Integrated regulation triggered by a cryophyte ω-3 desaturase gene confers multiple-stress tolerance in tobacco

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Abstract

ω-3 fatty acid desaturases (FADs) are thought to contribute to plant stress tolerance mainly through linolenic acid (C18:3)-induced membrane stabilization, but a comprehensive analysis of their roles in stress adaptation is lacking. Here, we isolated a microsomal ω-3 FAD gene (CbFAD3) from a cryophyte (Chorispora bungeana) and elucidated its functions in stress tolerance. CbFAD3, exhibiting a high identity to Arabidopsis AtFAD3, was up-regulated by abiotic stresses. Its functionality was verified by heterogenous expression in yeast. Overexpression of CbFAD3 in tobacco constitutively increased C18:3 in both leaves and roots, which maintained the membrane fluidity, and enhanced plant tolerance to cold, drought, and salt stresses. Notably, the constitutively increased C18:3 induced a sustained activation of plasma membrane Ca2+-ATPase, thereby, changing the stress-induced Ca2+ signaling. The reactive oxygen species (ROS) scavenging system, which was positively correlated with the level of C18:3, was also activated in the transgenic lines. Microarray analysis showed that CbFAD3-overexpressing plants increased the expression of stress-responsive genes, most of which are affected by C18:3, Ca2+, or ROS. Together, CbFAD3 confers tolerance to multiple stresses in tobacco through the C18:3-induced integrated regulation of membrane, Ca2+, ROS, and stress-responsive genes. This is in contrast with previous observations that simply attribute stress tolerance to membrane stabilization.

Keywords: ω-3 FAD gene, Ca2+ signaling, Chorispora bungeana, multiple-stress tolerance, ROS, stress-responsive gene.

Introduction

Environmental stresses, such as low temperature, drought, and salinity, severely limit plant growth and productivity. To withstand these abiotic stresses, plants have evolved both constitutive and inducible mechanisms that prevent or reduce adverse effects. As the outer boundary of plant cells, the cell membrane is the primary sensor of environmental stresses, and its stabilization is required for the survival of the plant (Zhang et al., 2005; Shi et al., 2008). Membrane stabilization, especially the maintenance of its integrity and function, is affected by lipid composition and the degree of fatty acid desaturation (Mikami and Murata, 2003; Shi et al., 2008). Therefore, fatty acid desaturation caused by fatty acid desaturases (FADs), represented mainly by an increase in linolenic acid (C18:3), is considered as one of the factors involved in the tolerance of plants to many environmental stresses (Zhang et al., 2005; Upchurch, 2008).

Three ω-3 FADs that catalyse the conversion of linoleic acid (C18:2) to C18:3 have been identified in Arabidopsis: two are plastidial desaturases, FAD7 and FAD8, and one is
a microsomal desaturase, FAD3. It is widely accepted that ω-3 FADs are involved in plant tolerance to various abiotic stresses. In tobacco, overexpression of ArFAD7 was reported to enhance cold tolerance (Kodama et al., 1995), whereas its antisense expression reduced salt and drought tolerance (Im et al., 2002). Expression of FAD8 was induced by low temperature in Arabidopsis (Román et al., 2015) and by salt treatment in maize roots (Berberich et al., 1998). FAD3 family members were up-regulated in the leaves of lima bean and soybean by drought (Zhang et al., 2011) and cold (Román et al., 2012), respectively. Overexpression of LeFAD3 in tomato conferred tolerance to chilling (Yu et al., 2009) and salinity stress (Wang et al., 2014a). Although previous studies have observed the role of ω-3 FADs in stress tolerance, the relevance of these proteins in stress tolerance has always focused on the C18:3-induced membrane stabilization. However, stress tolerance is a complex process that consists of a series of responses at different levels (Perez and Brown, 2014) and cannot be achieved by membrane stabilization alone. Therefore, our knowledge of how ω-3 FADs respond to environmental stresses is still limited.

It is known that stresses trigger a rapid increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)), and the excess [Ca\(^{2+}\)]\(_{cyt}\) will result in aggregation of proteins/nucleic acids and precipitation of phosphates, together with disintegration of membrane lipids, leading to cell death (Case et al., 2007; Huda et al., 2013b). Therefore, increased export of Ca\(^{2+}\) from the cell/intracellular organelles is needed to maintain [Ca\(^{2+}\)]\(_{cyt}\) balance and adapt to the changing environment (Dodd et al., 2010; Huda et al., 2013a, b). As one important exporter, the plasma membrane (PM) Ca\(^{2+}\)-ATPase participates in various stress responses through generating a stress-induced Ca\(^{2+}\) signature (Huda et al., 2013a, b; Sun et al., 2016). Considering its location, we asked whether this enzyme is affected by membrane unsaturation. The same question is raised for another important membrane-bound protein, PM H\(^{+}\)-ATPase, which contributes to nutrient transport by generating an electrochemical gradient (Haruta and Sussman, 2012) and indirectly drives other membrane-bound transporters (Palmgren, 2001).

Stresses also trigger a reactive oxygen species (ROS) burst, which is partially attributed to the [Ca\(^{2+}\)]\(_{cyt}\) elevation (Baxter et al., 2014; Perez and Brown, 2014). The excess ROS accumulation will cause cell damage through lipid/protein oxidation and nucleic acid degradation (Gill and Tuteja, 2010; Huda et al., 2013a). Therefore, an enhanced ROS-scavenging ability is required for plant stress tolerance. In fact, overexpression of a microsomal ω-3 FAD gene in tomato enhanced the activity of antioxidant enzymes and conferred tolerance to salinity stress (Wang et al., 2014a), but the mechanism of the enhancement was not clear.

On the other hand, non-toxic levels of [Ca\(^{2+}\)]\(_{cyt}\) and ROS are regarded as key players in plant stress signaling; they induce the expression of stress-responsive genes through signal transmission and amplification, and this results in stress tolerance (Dodd et al., 2010; Huda et al., 2013a; Baxter et al., 2014; Perez and Brown, 2014). In addition, exogenous C18:3 can modulate the expression of stress-responsive genes, especially mediated by ROS (Mata-Pérez et al., 2015), but there is no related study on endogenous C18:3.

*Chorispora bungeana* is a perennial crucifer inhabiting periglacial regions at altitudes of 3800–3900 m. Its growing environment is characterized by low temperatures and freeze–thaw conditions, lack of oxygen, high ultraviolet light, strong wind, and drought stress. Being closely related to Arabidopsis (Zhao et al., 2012), *C. bungeana* is good plant material for the study of abiotic stress. Previous studies have confirmed that certain physiological and molecular mechanisms, rather than the existence of special morphological characteristics, might contribute towards its high survival under severe environmental conditions (Fu et al., 2006; Zhang et al., 2006, Shi et al., 2008; Di et al., 2009; Wu et al., 2009; Zhang et al., 2009; Zhao et al., 2012). Moreover, we found that the cold tolerance of *C. bungeana* suspension-cultured cells was associated with the rapid increase in C18:3 under low temperatures (Shi et al., 2008), which was produced mainly by microsomal ω-3 FAD. However, the actual mechanism for the involvement of the ω-3 FAD gene in stress tolerance is unknown because it has not yet been isolated and characterized.

In this study, we report the isolation and characterization of the microsomal ω-3 FAD gene, *CbFAD3*, from *C. bungeana*. The expression pattern and the functionality of *CbFAD3* were analysed in *C. bungeana* suspension-cultured cells and yeast cells, respectively. We also studied the function of *CbFAD3* under abiotic stresses using transgenic tobacco plants expressing *CbFAD3* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Moreover, the transcriptome of *CbFAD3*-overexpressing plants was analysed by microarray. The experimental data demonstrated that overexpression of *CbFAD3* confers tolerance to multiple stresses in tobacco plants through an integrated regulation that involves more than membrane stabilization.

### Materials and methods

#### Plant material

The suspension-cultured cells and regenerated plants of *C. bungeana* were prepared as described by Shi et al. (2008) and Fu et al. (2006), respectively. About 1-cm tall seedlings were placed on half-strength Murashige and Skoog (MS) medium with 0.5 mg l\(^{-1}\) indole-3-butyric acid added for rooting. Regenerated plants having 2-cm-long roots were used for the experiments. Wild-type (WT) and transgenic tobacco (*Nicotiana benthamiana*) seeds were sterilized and germinated on MS medium. After 10-d germination, tobacco seedlings were transferred into soil (30% peat, 70% turf), and irrigated with water every 2 d. Plants were grown at 25 °C with a 16-h photoperiod for 1–4 weeks before use.

#### Experiential treatments and morphological characterization

For quantitative real-time PCR (qRT-PCR) analysis, *C. bungeana* suspension-cultured cells were exposed to 0 °C, or added to culture medium with 15% PEG6000 (~0.6 MPa) or 200 mM NaCl for various times (3, 6, 12, 24, and 48 h). For germination experiments, tobacco seeds were germinated under different temperatures (20, 18, 16, and 14 °C), or different concentrations of PEG6000 (5, 10, 15, and 17.5%) or NaCl (50, 100, 150, and 200 mM). Germination was observed at 2-d intervals up to 30 d during stress application. For survival experiments, 4-week-old tobacco plants were exposed to −2 °C for 3 d, or were not watered for 10 d, or irrigated with 300 mM NaCl for 21 d. Survival rates were measured after a 10-d period of recovery growth under normal conditions.

#### Cloning and bioinformatics analysis

A 424-bp fragment of *CbFAD3* was cloned from *C. bungeana* suspension-cultured cells using degenerate primers P1 and P2 (see Supplementary...
Table S1 at JXB online), designed on the basis of a conserved domain database from tobacco, *Brassica napus*, and Arabidopsis. The 5′ and 3′ ends of *CbFAD3* were amplified using specific primers (P3–P6; Supplementary Table S1) and the SMARTer™ RACE cDNA amplification kit (Clontech, Japan). The full-length cDNA of *CbFAD3* was obtained by assembling the fragments, and the sequence was verified by PCR (using primers P7 and P8; Supplementary Table S1) and nucleotide sequencing. The sequences were analysed using Clustal X2.0 (SFI, Ireland), DNAman 5.2.2 (LynnisonBiostof, Canada), and MEGA 3.1 (ASU, USA) software or by BLAST (http://ncbi.nlm.nih.gov/blast). The nucleotide and amino acid sequences of *CbFAD3* were submitted to the NCBI GenBank database with accession numbers KM591203 and AKN35208, respectively.

**qRT-PCR analysis**

The expression of *CbFAD3* in *C. bungeana* was detected using *CbACT* (AY825362) as the housekeeping gene (Dí et al., 2009; Wu et al., 2009; Zhang et al., 2009). The amplification specificity of each primer pair (P9 and P10 for *CbFAD3*, P11 and P12 for *CbACT*; Supplementary Table S2) was checked by gel electrophoresis and dissolution curve analysis. The relative gene expression (F) was normalized against the expression of a housekeeping gene, according to the formula:

\[
F = \frac{(E_{\text{target}})_{\text{housekeeping}}}{(E_{\text{housekeeping}})_{\text{target}} (\text{control} - \text{sample})}
\]

which is considered as an accurate and reproducible mathematical model (Pfaff, 2001). The amplification efficiencies (E) for both target and housekeeping genes were between 90 and 110%. The cycle threshold (Ct) for these genes was obtained from three independent biological experiments.

**Heterogonous expression in yeast**

The coding region of *CbFAD3* was cloned into pYES2.0 (Invitrogen, USA) using specific primers (P13 and P14; Supplementary Table S1), to construct the expression plasmid pYES2-CbFAD3. pYES2-CbFAD3 and pYES2.0 were transformed into *Saccharomyces cerevisiae* strain INVSc1 (Invitrogen, USA) using *S. cerevisiae* EasyComp transformation kit (Invitrogen, USA). The yeast transformants were selected and cultured according to the method of Román et al. (2012). When the OD_{600} of the culture reached 0.2–0.3, gene expression was induced by adding 2% (w/v) galactose. Yeast cells were harvested by centrifugation at 1500 g for 5 min at 4 °C and washed with distilled water. The extraction and SDS-PAGE of total yeast proteins were performed as described by Horvath and Riezman (1994). The production of C18:3 was induced by adding 2% (w/v) galactose, 50 μM C18:2 (Sigma-Aldrich, USA) and 0.1% (w/v) NP-40, and was measured after growth at 20 °C for 3 d.

**Transformation and generation of transgenic plants**

The coding region of *CbFAD3*, amplified using specific primers (P15 and P16; Supplementary Table S1), was cloned within the XbaI–Sad site of the binary vector pBI121 to replace the GUS gene and construct the recombinant plasmid, pBI121-CbFAD3, under the control of the CaMV 35S promoter. The pBI121-CbFAD3 plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The transgenic tobacco plants were generated using the *Agrobacterium*-mediated transformation method (Horsch, 1985). Five positive transgenic lines exhibiting 3:1 segregation ratio were identified by PCR using primers P17 and P18 (Supplementary Table S1; Supplementary Fig. S1A). The homozygous lines were obtained by backcrossing or self-pollination, and the expression level of *CbFAD3* was verified by qRT-PCR using primers P9 and P10 (Supplementary Table S2; Supplementary Fig. S1B). NILT25 (L18908) was used as the housekeeping gene (Schmidt and Delaney, 2010) using primers P19 and P20 (see Supplementary Table S2). Three independent homozygous T3 transgenic lines (L2, L3, and L4) showing higher expression levels were used in the experiments.

**Extraction and analysis of fatty acids**

Lipids and total fatty acids were extracted from 5 g of tobacco leaves or roots as described in our previous report (Shi et al., 2008). The total lipid and fatty acid composition of whole yeast cells was determined using the one-step method of Garcés and Mancha (1993). Fatty acid methyl esters of each sample were analysed with a gas chromatograph–mass spectrometer (6890N/5975C; Agilent, USA) fitted with a capillary column (Agilent DB-FFAP; 30 m×0.25 mm×0.5 μm). Hydrogen was used as a carrier gas with a linear rate of 1.1 ml min\(^{-1}\) and split ratio of 100:1. The injector and detector temperature was 200 °C, and the column temperature was programmed to increase from 70 to 230 °C (10 min holding) at a rate of 8 °C min\(^{-1}\). The voltage of the ionization source was 70 eV along with a solvent delay of 1.5 min. The ion source, quadrupole, and interface temperature were 230, 150, and 250 °C, respectively.

**Measurement of electrolyte leakage, chlorophyll fluorescence, malondialdehyde, H\(_2\)O\(_2\), and antioxidant enzymes**

Electrolyte leakage from tobacco leaves was measured using a conductivity meter (Mettler-Toledo, Switzerland) as described in our previous publication (Shi et al., 2008). The maximum efficiency of photosystem II photochemistry (F\(_{v}\)/F\(_{m}\)) of fully expanded leaves was measured using a PAM-2100 fluorometer (Walz, Germany). The contents of malondialdehyde (MDA) and H\(_2\)O\(_2\) of tobacco leaves were determined as described previously (Yang et al., 2012). To determine the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), tobacco leaves (0.5 g) were ground in 50 mM KH\(_2\)PO\(_4\) buffer (pH 7.8, containing 1% polyvinylpyrrolidone) at 0 °C. The homogenate was centrifuged at 15 000 g for 20 min at 4 °C. The supernatant was used to determine the enzyme activity according to the method described by Miao et al. (2010).

**Preparation of plasma membrane and analysis of membrane fluidity and enzymes**

PMs were isolated from 10 g of tobacco leaves or roots by aqueous two-phase partitioning as described in our previous report (Shi et al., 2008). The purity of PM vesicles was estimated according to the method of Widell and Larsson (1990). The enzymatic activity inhibited by vanadate, azide, nitrate, and molybdate was about 85, 1, 6.4, 9.7, and 2.3%, respectively, indicating that the PM vesicles were well purified. Protein content was determined by a dye–protein binding method using bovine serum albumin as a standard (Bradford, 1976). According to the protocols described in our previous publication (Shi et al., 2008), the fluidity of isolated PM was measured using a fluorescent probe (1,6-diphenyl-1,3,5-hexatriene, Sigma-Aldrich). The activity of PM H\(^{2+}\)-ATPase was determined by monitoring P\(_i\) release at 660 nm. The activity of PM Ca\(^{2+}\)-ATPase was measured as eosin-sensitive Mg-inosine triphosphate hydrolysis (Giacometti et al., 2012).

**Measurements of Ca\(^{2+}\) fluxes**

The net flux of Ca\(^{2+}\) was measured using a non-invasive micro-test technology (NMT, Younger USA Science & Technology Corp., USA). One-week-old seedlings of WT and transgenic tobacco grown in backfilling solution (0.05 mM CaCl\(_2\), 0.1 mM KCl, 0.1 mM MES, pH 6.0) were fixed under a microscope. A Ca\(^{2+}\)-selective microelectrode (3 mm aperture, XYPG120-2) was propelled to approximately 300–400 μm from the root apex. Steady-state Ca\(^{2+}\) fluxes were measured for 4 min. Then, PEG6000 and NaCl were applied to a final concentration of 15% and 200 mM, respectively, and measurements were taken for another 10 min. The data measured during the first minute were discarded because of the diffusion effects of stock addition. The concentration gradients were measured by moving the microelectrode a distance of 30 μm in 5 s. The corrected slope and intercept of micropipette was 26.32 and 33.33, respectively.

**Protoplast isolation and [Ca\(^{2+}\)]\(_{cyt}\) measurement**

Protoplasts were isolated from tobacco root epidermis using the enzymatic digestion procedure described by Rutschow et al. (2014).
The protoplast suspensions were then incubated with Fluo-3 AM (Sigma-Aldrich) at a final concentration of 5 μM at 30 °C for 45 min and washed with MMG solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES, pH 5.7) containing 2 mM CaCl2. The pre-incubated protoplast suspension was put in the middle of a poly-L-lysine-pretreated (0.003% w/v) slide (Sigma-Aldrich) at a final concentration of 15% and 200 mM, respectively, the detection started and continued at 3-s intervals for 5 min.

Microarray analysis

Two-week-old tobacco seedlings were treated with 200 mM NaCl for 6 h. Leaves from five independent plants were pooled as one biological sample, and three samples were hybridized separately for each line. The samples were sent to CapitalBio Corporation (Beijing, China) for microarray analysis. A microarray chip for N. tabacum (4 × 44 format; G2514F; Agilent) was scanned by an Agilent G2565CA microarray scanner, and analysed by GeneSpring GX software. The P-value was calculated with Student's unpaired t-test and corrected for false discovery rate (Benjamini–Hochberg). A total of 43,817 genes from the tobacco genome were detected; 351 genes exhibiting more than 2-fold enhancement (115 genes) or reduction (236 genes) in the transcript levels were considered to show significant alterations in expression. The raw data files were deposited in the NCBI GEO database with accession number GSE74260 (GSM1915764-1915769). To validate the microarray data, the increased expression of six genes was compared between transgenic and WT tobacco plants by qRT-PCR (see Supplementary Fig S2) using specific primers (P21–P32; Supplementary Table S2). NtL25 (L18908) was used as the housekeeping gene (Schmidt and Delaney, 2010) using primers P19 and P20 (see Supplementary Table S2).

Statistics

All data were analysed for variance using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), and statistical significance between samples was indicated when P<0.05.

Results

Isolation and analysis of CbFAD3 from C. bungeana

After full length verification, the complete CbFAD3 cDNA was obtained, having 1493 bp and an 1161-bp open reading frame (ORF) from 152 bp to 1312 bp. The ORF encodes a deduced protein of 387 aa, with a predicted molecular mass of 44.2 kDa and a pI value of 8.92. CbFAD3 contained four conserved transmembrane domains (TMD; Fig. 1A) and three histidine boxes (H1–H3; Fig. 1A), which are considered as highly conserved in all the membrane-bound FADs and ω-3 FADs, respectively (Los and Murata, 1998). The sequence analysis data showed that CbFAD3 exhibited high identity to other cruciferous microsomal ω-3 FADs, such as δ-FAD3 (94%), AtFAD3 (93%), BnFAD3 (92%), BjFAD3 (92%), BoFAD3 (92%), and SaFAD3 (92%). The phylogenetic tree showed that CbFAD3 was clustered in the same clade as the cruciferous FAD3s, and was closest to Arabidopsis AtFAD3 (Fig. 1B). These analyses indicate that CbFAD3 is a microsomal ω-3 FAD gene.

Expression pattern of CbFAD3 in C. bungeana

To analyse the expression of CbFAD3 in C. bungeana, qRT-PCR was performed. The data showed that CbFAD3 was expressed in all the tested tissues, with the highest expression in suspension-cultured cells, followed by that in roots and leaves, with the lowest expression observed in stems (Fig. 2A). This expression pattern is similar to that of AtFAD3 in Arabidopsis (Kodama et al. 1997). Using C. bungeana suspension–cultured cells, we found that the expression of CbFAD3 was significantly induced by various abiotic stresses, whereas there was no significant variation in the gene expression under normal conditions (Fig. 2B). The induced expression of CbFAD3 peaked at 3, 6, and 24 h under cold (0 °C), NaCl (200 mM), and PEG (15%) treatments, respectively, with the corresponding maximum increases of 6.3-, 4.6-, and 10-fold, respectively, compared with untreated controls. Consistent with previous findings that FAD3 genes involved in stress adaptation can be induced by abiotic stresses (Yu et al. 2009; Zhang et al. 2011; Román et al. 2012), these results suggest that CbFAD3 might be involved in response to cold, drought, and salt stresses.

Expression of CbFAD3 in yeast leads to conversion of C18:2 to C18:3

Yeast has been proven to be a suitable heterologous expression system for studying the functionality of microsomal ω-3 FAD genes (Dyer et al., 2001). To determine the functionality of CbFAD3, the ORF of CbFAD3 was expressed in S. cerevisiae under the galactose-inducible promoter of the pYES2.0 vector. The results of SDS-PAGE showed that a 44.2 kDa protein was induced in yeast cells transformed with pYES2–CbFAD3, but was not induced in the controls transformed with empty pYES2 vector (Fig. 3A). The fatty acid analysis of whole yeast cells clearly indicated that CbFAD3 could effect the conversion of C18:2 to C18:3 in CbFAD3-transformed yeast cells, whereas no C18:3 was detected in the controls (Fig. 3B). The percentage of C18:3 obtained from CbFAD3-transformed yeasts at 20 °C was 0.7% (see Supplementary Table S3), which was more than that (about 0.3%) from yeast-expressed Arabidopsis AtFAD3 (Kumar et al., 2012). These results confirm that CbFAD3 is a functional microsomal ω-3 FAD gene.

Overexpression of CbFAD3 in tobacco enhanced plant tolerance to multiple abiotic stresses

To clarify the role of CbFAD3 in stress tolerance, the germination and survival of transgenic tobacco were observed under different abiotic stresses. Under normal conditions, there were no significant differences in germination between transgenic and WT seeds. Although seed germination was inhibited by low-temperature, PEG and NaCl treatments, the germination rates of the transgenic lines were much higher than that of WT tobacco plants (Fig. 4A, B). In the chilling temperature condition (14 °C), the germination rates were 35.0–61.3% for transgenic seeds and only about 13.8% for WT controls. Similarly, with the high-PEG (17.5%) and high-salt (200 mM) media, the germination rates of transgenic seeds were more than 50% and 40%, respectively, whereas those of WT controls were around 20%.

There were no significant differences in growth between WT and transgenic plants under normal conditions, whereas
the survival rates were significantly higher in transgenic plants than in WT controls under abiotic stresses (Fig. 4C–E). Cold exposure (−2 °C for 3 d) led to WT tobacco becoming wilted and nipped, whereas it had a weaker influence on transgenic plants. After recovery, the survival rate of transgenic and WT plants was 68.9–76.7% and 35.2%, respectively. Likewise, an about 2.0-fold survival rate was also found in transgenic lines as compared with WT controls under drought (not watered for
Shi et al. found that overexpression of CbFAD3 in tobacco can enhance plant tolerance to multiple abiotic stresses. CbFAD3-overexpressing plants had improved physiological traits under abiotic stresses. To further confirm the enhanced stress tolerance caused by CbFAD3, various physiological traits important for stress responses were measured in the leaves of transgenic tobacco plants. As shown in Fig. 5, there were signs of increase in membrane stability and photosynthetic capacity in several, but not all, CbFAD3-overexpressing lines under normal conditions; however, these physiological traits were much better in all transgenic lines than in WT controls under abiotic stresses. During cold exposure, lower ion leakage (42.1–47.6%) and MDA accumulation (71.3–74.8%), and higher chlorophyll fluorescence ($F_v/F_m$, 143.3–154.6%) were observed in transgenic lines when compared with WT controls. Similar physiological phenomena were also observed in the transgenic plants under drought and salt treatments. These data show that CbFAD3-overexpressing plants demonstrate reduced damage and higher integrity of cellular membranes under various abiotic stresses.

CbFAD3-overexpressing plants had constitutive high membrane unsaturation

To identify the contribution of CbFAD3 to membrane unsaturation, the fatty acid compositions of transgenic tobacco plants were measured. Overexpression of CbFAD3 constitutively increased the fatty acid unsaturation, including the C18:3 content, C18:3/C18:2 ratio, and double bond index (DBI), in both leaves and roots of tobacco plants (Fig. 6A, B).
Under normal conditions, the average level of C18:3 in transgenic lines increased by about 20.8% in leaves and by 126.2% in roots compared with the levels in WT plants. The increases in C18:3/C18:2 ratio and DBI in transgenic lines were also greater in roots than in leaves. After 10 d of drought treatment, the levels of C18:3 in both leaves and roots of transgenic
plants were almost unchanged, whereas those of WT plants increased to the levels found in transgenic lines (Fig. 6A, B). The consumption of C18:2 in WT roots was compensated by the conversion of stearic acid (C18:0) and oleic acid (C18:1) to C18:2. Although the C18:3/C18:2 ratio in transgenic lines was higher than that in WT plants, the DBI in WT plants was equal to (in leaves) or more than (in roots) that in the transgenic lines. These results indicate that overexpression of *CbFAD3* in tobacco can constitutively increase the fatty acid unsaturation, especially the level of C18:3, to the same level as that induced by drought in WT plants.

**CbFAD3**-overexpressing plants continuously stabilized membrane fluidity and activated PM Ca$^{2+}$-ATPase

To evaluate the function of *CbFAD3* in membrane stabilization, the membrane fluidity and enzyme activities of transgenic tobacco plants were examined. The value $P$ calculated from fluorescence polarization indices is an indication of membrane fluidity (Mykytczuk et al., 2007); the higher the value, the lower is the fluidity. In *CbFAD3*-overexpressing tobacco plants, the constitutive fatty acid desaturation did not significantly change the membrane fluidity, which was already optimal for survival, under normal conditions, but maintained the fluidity under drought stress (Fig. 6A–C). Moreover, the drought-induced fatty acid desaturation in WT tobacco plants also maintained the membrane fluidity under drought stress except for a slight rigidification in the leaves.

The experimental data together with a correlation analysis showed that the sustained activation of PM Ca$^{2+}$-ATPase in leaves and roots of *CbFAD3*-overexpressing plants was exactly correlated with the constitutive accumulation of C18:3, and the inducible activation of PM Ca$^{2+}$-ATPase in WT plants was in accordance with the drought-induced increase in C18:3 (Fig. 6A, B, E; Table 1). However, the enzyme activities in both WT and transgenic tobacco plants did not correlate with membrane fluidity. The drought-induced inhibition of PM H$^{+}$-ATPase, which did not correlate with the membrane unsaturation and fluidity, was not different between WT and transgenic leaves, but was observably milder in the roots of transgenic lines (Fig. 6A, B, E; Table 1). These results suggest that the *CbFAD3*-induced constitutive accumulation of C18:3 might maintain membrane fluidity and activate PM Ca$^{2+}$-ATPase under any condition, although its role in the PM H$^{+}$-ATPase was complex.

**CbFAD3**-overexpressing plants changed the stress-induced Ca$^{2+}$ signaling during early stresses

To explore the role of *CbFAD3* in regulating Ca$^{2+}$ signaling, the net Ca$^{2+}$ flux and [Ca$^{2+}$]$_{\text{cyt}}$ of transgenic tobacco plants were determined under different stresses. Although both of the two Ca$^{2+}$ indices reflect a combined result of Ca$^{2+}$ influx through Ca$^{2+}$ channels and Ca$^{2+}$ efflux driven by Ca$^{2+}$-ATPase and Ca$^{2+}$ exchangers (Bose et al., 2011), they are not completely consistent with each other. This is because the net Ca$^{2+}$

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**Fig. 5.** Physiological comparisons between *CbFAD3*-transgenic and WT tobacco leaves under stress conditions. (A) Electrolyte leakage, reflecting the integrity of the cellular membranes. (B) MDA content, showing the state of lipid peroxidation. (C) $F_v/F_m$ ratio, representing the photosynthetic efficiency. Transgenic lines L2, L3, and L4 were used in these experiments, and for each line more than five plants were used in every experiment. Four-week-old tobacco plants were exposed to 0 °C, not watered, or treated with 200 mM NaCl for 3 d, and then measured. Values are means ±SE of three biological replicates for each experiment. Statistical significance between samples, determined by Student’s $t$-test, is indicated by different letters.
Integrated mechanism of CbFAD3 in stress tolerance

Flux detected by NMT presents a dynamic Ca\(^{2+}\) state just around the plasma membrane of cells that adjoin the micro-electrode, whereas the [Ca\(^{2+}\)]\(_{cyt}\) monitored using LSCM shows the cytoplasmic Ca\(^{2+}\) variation affected by the Ca\(^{2+}\) flux from both plasma membrane and tonoplast.

When exposed to 15% PEG, tobacco root tips exhibited a transient increase in Ca\(^{2+}\) efflux, which was 0.9–1.4 times larger in transgenic lines than in WT plants (Fig. 7A). The PEG stress also induced an instantaneous [Ca\(^{2+}\)]\(_{cyt}\) elevation in the root protoplasts, and the elevation in transgenic lines was only 64.0–77.8% of that in WT controls (Fig. 7B). The higher Ca\(^{2+}\) efflux (142.0–156.8%) and the lower [Ca\(^{2+}\)]\(_{cyt}\) elevation (60.9–71.9%) were also observed in transgenic lines under salt shock (200 mM NaCl) (Fig. 7C, D). These results revealed that the sustained activation of the PM Ca\(^{2+}\)-ATPase in CbFAD3-overexpressing plants changes the stress-induced Ca\(^{2+}\) signaling during early stresses.

CbFAD3-overexpressing plants enhanced ROS scavenging under abiotic stresses

To reveal the function of CbFAD3 in ROS scavenging, the activities of antioxidant enzymes and the level of H\(_2\)O\(_2\) were analysed in the leaves of transgenic tobacco plants. Under normal conditions, the ROS scavenging ability of transgenic plants was not significantly higher than that of WT plants, except for the SOD activity.  

**Table 1. Pearson correlation coefficients (two-tailed) between membrane unsaturation indices and physiological indices in leaves (L) or roots (R) before and after drought stress**

|               | C18:3 | C18:3/C18:2 | DBI  |
|---------------|-------|-------------|------|
| EL (L)        | 0.26  | −0.19       | 0.33 |
| MDA (L)       | 0.25  | −0.23       | 0.34 |
| Fv/Fm (L)     | −0.11 | 0.37        | −0.14|
| MF (L)        | −0.18 | −0.33       | 0.04 |
| MF (R)        | −0.11 | −0.18       | 0.14 |
| Ca\(^{2+}\)-ATPase (L) | 0.81*** | 0.69**      | 0.63* |
| Ca\(^{2+}\)-ATPase (R) | 0.95*** | 0.79***      | 0.82*** |
| H\(^{+}\)-ATPase (L) | −0.42 | 0.10        | −0.43|
| H\(^{+}\)-ATPase (R) | −0.31 | −0.29       | −0.30|
| SOD (L)       | 0.73*** | 0.37        | 0.69** |
| CAT (L)       | 0.53*  | −0.01       | 0.59** |
| POD (L)       | 0.45  | −0.05       | 0.50* |
| H\(_2\)O\(_2\) (L) | 0.28  | −0.19       | 0.36 |

The data from CbFAD3-transgenic (L2 and L3) and WT tobacco plants were used for the calculation (three replications, n=12 × 3). Significant positive correlation (R≥0.50, P<0.05) is indicated in bold. *P<0.05, **P<0.01, ***P<0.001.
However, the activities of SOD, CAT, and POD increased rapidly in stress-treated transgenic lines compared with that in the WT controls (Fig. 8A–C). Under stress conditions, the transgenic lines showed significantly less accumulation of $\text{H}_2\text{O}_2$ and MDA, and less ion leakage, as well as obviously higher photosynthetic activities (Fig. 5A–C; Fig. 8D), confirming reduced oxidative damage during the onset of the stresses. Furthermore, the correlation coefficients verified that the activities of these antioxidant enzymes had a certain degree of positive correlation with membrane unsaturation, including the C18:3 content and DBI (Table 1).
CbFAD3-overexpressing plants increased the expression of stress-responsive genes under stress condition

To elucidate the molecular mechanism of stress tolerance mediated by CbFAD3, increased expression of genes in transgenic plants compared with WT controls was identified under salt stress using microarray analysis. According to the search results of the NCBI database, 61 genes annotated with related functions were confirmed from the 115 genes in transgenic plants (Table 2); the others, annotated as 'uncharacterized gene/protein' in the database, are not shown. Among the confirmed genes, there were 59 that were directly or indirectly involved in plant stress response; these included genes for nitrogen/sulfur metabolism–related proteins (15 genes), cell wall/membrane–related proteins (11 genes), photosynthesis–related proteins (six genes), protein kinases (six genes), ROS–responsive proteins (five genes), genes for transcription factors (four genes), chromatin remodeling or DNA methylation proteins (three genes), signaling proteins (three genes), alkaloid biosynthesis–related proteins (three genes), cell cycle–related proteins (two genes), as well as one gene for disease resistance protein. Beside these, two pentatricopeptide repeat (PPR)–containing protein genes linked with the recovery of fertility were detected, which might ensure the fertility of transgenic plants. These results indicate that overexpression of CbFAD3 in tobacco can increase the expression of stress–responsive genes under stress conditions.

Discussion

As a consequence of overexpressing a FAD3 gene (Zhang et al. 2005; Yu et al. 2009; Wang et al. 2014a), constitutively increased C18:3 content, accompanied by enhanced multiple–stress tolerance (Fig. 4), was observed in CbFAD3–transgenic tobacco plants. Furthermore, the high level of C18:3 resulting from CbFAD3 overexpression in transgenic plants was approximately the same as that induced by drought stress in WT plants (Fig. 6A, B). Likewise, overexpression of AtFAD7 leads to the same consequence in tobacco plants as low–temperature–induced C18:3 production, and confers low–temperature tolerance in young tobacco leaves (Kodama et al. 1995). These results indicate that overexpression of ω–3 FAD genes can constitutively increase C18:3 to the same level as that induced by abiotic stresses and thereby enhance the plant’s stress tolerance.

Previous studies have suggested that the enhanced stress tolerance is due to the modification of membrane fluidity caused by fatty acid desaturation, which prevents stress–induced membrane rigidification/disruption and maintains the structural and functional integrity of cell membranes (Mikami and Murata, 2003; Los and Murata, 2004; Zhang et al., 2005; Upchurch, 2008; Yu, et al. 2009). Consistent with previous findings, fatty acid desaturation, in both transgenic and WT plants (Fig. 6A, B), helped the cell membranes to maintain optimal fluidity during abiotic stress (Fig. 6C). However, the distinct stress tolerance between these plants (Fig. 5) appears to weaken the dominant role of the maintenance of membrane fluidity in enhancing stress tolerance. More factors should, thus, be taken into consideration.

As an important shaper of Ca\(^{2+}\) signature in response to environmental stimuli, PM Ca\(^{2+}\)–ATPase, which is primarily regulated at the post–translational level, plays a crucial role in stress signaling and adaptation (Befagna et al., 2005; Bose et al., 2011; Shabala et al., 2011). Interestingly, the experimental results along with correlation analysis showed that the activity of PM Ca\(^{2+}\)–ATPase, in both CbFAD3–transgenic and WT plants, had a strong positive correlation with fatty acid unsaturation, especially with the level of C18:3, but was not correlated with membrane fluidity (Fig. 6A–C; Table 1). To the best of our knowledge, relevant studies have never been reported in plants, though there are findings reported from human and animal studies confirming that C18 unsaturated fatty acids increase the activity of PM Ca\(^{2+}\)–ATPase in neutrophils (Hwang et al., 2009), and membrane fluidity has no significant effect on the activity of sarcoplasmic reticulum Ca\(^{2+}\)–ATPase (Madden et al., 1981). The interpretation of the underlying mechanism is that certain unsaturated fatty acids may help form the active state of some membrane enzymes such as Ca\(^{2+}\)–ATPase by penetrating into the protein core to displace the native interactions and destabilize the native state (Grossfield et al., 2006; Hwang et al., 2009).

Although the C18:3–induced activation of PM Ca\(^{2+}\)–ATPase was found in both transgenic and WT plants (Fig. 6A, D; Table 1), the distinct stress tolerance between them suggests that the enhanced tolerance of transgenic plants might be associated with the sustained activation of PM Ca\(^{2+}\)–ATPase induced by the constitutive accumulation of C18:3. Indeed, this assumption is strongly supported by a recent finding that overexpressing a PM Ca\(^{2+}\)–ATPase gene (OsACA6) in tobacco plants, which is analogous to providing a sustained activation of PM Ca\(^{2+}\)–ATPase in the whole plant, enhances the tolerance of plants to drought, salt, and cold stresses (Huda et al., 2013a, 2014). As for the enhanced multiple–stress tolerance, Huda et al. (2013a) speculated that overexpression of OsACA6 might enhance Ca\(^{2+}\) efflux and shape the [Ca\(^{2+}\)]\(_{cyt}\) spike, thereby regulating the signaling mechanisms that promote ROS scavenging and the expression of stress–responsive genes. In our experiments, the enhanced Ca\(^{2+}\) efflux (Fig. 7A, B) and the lowered [Ca\(^{2+}\)]\(_{cyt}\) elevation (Fig. 7C, D) resulting from the sustained activation of PM Ca\(^{2+}\)–ATPase (Fig. 6D) were observed in CbFAD3–transgenic plants during the early stage of abiotic stresses. This confirms Huda’s speculation (Huda et al., 2013a) along with the enhanced ROS scavenging (Fig. 8) and the increased expression of stress–responsive genes (Table 2). More importantly, all these results provide strong evidence for our assumption that the C18:3–induced sustained activation of PM Ca\(^{2+}\)–ATPase, which regulated the stress–induced Ca\(^{2+}\) signaling through enhancing Ca\(^{2+}\) efflux and shaping of the [Ca\(^{2+}\)]\(_{cyt}\) spike, is required for the multiple–stress tolerance of CbFAD3–overexpressing plants.

It is known that stress–induced [Ca\(^{2+}\)]\(_{cyt}\) elevations vary in magnitude, frequency, and shape depending on the type and severity of stress and thus create a unique stress–specific Ca\(^{2+}\) signature that is subsequently decoded by signal transduction networks (Bose et al., 2011). Given that the stress–induced [Ca\(^{2+}\)]\(_{cyt}\) spikes in CbFAD3–transgenic plants were changed in magnitude and shape compared with those in WT controls.
(Fig. 7C, D), it is reasonable to assume that a proper regulation of the stress-specific Ca\textsuperscript{2+} signature might enhance plant stress tolerance through triggering distinct downstream responses. Although the exact criteria for the regulation are not clear, it needs at least to control the stress-induced [Ca\textsuperscript{2+}] cyt elevation within non-toxic levels (Dodd et al., 2010; Huda et al., 2013a), and retain the signaling role of the elevation (Case et al., 2007; Huda et al., 2013b).

Considering the cytotoxicity of the excess ROS resulting from the environmental stimulus, plant stress tolerance is often attributed to the enhanced ROS-scavenging ability (Gill and Tuteja, 2010; Huda et al., 2013a). Wang et al. (2014a) found that overexpression of LeFAD3 in tomato could enhance antioxidant enzyme activities and then salt stress tolerance. Similar phenomena (Figs 4, 8) were also found in CbFAD3-overexpressing tobacco plants. Correlation analysis showed that the antioxidant enzyme activities, especially the SOD activity, had a certain degree of positive correlation with the level of C18:3 and DBI (Table 1). A more than 2-fold increase in SOD mRNA was detected in transgenic plants compared with WT controls (Table 2; Supplementary Fig. S2), suggesting the existence of transcriptional or post-transcriptional regulation of SOD. Given that PM Ca\textsuperscript{2+}-ATPase, which could be activated by C18:3 accumulation (Fig.6A, D; Table 1), is an important regulator in switching off the signal triggering ROS production (Beffagna et al., 2005; Shabala et al., 2011; Huda et al., 2013a), at least a partial contribution of the PM Ca\textsuperscript{2+}-ATPase to the activation of antioxidant enzymes cannot be ruled out. Additionally, because C18:3 plays a role in plant tolerance by serving as a sink of ROS (Mène-Saffrané et al., 2009), CbFAD3-transgenic plants with the constitutive accumulation of C18:3 might possess more non-enzymatic antioxidant ability to counter the oxidative burst during the early stress response. It has been suggested that cross-tolerance, the enhanced ability of a plant to tolerate multiple stresses, results partly from the overlap between the ROS signaling mechanisms (Perez and Brown, 2014). Therefore, the multiple-stress tolerance of CbFAD3-overexpressing plants was associated with enzymatic and non-enzymatic ROS management brought about by the constitutively increased C18:3 content as well as by the sustained activation of PM Ca\textsuperscript{2+}-ATPase.

A recent study confirmed that exogenous C18:3 can modulate the expression of stress-responsive genes, especially mediated by ROS (Mata-Pérez et al., 2015). This prompts speculation that the constitutive accumulation of C18:3 may also affect the increased expression of stress-responsive genes in CbFAD3-overexpressing plants (Table 2). Microarray analysis showed that the highest increased expression (15.02-fold) belonged to the gene for cytochrome c oxidase, a respiration- and ROS-related protein, which is controlled by the C18:1 and C18:3 content of membranes, and has a critical role in chilling and salt tolerance (De Santis et al., 1999; Yan et al., 2005). Among the other genes, the genes for protein kinases, ROS-responsive proteins, transcription factors, signaling proteins, disease resistance proteins,
Table 2. Transcripts significantly increased in CbFAD3-overexpressing tobacco seedlings compared with those of WT controls under salt stress conditions

| Number | Probe name    | Accession no. | Gene annotation                                      | Fold (log) | Function                                      | Reference                  |
|--------|---------------|---------------|------------------------------------------------------|------------|-----------------------------------------------|----------------------------|
| 1      | A_95_P204097 | EH618725      | Cytochrome c oxidase                                  | 15.02      | Chilling and salt tolerance                   | De Santis et al. (1999)    |
| 2      | A_95_P267086 | FG169445      | Monocopper oxidase-like protein                       | 14.00      | Plant growth (i.e. cell wall expansion)       | Yan et al. (2005)          |
| 3      | A_95_P117392 | DV160317      | Disease resistance protein RGA2                      | 12.64      | Defense against pathogen attack              | Loutre et al. (2009)       |
| 4      | A_95_P238494 | FG167609      | Asparagine synthetase                                 | 12.55      | Response to abiotic stresses                 | Wang et al. (2005)         |
| 5      | A_95_P113687 | CV021257      | Caffeoyl-CoA O-methyltransferase                      | 11.31      | Response to salt and water deficit stresses  | Senthil-Kumar et al. (2010) |
| 6      | A_95_P268896 | DW003469      | YLS2-like protein                                     | 11.26      | Response to salt stress                      | Aghaei and Komatsu (2013)  |
| 7      | A_95_P254509 | EB438355      | Histidine kinase                                      | 10.89      | Plant growth and stress responses             | Cao et al. (2015)          |
| 8      | A_95_P016001 | EB678560      | Non-specific lipid-transfer protein                   | 10.75      | Response to abiotic stresses                 | Gangadhar et al. (2016)    |
| 9      | A_95_P281518 | FG645498      | Chromatin-remodeling complex-like                     | 10.30      | Response to drought, cold and salt stresses  | Kim et al. (2010)          |
| 10     | A_95_P035683 | BP128538      | Zinc finger protein                                   | 9.88       | Plant growth and stress responses             | Yang et al. (2014)         |
| 11     | A_95_P234479 | FG170626      | Methyltransferase-like protein                        | 9.03       | Response to abiotic/biotic stresses          | Sahu et al. (2013)         |
| 12     | A_95_P054511 | BP133457      | Cytochrome P450 -like                                | 6.68       | Plant development and abiotic stress tolerance | Perez and Brown (2014)     |
| 13     | A_95_P084005 | BP528907      | MYB transcription factor                              | 5.48       | Plant growth, development and stress response | Zhang et al. (2012)        |
| 14     | A_95_P134852 | EB437708      | SNF1-related protein kinase                           | 4.31       | Response to salt and drought stresses        | Xu et al. (2009)           |
| 15     | A_95_P006516 | EH616694      | Glycine-rich cell wall structural protein             | 3.70       | Salt tolerance and response to pathogen attack | Li et al. (2009)           |
| 16     | A_95_P170234 | EH664856      | APS reductase-like protein                            | 3.58       | Plant development and cold stress response   | Zheng et al. (2013)        |
| 17     | A_95_P259371 | FG171287      | APS reductase-like protein                            | 3.57       | Plant development and cold stress response   | Phartiyal et al. (2008)    |
| 18     | A_95_P316268 | AY772945      | Pectin methylesterase                                 | 3.45       | Cell wall architecture and response to chilling stress | Qu et al. (2011)         |
| 19     | A_95_P099313 | BP534878      | Methyltransferase-like protein                        | 3.33       | Response to abiotic/biotic stresses          | Sahu et al. (2013)         |
| 20     | A_95_P047176 | BP131588      | Ycf3 protein                                          | 3.14       | PSI assembly and drought tolerance           | Muchero et al. (2010)      |
| 21     | A_95_P037263 | BP128958      | GST-like protein                                      | 3.06       | Plant development and abiotic stress tolerance | Perez and Brown (2014)     |
| 22     | A_95_P000116 | FG157904      | APS reductase-like protein                            | 2.98       | Plant development and cold stress response   | Phartiyal et al. (2008)    |
| 23     | A_95_P192712 | EB432744      | Calcium-binding protein                               | 2.91       | Response to environmental stresses           | Tsou et al. (2012)         |
| 24     | A_95_P173162 | EH665543      | Aspartate aminotransferase                            | 2.81       | Nitrogen metabolism and synthesis of amino acids | Zhou et al. (2009)       |
| 25     | A_95_P221852 | DV158128      | Blue copper protein                                   | 2.68       | Plant development and response to salinity and heavy metal stress | Ruan et al. (2011)        |
| 26     | A_95_P173657 | EH665660      | Leucine-rich receptor-like kinase                     | 2.56       | Protein phosphorylation                      | Moscatiello et al. (2006)  |
| 27     | A_95_P083300 | BP528727      | Ycf2 protein                                          | 2.54       | PSI assembly and drought tolerance           | Muchero et al. (2010)      |
| 28     | A_95_P078860 | BP527625      | Receptor-like protein kinase                          | 2.52       | Response to abiotic stresses                 | Ye et al. (2017)           |
| 29     | A_95_P128347 | EB428011      | Pectate lyase                                         | 2.52       | Response to abiotic stresses                 | Palusa et al. (2007)       |
| 30     | A_95_P122212 | DW002241      | Golgin subfamily protein                              | 2.51       | Golgi formation and membrane trafficking     | Latijnhouwers et al. (2007) |
| 31     | A_95_P034973 | BJ999201      | RNA-binding protein                                   | 2.51       | Tolerance to salt and drought stress         | Ambrosone et al. (2015)    |
Table 2. Continued

| Number | Probe name  | Accession no. | Gene annotation                        | Fold (log$_2$) | Function                                      | Reference                  |
|--------|-------------|---------------|----------------------------------------|----------------|-----------------------------------------------|----------------------------|
| 32     | A_95_P297413 | FG152847      | ICR1-like protein                      | 2.51           | Plant growth                                  | Li et al. (2008)           |
| 33     | A_95_P035398 | BP128462      | Maturase K gene                        | 2.46           | Splicing of chloroplast group II introns      | Vogel et al. (1999)        |
| 34     | A_95_P157547 | EH615593      | Reticuline oxidase-like protein        | 2.41           | Cell wall architecture and response to pathogen attack | Belchi-Navarro et al. (2013) |
| 35     | A_95_P159832 | EH618298      | CCR-like protein                       | 2.40           | Abiotic stress tolerance                      | Ghosh et al. (2014)        |
| 36     | A_95_P190577 | EH615701      | LH1-like protein                       | 2.38           | Ethylene responses                            | Shin et al. (2015)         |
| 37     | A_95_P005336 | BP130308      | Pectin methyltransferase inhibitor     | 2.36           | Anti-fungal activity disease resistance and stress tolerance | An et al. (2008)           |
| 38     | A_95_P053311 | EB425896      | Sulphur deficiency-induced protein     | 2.28           | Utilization of stored sulfate                 | Howarth et al. (2009)      |
| 39     | A_95_P018051 | DV160720      | SOD                                    | 2.27           | Plant development and abiotic stress tolerance | Perez and Brown (2014)     |
| 40     | A_95_P186302 | EB436456      | APS reductase-like protein             | 2.26           | Plant development and cold stress response    | Phartiyal et al. (2008)    |
| 41     | A_95_P183277 | DW001571      | Putrescine N-methyltransferase         | 2.26           | Biosynthesis of alkaloid and wound response   | Sachan and Falcone (2002)  |
| 42     | A_95_P155202 | FG191506      | Transcription initiation factor        | 2.26           | Plant growth, development and abiotic stress tolerance | Singh et al. (2013)       |
| 43     | A_95_P134087 | EB435916      | APS reductase-like protein             | 2.23           | Plant development and cold stress response    | Phartiyal et al. (2008)    |
| 44     | A_95_P145077 | EB448622      | TRB-like protein                       | 2.22           | Telomere formation                            | Schrumpfová et al. (2014)  |
| 45     | A_95_P164817 | EH624155      | Receptor-like protein kinase           | 2.22           | Response to abiotic stresses                  | Ye et al. (2017)           |
| 46     | A_95_P130417 | EB430467      | PPR-containing protein                 | 2.19           | Recovery of fertility                          | Bentolila et al. (2002)    |
| 47     | A_95_P284013 | FG139134      | Receptor-like protein kinase           | 2.19           | Response to abiotic stresses                  | Ye et al. (2017)           |
| 48     | A_95_P255821 | X06134        | Nitrate reductase                      | 2.19           | Stress tolerance and plant growth             | Zhang et al. (2014)        |
| 49     | A_95_P131462 | FG191106      | PPlase-like protein                    | 2.18           | Salt stress response                          | Wang et al. (2014)         |
| 50     | A_95_P023626 | AW032686      | Chloroplast NAD(P)H dehydrogenase      | 2.17           | Photosynthesis and growth under cold stress   | Yamori et al. (2011)       |
| 51     | A_95_P058111 | BP134390      | PPR-containing protein                 | 2.15           | Recovery of fertility                          | Bentolila et al. (2002)    |
| 52     | A_95_P107022 | CV018176      | Sodium-coupled neutral amino acid transporter | 2.15       | Plant growth, development and salt stress response | Ortiz-Lopez et al. (2000) |
| 53     | A_95_P136752 | EB680256      | Granule-bound starch synthase          | 2.14           | Amylose synthesis                             | Popova et al. (2003)       |
| 54     | A_95_P221202 | BP128310      | GDSL esterase/lipase                  | 2.12           | Lipid metabolism, plant development, biotic and abiotic stress responses | Seung et al. (2015)       |
| 55     | A_95_P248432 | AM794263      | Chloroplast ribosomal protein          | 2.09           | Abiotic stress resistance                      | Liu et al. (2014)          |
| 56     | A_95_P121002 | DW000972      | Glycine dehydrogenase                  | 2.09           | Photosynthesis and plant growth               | Timm et al. (2012)         |
| 57     | A_95_P249287 | AF149251      | Secretory peroxidase                   | 2.08           | Membrane protective function                  | Lühtrie et al. (2011)      |
| 58     | A_95_P134072 | HO663864      | APS reductase-like protein             | 2.08           | Plant development and cold stress response    | Phartiyal et al. (2008)    |
| 59     | A_95_P161877 | EH620463      | UCH-like protein                       | 2.08           | Ubiquitin recycling and protein regulation    | Isono and Nagel (2014)     |
| 60     | A_95_P232903 | AM847814      | Tropinone reductase homolog            | 2.03           | Biosynthesis of alkaloid                      | Kushwaha et al. (2013)     |
| 61     | A_95_P315843 | FG189231      | Cyclin                                 | 2.01           | Drought stress response                       | Zhou et al. (2013)         |

The WT and transgenic tobacco plants were grown in the greenhouse for 2 weeks and treated with 200 mM NaCl for 6 h; total RNA was extracted from seedlings to perform gene expression profiling by microarray analysis. Transcripts exhibiting more than 2-fold increase in OsFAD3-overexpressing seedlings compared with those of WT controls were considered to show significant changes. Values are means from three independent trials for transgenic and WT plants.

Chromatin remodeling proteins, and DNA methylation proteins, which are induced by Ca$^{2+}$ or ROS signaling and in turn affect the signal pathway and gene expression, play a key role in stress tolerance (Moscipelli et al., 2006; Xu et al., 2009; Kim et al., 2010; Zhang et al., 2012; Aghaei and Komatsu, 2013; Sahu et al., 2013; Perez and Brown, 2014; Yang et al., 2014; Cao et al., 2015). Moreover, nitrogen/sulfur metabolism-related proteins and alkaloid biosynthesis-related proteins are confirmed to participate in the stress response for generating diverse physiologically active substances (Sachan and Falcone, 2002; Wang et al., 2005; Phartiyal et al., 2008; Aghaei and Komatsu, 2013), and the increased expression of their genes was detected. Notably, the increased transcript accumulation of cell wall/membrane-related proteins and photosynthesis-related proteins provides...
a rational explanation for maintaining membrane and photosynthetic status in transgenic plants under abiotic stresses (Fig. 5). Also, the data showed an increased mRNA level of cell cycle-related proteins, which can regulate the drought stress response by inhibiting ROS accumulation (Zhou et al., 2013). These results demonstrate that the multiple-stress tolerance of CbFAD3-overexpressing plants was related to the increased expression of stress-responsive genes, most of which not only are coordinated with one another, but are affected by C18:3 or the C18:3-induced regulation of Ca^{2+} and ROS.

In conclusion, CbFAD3 is a C. bungeana ω-3 FAD gene involved in stress adaptation. Overexpression of CbFAD3 in tobacco enhanced multiple-stress tolerance through C18:3-induced integrated regulation, including membrane stabilization, [Ca^{2+}]_{cyt} modification, ROS management, and the increased expression of stress-responsive genes (Fig. 9). These results reveal a comprehensive mechanism for the involvement of CbFAD3 in response to environmental stresses and should provide a potential target for crop improvement.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Molecular analysis of CbFAD3-overexpressing tobacco plants.

Fig. S2. qRT-PCR analysis for the increased expression of six genes in CbFAD3-overexpressing tobacco plants under salt treatment.

Table S1. Information for primers used for gene cloning and vector construction.

Table S2. The accession number of ω-3 FADs included in alignment.

Table S3. Information for primers used for qRT-PCR.

Table S4. Fatty acid composition of S. cerevisiae cells overexpressing CbFAD3 grown at 20 °C.

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