsFAD2 plants showed significantly enhanced cold tolerance at the reproductive stage, increasing grain yield by 46% over controls in the greenhouse under cold conditions. These results indicated that OsFAD2 is involved in fatty acid desaturation and maintenance of the membrane lipids balance in cells, and could improve the tolerance of yeast and rice to low temperature stress.

Introduction

Rice (*Oryza sativa* L.) originated in tropical and subtropical climates and is very sensitive to low temperatures. Expansion of rice cultivation into regions that experience periodic or sustained low temperatures has increased the risk of crop loss through chilling injury. Tolerance to low temperatures during seed emergence and the early growth stages is an important characteristic for rice seedling establishment, particularly where direct sowing cultivation is practiced in colder regions, such as Heilongjiang Province, in the northeast region of China. Therefore, studies that aim to enhance the tolerance of rice to low temperature are of strategic significance. In general, plants with acquired chilling tolerance accumulate intracellular sucrose and monosaccharide, and are able to desaturate membrane phosphatides (Wu et al. 1997; Browse and Xin 2001).

Polyunsaturated fatty acids in plant lipids are mainly linoleic and α-linolenic acids, which have a crucial role in a wide range of physiological processes as structural components of membrane lipids (Weber 2002). In addition to their important physiological role, these fatty acids are also essential for human health and nutrition, because they cannot be synthesized in the body and must be provided by the diet. Consequently, the desaturation of fatty acids is an important aspect in oil biochemistry, as it influences the quality of plant oils (Knutzon et al. 1992; Mikkilineni and Rocheford 2003).

The fatty acid desaturase FAD2 is the Δ-12 fatty acid desaturase that introduces a double bond at the Δ-12 position of oleic acid to form linoleic acid in the endoplasmic reticulum (ER) (Miquel and Browse 1992; Okuley et al. 1994). The FAD2-catalyzed pathway is probably the primary route for production of polyunsaturated lipids (Shanklin and Cahoon 1998). There are two distinct Δ-12 desaturases, one on the plastidial membrane and the other on the ER; both have distinct genes with specialized signals for their respective localization (Ohlrogge and Browse 1995; Dyer and Mullen 2001; McCartney et al. 2004). The first gene encoding a plant microsomal Δ-12 fatty acid desaturase was isolated from *Arabidopsis*

(Okuley et al. 1994). Since then, a large number of FAD2 genes have been isolated from different plant families, including maize (Mikkilineni and Rocheford 2003), soybean (Heppard et al. 1996), marigold (Qiu et al. 2001), cotton (Zhang et al. 2009), olive (Hernandez et al. 2005), purslane (Teixeira et al. 2009), and tropaeolum (Mietkiewska et al. 2006).

Plants often encounter abiotic stresses such as low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. Even with the best available land and agricultural practices, the impacts of these stresses can significantly reduce the productivity of food and fiber crops (Teixeira et al. 2009). More complete knowledge of fatty acid desaturation, mobilization, and regulation could significantly aid the development of effective strategies for managing abiotic and biotic stresses for these plants (Upchurch 2008).

In this study, the *OsFAD2* gene was isolated and shown to encode a functional fatty acid desaturase protein. Its spatial expression in rice was investigated using in situ hybridization. Yeast cells overexpressing *OsFAD2* showed a higher survival rate after freeze–thaw treatment. Furthermore, we observed that transgenic rice overexpressing *OsFAD2* showed an increased germination rate under low temperature, and its resistance to chilling was improved, which supported a role for the OsFAD2 protein in plant chilling resistance. It also could increase grain yield significantly under greenhouse cold conditions.

Materials and methods

Plant material and growth conditions

Two rice varieties, the Indian landrace rice cultivar, *indica* Kasalath, and *japonica* rice (*Oryza sativa* cv. Nipponbare) were used. The Kasalath was used only for transgenic rice to carry out the physiology experiments. The *japonica* rice variety was used in all the other experiments. Rice plants were grown on wet gauze in a growth chamber (SPX-250-GB, Shanghai, China) at 28°C under a photoperiod of 16 h light/8 h dark. Standard *A. thaliana* ecotype Columbia and *fad2-1* mutant plants were grown in soil in the greenhouse (16 h light/8 h dark, 60% relative humidity, 20–23°C). For physiological experiments,

the seeds of T_3 transgenic lines and wild-type rice were used. The following experiments were performed using rice seedlings grown until the third leaf emerged.

Plasmid construction and transformation of Arabidopsis

To amplify a full-length cDNA of rice *FAD2*, reverse transcription (RT)-PCR was carried out using the primers 5′-ATGGGTGCCGGCGGCAG-3′ (forward) and 5′-TTAGAACTTGTTGTTGTACC-3′ (reverse), which were designed from the open reading frame (ORF) of the predicted Δ-12 fatty acid desaturase gene (GenBank accession no. FJ768953). Five micrograms each of total RNA isolated from rice leaves was transcribed with the PrimeScript™ RT Reagent Kit (TaKaRa, Kyoto, Japan) for the first-strand cDNA synthesis. RT–PCR was then performed using the two primers shown above. The PCRs were carried out for 30 cycles of 94°C for 40 s, 55°C for 40 s, and 68°C for 1.5 min in a volume of 20 μl. The PCR products were subcloned into the pGEM-T vector (Promega) for sequencing.

The *OsFAD2* cDNA ORF, with a *Spe*I restriction site at the 5′ end and a *Bstp*I site at the 3′ end, was obtained by PCR as previously described. The *Spe*I and *Bstp*I fragment was inserted into the *Spe*I/*Bstp*I sites of pCAMBIA1304, under the control of a cauliflower mosaic virus (CaMV) 35S RNA promoter. The 5′ promoter and leader sequence from the *cor15a* gene (0.98 kb) was synthesized from *Arabidopsis thaliana* genomic DNA by PCR. The cor15a promoter was used to displace the 35S RNA promoter of pCAMBIA1304-35S::OsFAD2 recombinant vector. The pCAMBIA1304-35S::OsFAD2 and pCAMBIA1304-cor15aP::OsFAD2 recombinant vectors were subsequently transformed into *Agrobacterium* GV3101 by the freeze–thaw method. The constructs were then introduced into *indica* rice Kasalath by *Agrobacterium*-mediated transformation (Hiei et al. 1994). To further confirm the transgenic rice plant, seeds of the T_0 generation were germinated in half-strength MS medium containing 75 mg/l hygromycin. The T_3 generation of transgenic rice plants was used in subsequent experiments.

The *Arabidopsis fad2-1* mutant seeds were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University and were derived from the Columbia wild-type (Miquel and Browse 1992). The *Arabidopsis fad2-1* mutant plants were propagated in soil for seed production (under a 16 h light/8 h dark cycle at 20–22°C). The *Arabidopsis fad2-1* mutant plants were transformed by the floral dip method (Clough and Bent 1998) when they were approximately 6 weeks old. Seeds were harvested and screened on hygromycin (25 mg/ml). Transformants were identified as seedlings that produced green leaves and well-established roots on the selection medium. The transformants were transplanted into soil and the T_3 generation of transformed plants was used for analysis. Transformation of the putative transgenic *Arabidopsis* plants with the *OsFAD2* constructs was confirmed by RT–PCR amplification of cDNA from the transgenic plants. *Arabidopsis* primers actin-F: 5′-AGGTAATCAGTAAGGTCACGG-3′ and actin-R: 5′-GGATGGCTGGAAGAGGAC-3′ were used for control amplification of actin (Changhua et al. 2009). The entire 1.2-kb coding region of the *OsFAD2* gene was amplified using the same extract and forward 5′-ATGGGTGCCGGCGGCAG-3′ and reverse 5′-TTAGAACTTGTTGTTGTACC-3′ primers.

Sequence alignment and phylogenetic analysis

The deduced amino acid sequence of OsFAD2, and those of other FAD2, were aligned using the multiple sequence alignment ClustalW package. A phylogenetic tree was constructed using the neighbor-joining method provided by the computer program Phylip3.68. Bootstrapping (1,000 replicates) was performed to quantify the relative support for branches of the inferred phylogenetic tree.

In situ hybridization

Leaves dissected from rice grown in the dark for 4 days and at 15°C for 4 days, respectively, were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight, followed by dehydration in a graded ethanol series, and cleaned in xylene. The hybridization progressed according to a previously described method (Wang et al. 2006). The washes, blocking, antibody incubation and detection were performed according to the procedure for In Situ Hybridization to Chromosomes, Cells, and Tissue Sections (Roche manufacturer's handbook).

Treatment with exogenous factors and real-time RT–PCR

To characterize the effects of different hormones on *OsFAD2* expression, 2-week-old seedlings of rice (*Oryza sativa* cv. Nipponbare) were cultured in aqueous solutions containing 5 mM gibberellins (GA$_3$), 10 mM 6-benzyladenine (6-BA), 10 mM kinetin (KT), respectively, at 28°C for 24 h; seedlings cultured in distilled water at 28°C were used as controls. All the treatments were repeated three times, and the results represented the means ($\pm$SD) of those three independent experiments. After the treatments, the shoots were immediately frozen in liquid nitrogen for RNA extraction and real-time RT–PCR analysis. Quantitative RT–PCR was conducted to detect the expression pattern of *OsFAD2*. The *OsFAD2* primers were: forward 5′-CGCCTGCGAAATCTACCAACG-3′ and reverse 5′-TTCTGCTGCTCCTCCCGCTCCT-3′). Primers for *rac1* (the gene of GTP binding protein; GenBank accession no. x16280) were 5′-CTGCGATA ATGGAACTGGT-3′ and 5′-ACAATGCTGGGGAA GACA-3′, which were designed specifically for real-time PCR and generated an 80-bp product. A SYBR® Premix Ex TaqTM Kit (Takara, Japan) was used according to the manufacturer's instructions to amplify the previously synthesized cDNA. Test samples were cDNA from developing seed, root, leaf, and stem. A two-step real-time PCR program was run on the Mastercycler ep realplex cycler (Eppendorf, Germany), and comprised 40 cycles of amplification at 95°C for 15 s and 60°C for 15 s. After 40 cycles, the melting curve was assessed to ensure amplification specificity. The Ct value was calculated automatically by the cycler, and we calculated the relative expression quantity of the samples according to their Ct values. The constitutively expressed gene *rac1* was used as an internal standard. Data are presented as the means ($\pm$SD) of three independent experiments.

The functional assay of OsFAD2 in oocytes

mRNA synthesis of *OsFAD2* cDNA: The full-length cDNA of *OsFAD2* was amplified with primers described previously and then subcloned into the *Eco*RV and *Spe*I sites of the oocyte expression vector pT7TS (Cleaver et al. 1996). All of the subclones were sequenced to confirm their authenticity. mRNA

synthesis was performed as described previously (Tong et al. 2005).

Oocyte preparation, mRNA injection, and electro-physiological testing were performed as described previously (Tong et al. 2005). Small modifications in the oocyte preparation and mRNA injection were used. The collagenase concentration was reduced from 0.03 to 0.05 g/ml, and the oocytes were pretreated in Ca^{2+}-free ND96 (Liu and Tsay 2003) for 8–15 min. The treatment time course was dependent on the individual batch of oocytes and the thickness of the cell coat. The treatment was stopped by adding Modified Barth's Medium (Amiard et al. 2007; with Ca^{2+}), as soon as the oocyte was softened. The oocytes were injected immediately, or after incubation at 18°C overnight. For gene expression in oocytes, 50 ng of mRNA was injected; 50 ng of water was injected as a control. After injection, all oocytes were incubated in MBS, pH 7.4, at 18°C for 3–5 days, before testing for pH change.

Oleic acid desaturation

Oocytes were grown at 18°C for 48 h in an incubator, and then transferred to ND96 containing 10 mM oleic acid for 2 h, adding 0.5 mM NaH$_2$PO$_4$ into the MBS (adjusted to pH 7.4). Between 30 and 40 oocytes were used for each oleic acid desaturation experiment. At the end of desaturation, oocytes were washed in ice-cold MBS, and then placed into 0.25 ml of water for the calibration. Oocytes that had changed color were selected for the further experiments. A pH-responsive fluorescence dyestuff was used to monitor the color change. All the selected oocytes were lysed using a sonicator for 10 s, and a 10-fold diluted sample was assayed to measure the fluorescence intensity accumulated in each cell, using a SpectraMax M5 spectrophotometer (Molecular Devices).

Expression of *OsFAD2* in yeast (*Saccharomyces cerevisiae*)

For the functional expression of the *OsFAD2* gene in yeast, the 1.2-kb ORF was amplified by PCR using KOD Plus DNA polymerase (Toyobo), and subcloned into the *Eco*RI and *Xba*I sites of the yeast-bacterial shuttle vector pYES2/NT(C) (Invitrogen), which has the GAL1 promoter for inducible expression of genes. The *Saccharomyces cerevisiae* strain

INVScI (Invitrogen) was transformed with both pYES2/FAD2 DNA and pYES2 NT(C) vector DNA (as a control) by the lithium acetate method and selected on minimal agar plates lacking uracil (Invitrogen manual).

INVScI cells were pre-cultivated aerobically for 12–14 h in yeast extract peptone dextrose (YEPD) medium (30°C, 250 rpm) to an OD_{600} of 1.0–1.8. The cultures were centrifuged, washed once with $1\times$ PBS, and inoculated into Induction Medium (SC medium with filter-sterilized 20% galactose and filter-sterilized 10% raffinose), the initial OD_{600} of which was adjusted to 0.05. Cultures were cultivated aerobically with a speed of 250 rpm at 30°C. The samples were centrifuged and washed once with $1\times$ PBS. Five series of ten-fold dilutions were taken until the cell numbers were 1,000 cells/ml. Aliquots (10 μl) from each dilution were spotted in triplicate onto YEPD medium plates and then incubated at 30°C for 2 days. For cold treatment, cultures cultivated until the OD_{600} reached 1.0 were plated on YEPD medium and shifted to 4°C for 4 weeks before being incubated at 30°C for 2 days for recovery and photography.

Lipid extraction and fatty acid analysis

Yeast transformants were grown in SC-U (synthetic complete minus uracil) medium (Adams et al. 1998) at 30°C, washed and suspended in galactose induction medium (SC-U medium containing 2% galactose and 2% raffinose), and grown for three generations. The yeast cells were pelleted and washed three times with distilled water to remove media or metabolites that could potentially interfere with the lipid analyses. Cell pellets were mixed with 3 ml of 2.5% methanolic H_2SO_4 for ester verification, incubated at 80°C for 2 h and extracted in 2 ml of hexane and 1 ml of 0.9% NaCl. The hexane extracts were dried in a nitrogen current, reconstituted in 25 μl of hexane, and analyzed by gas chromatography.

Fatty acid methyl esters of *Arabidopsis* and rice leaf samples were prepared as previously described (Miquel and Browse 1992). Samples (1 μl) were separated by gas chromatography on a 30 m × 0.32 mm capillary column (HP6890 Plus Hewlett-Packard, Wilmington, DE, USA) and quantified using a flame ionization detector. The gas chromatograph was programmed for an initial temperature of 150°C for 3 min followed by an increase of 15°C/min to

210°C; the final temperature was maintained for a further 12 min.

Low temperature treatment, electrolyte leakage test, and chlorophyll fluorescence measurement

Two-week-old rice seedlings were cultured hydroponically in nutrient solution. The chilling treatment was carried out by exposing the plants to a growth chamber at $10 \pm 0.5°C$ for 8 days. For cold-tolerance germination testing, transgenic and wild-type seeds were germinated on Murashige and Skoog (MS) medium at 28°C (control) and at 4°C for 4 days, and then allowed to recover at 28°C for 3 days. The germination ratio was calculated with a relative value. The electrolyte leakage test was performed as reported by Changhua et al. (2009). The relative electrical conductivity (REC) was calculated as follows: REC (%) = C1/C2 × 100. The F_v/F_m values were then measured as described previously (Wenbin et al. 2009).

Cold treatments and grain yield analysis of rice plants in the field

To evaluate yield components of transgenic plants under normal field conditions, T_3 generation lines of the 35S::FAD2 and cor15aP::FAD2 plants, together with NT controls, were transplanted to a paddy field at Fudan University, Shanghai, China. A randomized design was employed with three replicates. Yield parameters were scored for 20 plants per transgenic line with three replicates. Plants located at borders were excluded from data scoring. To evaluate yield components of transgenic plants under greenhouse cold conditions, each of the 35S::FAD2 and cor15-aP::FAD2 plants and NT controls were transplanted to a removable 0.5-m-deep container filled with natural paddy soil. The experimental design, transplanting spacing, and scoring of agronomic traits were as described by Oh et al. 2009. When the plants grown under normal conditions had reached florescence, the containers were transferred to the greenhouse at 20°C for 2 days. The plants were then allowed to continue growing under normal conditions. When the plants grown under normal conditions had reached maturity and the grains had ripened, they were harvested and threshed by hand (separation of seeds from the vegetative parts of the

plant). The imperfect and filled grains were then separated, and the weights counted independently. The following agronomic traits were scored: flowering date, panicle length, number of tillers, number of panicles, spikelets per panicle, filling rate (%), total grain weight (g), and 1,000 grain weight (g).

Statistical analysis

Two-sample t test analysis was performed using Minitab 15 for Windows.

Results

Isolation and sequence analysis of OsFAD2 from Oryza sativa

To amplify the predicted ORF of the rice putative Δ-12 fatty acid desaturase by RT–PCR, primers were designed based on the 5′ and 3′ ends of the rice ORF sequence. Poly(A)$^+$ RNA from leaves of rice was reverse transcribed, and the cDNA products were used as templates for PCR. Sequencing of the PCR product showed that it contained a 1,167-bp putative ORF, encoding 388 amino acid residues with a theoretical molecular mass of 44.34 kDa. An alignment of the predicted protein sequence of OsFAD2 with Δ-12 fatty acid desaturases of other species showed that there is high similarity among them (Electronic Supplementary Material Fig. 1). Three conserved histidine-rich motifs (HXCGH, EXXXXXHXXHH, and HXXHH) are contained in the putative protein. These conserved motifs are found in all membrane-bound desaturases, and contribute to an iron-binding site required for enzyme activity. There was an aromatic amino acid enriched signal at the C-terminus of the protein (–YNNKL), which has been reported to be necessary and sufficient for retaining enzymes in the ER.

To elucidate the phylogenetic relationships of the microsomal FAD2 proteins, their deduced amino acid sequences were aligned with other homologous Δ-12 desaturase sequences and a neighbour-joining tree was constructed (Supplemental Fig. 2). OsFAD2 was positioned in a subgroup with FAD2 enzymes that exhibit a "housekeeping" pattern of expression. Taken together, these results suggest that OsFAD2 is a member of the plant housekeeping type FAD2 gene family.

Expression analysis of OsFAD2 gene in rice organs and tissues

Regulation of the expression of FAD2 genes in plants is still not well understood, and the tissue- and development-specific expression patterns of these genes will help us to understand the mechanisms involved. The expression of the OsFAD2 transcript in different tissues including root, seed, stem, and leaves was assessed by real-time RT–PCR. The OsFAD2 gene was expressed in all plant organs (Fig. 1a), including non-photosynthetic organs, such as developing seeds, stems, and roots, with the highest expression in the leaves.

We performed in situ hybridization using digoxigenin-labeled antisense RNA as a probe to investigate the detailed spatial tissue expression pattern. The results were consistent in all samples analyzed. Young leaves and roots were transversally sectioned and hybridized to the antisense probe. A T7 sense probe was used as a control (Fig. 1c a, e, i and c, g, k). Transcripts were detected in leaves and roots of seedlings grown at 15°C for 4 days in the dark, mainly in the epidermis and mesophyll cells (Fig. 1c, d and j). In the leaves, the accumulation of OsFAD2 mRNA increased in mesophyll cells when the seedlings were exposed to 15°C for 4 days and dark conditions (Fig. 1c, d and l). However, in the roots, OsFAD2 mRNA increased in epidermis cells under the same conditions (Fig. 1c, b and j). The results demonstrated that the OsFAD2 gene was constitutively expressed in thylakoid-rich photosynthetically active cell types and nonphotosynthetic tissue (Fig. 1c), and was induced by moderate low temperature and dark conditions.

Cytokinin regulates the transcript levels of OsFAD2

To investigate whether hormones are involved in the regulation of OsFAD2, we harvested rice seedlings treated with various factors and detected the transcription levels of OsFAD2 by real-time quantitative RT–PCR. Expression levels of OsFAD2 were markedly increased in shoots treated with 6-BA, and significantly decreased by treatment of with KT and GA$_3$ (Fig. 1b). To further confirm the regulatory effects of cytokinin on OsFAD2 transcripts, we
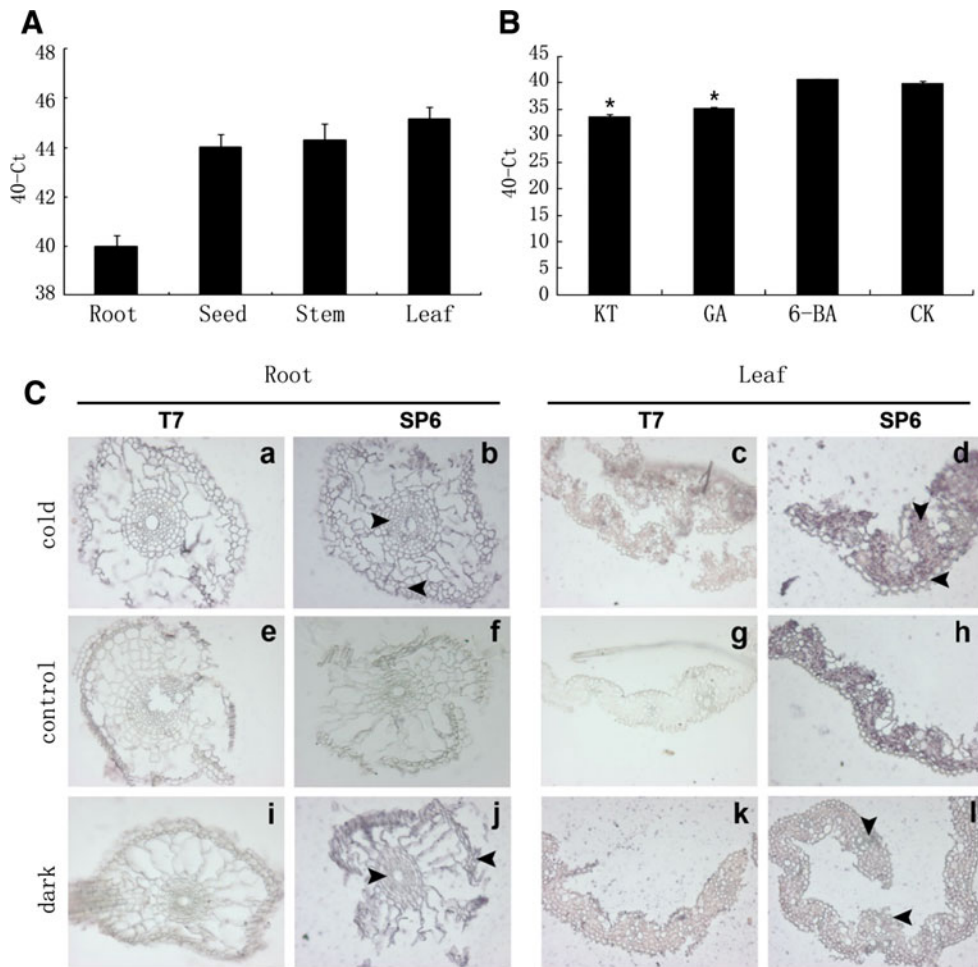
Fig. 1 Expression of *OsFAD2* in different tissues of *Oryza sativa*. Real-time PCR was employed to quantify the relative amounts of *OsFAD2* transcripts in young seeds, young stems, young roots, and young leaves (**a**). Location of *OsFAD2* transcripts in rice roots and leaves as affected by dark and cold treatment by in situ hybridization. Sections of the treated leaves and roots hybridized with antisense probe or sense probe. **e**, **f**, **g**, and **h** are control. Epidermis (*left arrow*); mesophyll cells (*down arrow*); vascular bundle (*right arrow*). To characterize the effects of different hormones on *OsFAD2* expression, real-time PCR was used to quantify the relative amounts of *OsFAD2* transcripts in rice shoots when treated with different hormones (**c**). Two-sample *t* test; * = significant difference from wild-type at $P < 0.05$

performed gas chromatography assays on the shoots of rice seedlings treated with cytokinin. The 18:2/18:1 value was usually used to represent the FAD2 fatty acid desaturase function. The 18:2/18:1 value is higher in 6-BA treatment (12%/1.7%) than in WT (14.9%/2.2%). The 18:2/18:1 value is lower in KT and GA$_3$ treatments than in WT. Analysis of acyl chain saturation by gas chromatography indicated an increase in di-unsaturated acyl chain species after 6-BA treatment and a decline after KT and GA$_3$ treatments (Table 1).

Fatty acid desaturase activity analysis of the *OsFAD2* gene in yeast cells, *Xenopus* oocyte cells, and in an *Arabidopsis* mutant line

To study the function of the protein encoded by the *OsFAD2* gene, the coding region was cloned into the yeast expression vector pYES2/NT(C) and transformed into yeast *S. cerevisiae* (strain InvScI) cells. Yeast cells transformed with the plasmid containing the *OsFAD2* ORF were capable of producing linoleic acid (Fig. 2a). No linoleic acid was produced by the

Table 1 Summary of fatty acid compositions of shoots of 2-week-old rice seedlings after treatment with different hormones

Fatty acid	CK (%)	KT (%)	GA (%)	6BA (%)
14:0	9.5 ± 0.30	1.4 ± 0.60	1.9 ± 0.70	2.8 ± 1.17
16:0	23.4 ± 2.12	19.5 ± 3.64	17.4 ± 2.17	17.8 ± 1.35
18:0	8.7 ± 0.37	6.2 ± 1.94	4.9 ± 0.65	5.6 ± 0.75
18:1	2.2 ± 1.3	1.4 ± 0.38	1.6 ± 0.54	1.7 ± 0.47
18:2	14.9 ± 2.25	7.8 ± 0.42	9.5 ± 1.33	12.0 ± 3.03
18:3	41.3 ± 3.56	61.7 ± 5.99	64.7 ± 4.57	60.2 ± 4.90

Fatty acids methyl esters were separated and quantified by gas chromatography with flame ionization detection. All values are given as mol% of fatty acids. The value for each individual plant is the average of three samples ± SD

control yeast cells transformed with the pYES2/NT(C) shuttle vector alone (Fig. 2b).

To confirm that OsFAD2 has the ability to desaturate oleic acid, *OsFAD2* linear DNA was transcribed into mRNA in vitro and expressed in *Xenopus* oocytes. After incubated with oleic acid for 2 h, the efflux activity for oleic acid was significantly higher in the oocytes injected with *OsFAD2* mRNA than in oocytes injected with water (Fig. 2c). The phenomenon was consistent with the results of fluorescence intensity accumulation in each cell using an ELISA reader (Fig. 2d). The fluorescence intensity was dramatically higher in the oocytes injected with *OsFAD2* mRNA than in oocytes injected with water. Fluorescence reached a peak 1 h after injection with *OsFAD2* mRNA. This result indicated that OsFAD2 is able to catalyze the desaturation of oleate (18:1) to linoleate (18:2) in the heterologous expression system.

To further investigate the function of OsFAD2, the *O. sativa* cDNA for the FAD2 polypeptide was expressed in the *Arabidopsis fad2* mutant line (designated as *fad2-1* by Miquel and Browse (1992), under the control of a cauliflower mosaic virus (CaMV) 35S RNA promoter. T$_3$ plants for three of the transgenic *Arabidopsis* lines were obtained by screening successive generations of 35S::OsFAD2 transformants on hygromycin. RT–PCR analyses of the 4-weeks-old transformants confirmed that *OsFAD2* was constitutively expressed in the three transgenic *Arabidopsis* lines (Fig. 2e). Three hygromycin-resistant T$_3$ plants displaying elevated proportions of 18:2 leaf fatty acid

(in the range 2.02–2.38%, compared to the low 18:2 *fad2-1* profile) were selected. As shown in Table 2, significant changes in fatty acid composition were found. In all transgenic *fad2-1* lines overexpressing *OsFAD2*, it was possible to find individual plants with leaf fatty acid compositions restored to wild-type ratios of 18:2/18:1, indicating a functional Δ-12 desaturase activity. Our results clearly demonstrated that the rice *FAD2* gene encodes a functionally conserved desaturase that can compensate for the absence of the FAD2 enzyme in this *fad2* mutant.

Upregulation of *OsFAD2* gene improved chilling injury tolerance in yeast and rice

The effects of low temperature on the viability of recombinant INVScI cells was evaluated by observing the growth of 10-fold serial dilutions of cultures spotted on the YEPD medium plate. The YEPD plates spotted with five serial dilutions were grown at 4°C for 4 weeks before being shifted to 30°C for 2 days of recovery. The results showed that overexpressing *OsFAD2* could enhance INVScI transformant cells' viability under cold stress compared to controls (Fig. 2f).

When the seeds of *OsFAD2* transgenic plants were germinated on MS medium and then shifted to 4°C for 4 days, before recovering at 30°C for 3 days, the transgenic seeds had a significantly higher germination rate than the wild-type seeds (Fig. 3b). However, they showed no difference in germination rate on normal MS medium (Fig. 3a, upper). This result further supported the hypothesis that overexpression of *OsFAD2* could enhance cold tolerance in rice.

We also analyzed the function of OsFAD2 in transgenic rice plants. OsFAD2-overexpressing and cor15aP::FAD2 transgenic rice showed no obvious phenotype under normal conditions (Fig. 3c). However, when exposed to 10°C, the wild-type plants soon withered and showed growth retardation, while the transgenic lines grew well and only the leaf tips showed slight coiling (Fig. 3d). We measured chlorophyll fluorescence as an indicator of chilling tolerance after cold treatment (10°C). The F_v/F_m values represent the maximum photochemical efficiency of PSII in a dark-adapted state, where F_v stands for variable fluorescence and F_m stands for maximum fluorescence. Values for F_v/F_m were 0.78 ± 0.02 before stress induction (Fig. 3e).
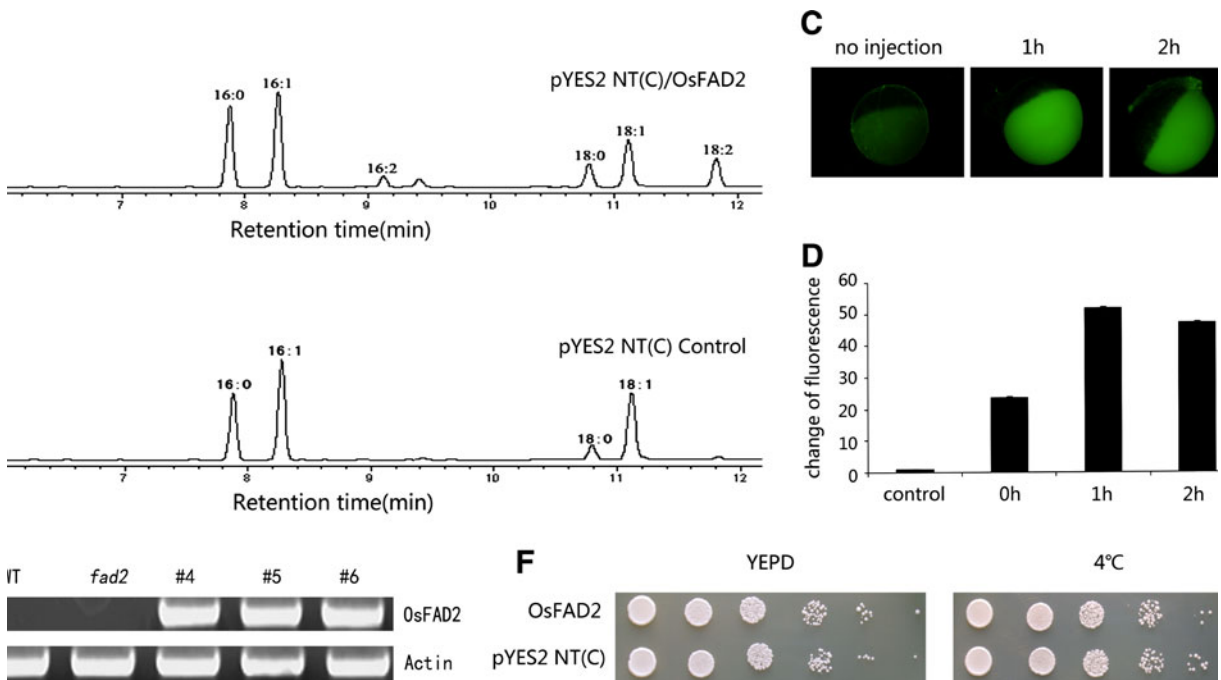
Fig. 2 Fatty acid desaturase activity analysis of the *OsFAD2* gene in yeast cells, *Xenopus* oocyte cells and an *Arabidopsis* mutant line. **a** Analysis of fatty acid methyl esters extracted from yeast transformants using gas chromatography with flame ionization detection (*FID*). *Saccharomyces cerevisiae* strain INVScI cells transformed with the recombinant plasmid pYES2 NT(C)/OsFAD2, grown in SC-U medium containing 2% galactose and 2% raffinose at 30°C for three generations. **b** Analysis of the pYES2 NT(C) control. *Saccharomyces cerevisiae* INVScI cells transformed with the shuttle vector pYES2 NT(C) were grown in SC-U medium containing 2% galactose and 2% raffinose at 30°C for three generations. The peak with a retention time of linoleic acid (C18:2) is seen in the yeast cells transformed with (**a**) the *OsFAD2* gene as expected, and not in the control cells transformed with (**b**) only vector DNA. **c** The oleic acid desaturation of oocytes grown at 18°C for 48 h in an incubator, then transfered to ND96 containing 10 mM oleic acid for 2 h adding 0.5 mM NaH$_2$PO$_4$ into the MBS (adjusted to pH 7.4). The changes in fluorescence intensity were monitored by fluorescence microscopy. **d** The color-changed oocytes were lysed using a sonicator for 10 s, and a 10-fold diluted sample was assayed to measure the fluorescence intensity accumulated in each cell using an ELISA Reader. The data are means ± SD of 30 independent oocytes injected with *OsFAD2* mRNA. The same number of water-injected oocytes was used as a control. Four-weeks old *Arabidopsis fad2-1* knockout plants, wild-type (Columbia ecotype plants) or T$_3$ plants of *fad2-1* transformed with the rice *FAD2* gene. **e** RT–PCR analysis of cDNA isolated from *OsFAD2*-transformed *Arabidopsis fad2-1* plants. A control *fad2-1* mutant and col-0 (wild-type) are included for comparison. Amplification of actin (ACT-11) gene product was introduced as a control. The rice *FAD2* is associated with restoration of wild-type growth in the *fad2-1* mutant system. **f** Samples (5 μl) of 10-fold serial dilutions of the control strain and the *FAD2* strain were spotted on a control YEPD plate. For cold treatment, samples (5 μl) of 10-fold serial dilutions were plated on a YEPD plate and shifted to 4°C for 4 weeks before being incubated at 30°C for 2–3 days and photographed

Following the 8-days cold treatment, the F_v/F_m values for the wild-type were reduced significantly to 0.2 ± 0.007 (Fig. 3e). In contrast, the F_v/F_m values for the 35S-overexpressing *FAD2* and cold-induced *FAD2* were 0.45 ± 0.015 and 0.37 ± 0.011, respectively. These ratios were notably higher than those calculated for the wild-type, which indicates that the transgenic plants had a higher degree of cold tolerance at the vegetative stage. REC, a key parameter of stress injury, of the *OsFAD2*-overexpressing rice and

cor15aP:FAD2 was lower than that of the control plants (Fig. 3f), suggesting that overexpression of *OsFAD2* increased the tolerance of rice to chilling stress. The expression level of *OsFAD2* in the transgenic lines of 35::FAD2 and cor15aP::FAD2 is shown in normal growth conditions and cold stress in Fig. 3g. Under normal conditions, the mRNA level of *OsFAD2* in the wild-type was lower than in transgenic lines. When cold treatment was applied, the mRNA level of *OsFAD2* increased.

Table 2 Fatty acid compositions of leaves of *Arabidopsis* wild-type (Columbia ecotype), *fad2-1* knockout mutant, and the *fad2* mutant lines transformed with OsFAD2-pCAMBIA1304 constructs

Fatty acid	Wild type (%)	*fad2-1* mutant (%)	*OsFAD2*-pCAMBIA1304 transformed *Arabidopsis fad2-1* plant		
			#4 (%)	#5 (%)	#6 (%)
14:0	5.00 ± 0.07	4.28 ± 0.41	10.13 ± 3.78	15.27 ± 3.30	13.71 ± 4.76
16:0	8.73 ± 0.90	9.17 ± 0.99	11.86 ± 2.56	11.54 ± 2.13	11.96 ± 2.74
18:0	3.45 ± 0.11	4.22 ± 1.06	7.90 ± 1.71	8.23 ± 2.75	8.46 ± 4.12
18:1	0.12 ± 0.02	0.93 ± 0.14	0.51 ± 0.11	0.33 ± 0.14	0.58 ± 0.12
18:2	2.11 ± 0.13	0.82 ± 0.04	2.02 ± 0.14	2.30 ± 0.69	2.38 ± 0.32
18:3	80.78 ± 1.95	80.12 ± 6.33	67.57 ± 4.52	61.86 ± 7.22	62.90 ± 3.01

Three T_3 plants sampled have wild-type 18:1/18:2 ratios, confirming the functional activity of the rice FAD2 polypeptide in *Arabidopsis* plants. Fatty acids methyl esters were separated and quantified by gas chromatography with flame ionization detection. All values are given as mol% of fatty acids. The value for each individual plant is the average of three samples ± SD

Overexpression of OsFAD2 increases rice grain yield under greenhouse cold conditions

A phenotypic evaluation of 35S::FAD2, cor15aP::FAD2 and NT control plants revealed no major differences at the vegetative growth stage of the entire plants. We evaluated yield components of the transgenic plants under normal and greenhouse cold conditions. The T_3 generation rice lines of each of the 35S::FAD2 and cor15aP::FAD2 plants, together with NT controls, were transplanted to a paddy field and grown to maturity. Yield parameters were scored for 20 plants per transgenic line in three replicates. The grain yield of the 35S::FAD2 plants remained similar to that of the NT controls under normal field conditions. In the cor15aP::FAD2 plants under the same field conditions, however, total grain weight was increased by 4.6% compared with the NT controls, which was due to increased numbers of filled grains and total spikelets. These observations prompted us to examine the yield components of the transgenic rice plants grown under greenhouse cold conditions. The 35S::FAD2 and cor15aP::FAD2 plants and NT controls were transplanted to a removable 0.5-m-deep container filled with natural paddy soil. When the plants grown under normal conditions had reached florescence stage, the containers were transferred to the greenhouse at 20°C for 2 days. The plants were then allowed to continue growing under normal conditions till the plants had reached maturity and the grains had ripened. The level of cold stress imposed under greenhouse conditions was equivalent to that which gives 30% of total grain weight obtained under normal growth conditions, which was shown by

the difference in levels of total grain weight of NT plants between the normal and cold conditions (Table 3). Statistical analysis of the yield parameters scored showed that the decrease in grain yield under cold conditions was significantly smaller in the 35S::FAD2 plants than in the NT controls. Specifically, in the cold-treated 35S::FAD2 plants, the number of filled grains was 32% higher than the cold-treated NT plants, which resulted in a 46% increase in the total grain weight, depending on transgenic line (Table 3). In the cold-treated cor15-aP::FAD2 plants, the number of filled grains was 15% higher than the cold-treated NT plants, which resulted in an 18% increase in the total grain weight, depending on transgenic line (Table 3). These observations suggest that overexpression of *OsFAD2* enhances cold tolerance of transgenic plants at the reproductive stage.

Discussion

Many studies have focused on the function of microsomal Δ-12 fatty acid desaturases in numerous plants (Qiu et al. 2001; Mikkilineni and Rocheford 2003; Mietkiewska et al. 2006). Nevertheless, their biological functions are still not well understood. In this study, we isolated and characterized a full-length cDNA clone that encodes OsFAD2 from *O. sativa*. The highest DNA sequence identity found in GenBank was to housekeeping-type gene *FAD2*, and this was validated by the results of alignment and phylogenetic analysis with microsomal Δ-12 fatty acid desaturases sequences from other plants. All known desaturases are characterized by the presence
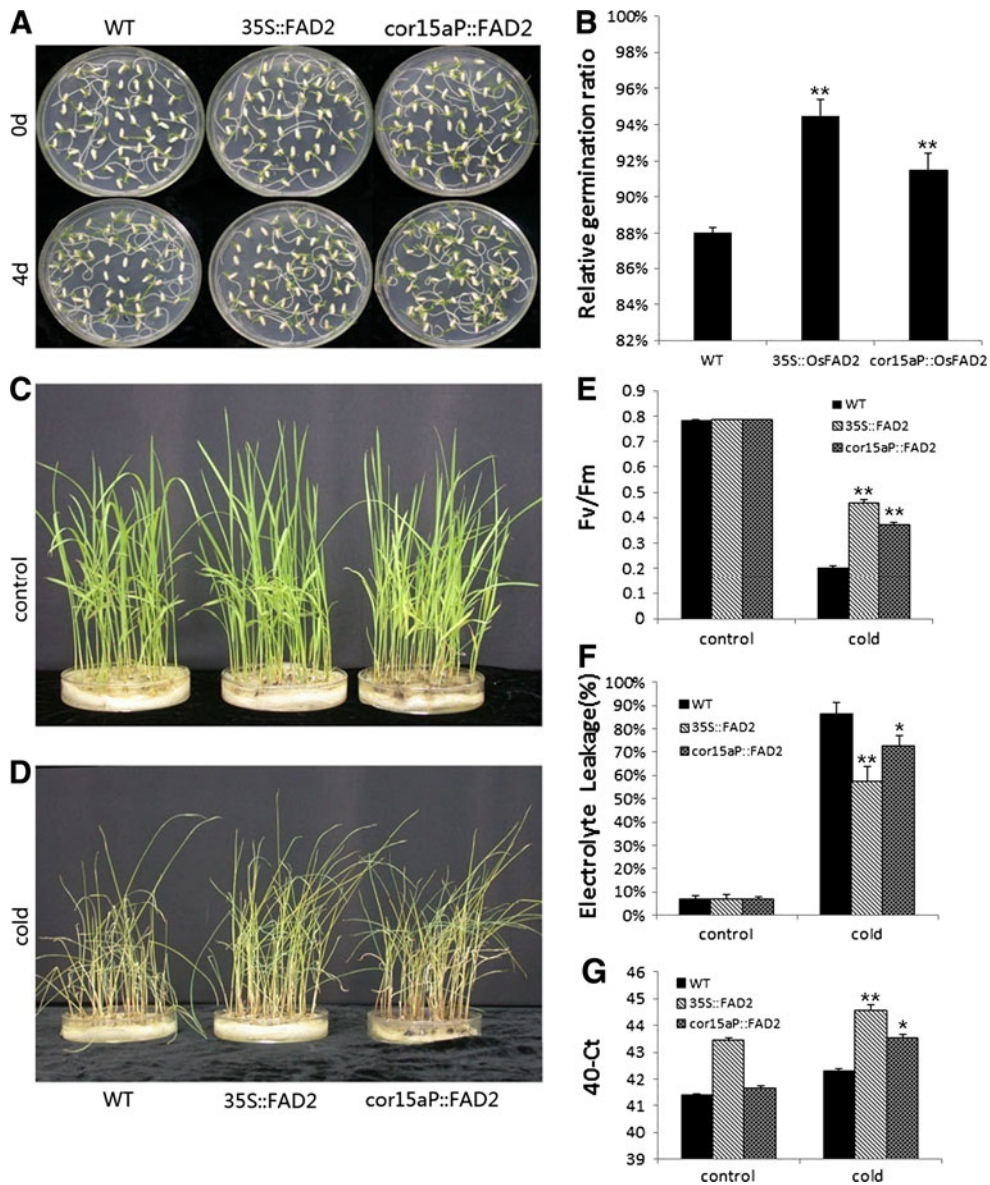
Fig. 3 Upregulation of *OsFAD2* gene improved chilling injury tolerance of rice. **a** Increased germination rate of the *OsFAD2* transgenic seeds in chilling condition. Germination status of over-expressed transgenic lines (35S::FAD2), non-overexpressed transgenic (cor15aP::FAD2), and wild-type line (Kasalath) in medium which were incubated at 4°C for 4 days and then allowed to recover at 30°C for 3 days and photographed. **b** Quantification of the seed germination performance in (**a**) (*error bar* indicates standard error of four replicates). **c, d** Phenotypic differences in various rice varieties by chilling treatment. Different phenotypes are shown in three rice varieties. Rice seedlings were either untreated (28°C) or treated at 10°C for 8 days and then photographed. **e** Cold tolerance of *OsFAD2*-overexpressing cor15aP::FAD2 plants as judged by the measurement of chlorophyll fluorescence. Changes in the chlorophyll fluorescence of the extended leaves under cold stress was measured. Functional damage to photosynthesis was estimated by measuring the mean and standard error of F_v/F_m values. The data are means ± SD for six independent samples. **f** The relative electrical conductivity (REC) of the leaves after chilling stress treatment. **g** The expression level of *OsFAD2* in the transgenic lines of 35::FAD2 and cor15aP::FAD2 is shown in normal growth conditions and cold stress. Two-sample *t* test; *, ** Significant difference from wild-type at $P < 0.05$ and $P < 0.01$, respectively)

Table 3 Agronomic traits of the 35S::FAD2 and cor15aP::FAD2 transgenic rice plants under normal and greenhouse cold conditions

Treatment	Constructs	Culm length (cm)	Panicle length (cm)	No. of panicles (/hill)	No. of spikelets (/panicle)	No. of total spikelets (/hill)	Filling rate (%)	No. of filled spikelets (/hill)	Total grain weight (g)	1,000 grain weight (g)
Normal	WT	154.38	29.11	13.11	170.62	2,190.16	0.94	2,101.90	34.00	16.26
	35S::FAD2	155.62	28.19	13.05	160.29	2,071.50	0.93	1,949.10	34.93	16.87
	Δ%	0.80	-3.14	-0.46	-6.06	-5.42	-1.34	-7.27	2.74	3.75
	cor15aP::FAD2	152.45	28.28	13.22	188.24	2,391	0.9322	2,152.2	32.21	16.53
	Δ%	-1.25	-2.84	0.84	10.33	9.17%	-1.16	2.39	-5.26	1.66
Cold	WT	154.34	22.29	12.32	110.75	1,329.00	0.42	560.22	9.78	16.03
	35S::FAD2	156.02	23.03	12.16	108.00	1,296.00	0.57	736.72	14.34	16.75
	Δ%	1.09	3.32	-1.30	-2.48	-2.48	35.00	31.51	46.63	4.49
	cor15aP::FAD2	155.22	25.53	12.27	111.44	1,311.00	0.46	642.11	11.55	16.24
	Δ%	0.57	14.54	-0.41	0.62	-1.35	9.29	14.62	18.10	1.31

Each data point represents the percentage of the mean values ($n = 20$)

of three histidine clusters at conserved locations in the protein sequence. These histidine clusters are thought to comprise the catalytic center of the enzyme, because they might act as ligands to a di-iron cluster in the catalytic site (Shanklin and Cahoon 1998). An aromatic amino acid enriched signal was found at the C-terminus of the OsFAD2 protein (–YNNTL); this motif has been reported to be both necessary and sufficient for retaining enzymes in the ER (McCartney et al. 2004). To examine the OsFAD2 expression patterns among tissues, quantitative real-time RT–PCR was conducted. Figure 1a shows that OsFAD2 was transcriptionally active in all tested tissues, which is in agreement with previous reports (Niu et al. 2007; Teixeira et al. 2009) that the OsFAD2 gene might have an extensive role in membrane lipid desaturation in photosynthetic tissues, such as leaves and stems, and also, to a lesser extent, in seed storage lipid desaturation. In addition, OsFAD2 mRNA accumulated in both leaves and roots at 28 and 15°C; the highest expression was observed in leaves at 15°C under dark treatment (Fig. 1b). The result indicated that an increase in mRNA levels occurred in response to the applied treatment. It was reported that the expression of cotton FAD2 gene increased in response to chilling temperature (Kargiotidou et al. 2008). In addition, cytokinin also regulated the transcription levels of OsFAD2. 6-BA treatment could increase the OsFAD2 mRNA level, accompanied by an increase in di-unsaturated acyl chain species. As auxin is related

to the upregulation of housekeeping-type Δ-12 fatty acid desaturase mRNA levels, a higher expression pattern of FAD2 in young tissues suggests a correlation between the level of membrane desaturation and cell elongation (Martínez-Rivas et al. 2001).

Many previous experiments on plant FAD2 function introduced their coding sequences into S. cerevisiae to investigate their possible in vivo functions. Heterologous expression of an A. thaliana FAD2 protected S. cerevisiae from ethanol stress (Kajiwara et al. 1996), and the expression of target gene from sunflower (Helianthus annuus) enhanced the tolerance of S. cerevisiae to freezing and salt stress (Rodriguez-Vargas et al. 2007). In this study, by the introduction of an inducible OsFAD2 gene into S. cerevisiae, we succeeded in producing an S. cerevisiae strain capable of endogenously synthesizing polyunsaturated fatty acids. Our results showed that the OsFAD2 gene functions normally in S. cerevisiae. The FAD2 desaturase protein found in many plants only catalyzes the conversion of 18:1-PC to 18:2-PC, because 16:2-PC is not found in the plant ER membrane (Hernandez et al. 2005; Lu et al. 2007). However, the S. cerevisiae FAD2 transformants synthesized 16:2-PC in addition to 18:2-PC, as 16:1-PC is present in the yeast ER. Thus, the FAD2 protein expressed in S. cerevisiae is capable of recognizing both 18:1-PC and 16:1-PC as substrates (Covello and Reed 1996). On the other hand, the enzyme appears to react preferentially with a specific substrate structure, because the ratio in PC of 18:2 to

18:1 was much greater than that of 16:2 to 16:1 (data not shown). Again, yeast cell survival following freezing storage increased if the cells were grown at 4°C instead of 30°C, which confirms that death during freezing can be prevented or alleviated by growth at low temperatures (Watanabe et al. 2004).

Previously, the *Xenopus* oocyte system was used to study the transport activity of numerous kinds of plant transporters. For example, the transport activity for arsenite was determined in *Xenopus* oocytes expressing the rice silicic acid transporter Lsi1 (Ma et al. 2008). In addition, when expressed in *Xenopus* oocytes, *A. thaliana* NIP1;1 was capable of transporting As(III) (Kamiya et al. 2009). In this paper, the heterologous expression system was successfully adopted for the first time to study the desaturase activity of plant Δ-12 fatty acid desaturase. The results indicated that OsFAD2 is able to catalyze the desaturation of oleate (18:1) to linoleate (18:2) in *Xenopus* oocytes. We also compared the membrane potential of the wild-type, constitutive expression and cold-induced expression of *OsFAD2* transgenic lines in roots. Electrophysiological measurements showed that the plasma membrane potential was lower than in transgenic rice lines. This result indicated that *FAD2* transgenic rice lines possessed increased fatty acid desaturase activity.

The function of the *OsFAD2* gene was further examined in the *Arabidopsis* knockout mutant (*fad2-1*). The *OsFAD2* gene effectively complemented the *Arabidopsis* mutant, as assessed by the mRNA level and level of linoleic acid. Overall, our results clearly demonstrated that the *OsFAD2* gene encodes a functionally conserved desaturase that can compensate for the absence of the FAD2 enzyme in this heterologous plant system, in agreement with previous reports (Mietkiewska et al. 2006; Zhang et al. 2009).

Many studies have demonstrated constitutive overexpression of tri-unsaturated fatty acids conferring cold-resistance capacity in plants (Wang et al. 2006; Dominguez et al. 2010). However, there has been no study on the overexpression of di-unsaturated fatty acids in plants. In this study, the *OsFAD2* coding sequence was introduced into rice by *Agrobacterium*-mediated transformation and overexpressed under the control of a CaMV 35S promoter and the control of a cold-inducible promoter, cor15a, from *A. thaliana*. The cor15a promoter is inactive, or

very weakly active, in most tissues and plant organs maintained under temperatures associated with active growth. In response to low temperature, it becomes highly active in the shoots, but not in the roots (Baker et al. 1994). The cor15a promoter has been successfully adopted to drive different plant genes in the face of numerous environmental conditions (Khodakovskaya et al. 2005, 2006). Using the germination rate calculation, electrolyte leakage measurement, F_v/F_m assay and the expression level of *OsFAD2* in the transgenic lines of 35::FAD2 and cor15aP::FAD2 under cold conditions, we established that, in comparison with seedlings or germinating seeds, transgenic rice plants have a higher level of basal tolerance to low temperature. More importantly, the 35S::FAD2 plants showed significantly enhanced cold tolerance at the reproductive stage, increasing grain yield by 46% over controls in the field under cold conditions. These results were consistent in our evaluations. In contrast, grain yield in 35S::FAD2 and cor15aP::FAD2 plants in the field remained similar to that of controls under normal conditions. In summary, constitutive expression and cold-induced expression of *OsFAD2* in rice conferred higher resistance to cold stresses on the transformant at both the vegetative and productive stages.

Rice is the primary food staple for about half the world's population. As the population increases, new means for improving productivity are required, such as developing crops that are more tolerant to abiotic stresses like low temperature. Identification of stress-tolerant tissues and the elucidation of the tolerance mechanism would greatly improve the agronomic traits of stress-sensitive rice and maize, which share genomic synteny. In this report, analysis of the function on the *OsFAD2* gene suggested that it was able to function in both eukaryotic yeast cells and animal *Xenopus* oocytes cells. Furthermore, it also conferred stress resistance, not only to yeast, but also to plants grown under unfavorable temperature conditions. The results presented here will provide direction for future studies to elucidate the genetic regulation of lipid desaturation in *Oryza sativa* under unfavorable conditions.

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