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ORIGINAL ARTICLE

The Role of the *OsCam1-1* Salt Stress Sensor in ABA Accumulation and Salt Tolerance in Rice

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Abstract Involvement of the salt-inducible calmodulin gene, *OsCam1-1*, in abscisic acid (ABA) biosynthesis during salt stress was studied in the 'Khoa Dawk Mali 105' (KDML105) rice cultivar (*Oryza sativa* L.). FL530-IL, an isogenic salt-resistant line derived from the KDML105 cultivar, accumulated a 2.9-fold higher concentration of ABA in the leaves after salt stress treatment than that for KDML105. A twenty-four and a seven- fold higher level of *OsCam1-1* transcripts were detected in the leaves of the FL530-IL and KDML105 rice cultivars, respectively, after 30 min of salt stress compared to non-salt-stressed plants. Transgenic rice lines that constitutively over-express the *OsCam1-1* gene were found to up-regulate *ABA aldehyde oxidase* and *9-cis-epoxycarotenoid dioxygenase 3*, two genes involved in ABA biosynthesis, and to have a higher ABA content, when compared to the wild type and the control transgenic lines without *OsCam1-1* over-expression. In addition, transgenic plants over-expressing *OsCam1-1* were more tolerant to salt stress, with, for example, a better ability to maintain their shoot and root mass (as dry weight) during salt stress, than the control plants. These data indicate that *OsCam1-1* signaling is likely to play an important role in ABA biosynthesis, and the level of *OsCam1-1* gene expression and ABA accumulation probably contribute to salt resistance in rice.

Keywords Abscisic acid, Calmodulin, *Oryza sativa*, Rice, Salt stress

Abbreviations abscisic acid- ABA, calcineurin B-like protein – CBL, calcium-dependent protein kinase – CDPK, calmodulin – CaM, Khoa Dawk Mali 105 - KDML105, methyl jasmonate - MJ

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Introduction

Salinity stress is one of the major abiotic stresses that seriously limits plant growth and development and can lead to a significant reduction in crop yield. The effects of salinity on plants are due to the ionic toxicity and the osmotic stress. Ionic and osmotic stress signals are sensed and decoded by all plants via distinct and interconnecting signaling pathways that act as relay mechanisms for the control of unique and stress-specific responses. These pathways are controlled by genetic programs, coordinate determinants and processes required for adaptation (Bressan et al. 2008). It has been proposed that after the perception of extracellular stress signals by membrane receptors, complex intracellular signaling cascades are activated. These include the calcium signaling network (Mahajan et al. 2008) and the accumulation of the plant hormone abscisic acid (ABA) (Bressan et al. 2008). Both intracellular calcium ion (Ca^{2+}) and ABA levels have been reported to be key messengers in salt-stress responses (Ghassemian et al. 2008; Guo et al. 2008; Hong-Bo et al. 2008; Mahajan et al. 2008).

Transient Ca^{2+} elevation is sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins that usually contain the 'EF-hand' motif(s), a helix-loop-helix structure (for review: Krebs and Heizmann 2007). Three major classes of EF-hand Ca^{2+} sensors have been characterized in plants, which include calmodulin (CaM) (for review: McCormack et al. 2005; Kim et al. 2009), calcium-dependent protein kinase (CDPK) (for review: Klimecka and Muszynìska 2007) and calcineurin B-like protein (CBL) (for review: Batistic and Kudla 2009; Luan 2009).

CaM is found in both the apoplast and the cytosol as well as in the endoplasmic reticulum and the nucleus of plant cells. CaM has been implicated in the Ca^{2+}-dependent

responses to light, gravity, mechanical stress, phytohormones, pathogens, osmotic stress, salinity, heavy metals, xenobiotics, anoxia, oxidative stress, heat stress and chilling (Zielinski 1998; Reddy 2001; Snedden and Fromm 2001; Fasano et al. 2002). In rice, *OsCam1-1*, *OsCam1-2* and *OsCam1-3* all encode for the identical protein, OsCaM1, whereas *OsCam2* and *OsCam3,* which encode a protein of only two amino acid differences between each other, have 98.7% amino acid sequence identity with OsCaM1 (Boonburapong and Buaboocha 2007). The expression of *OsCam1-1* gene transcripts has been shown to rapidly (1 h after stress) and highly increase in response to salt stress (0.15 M), and then to slowly decrease two and four h after treatment (Phean-o-pas et al. 2005), suggesting that the OsCaM1-1 isoform probably plays an important role in the Ca^{2+}-mediated salt-stress response.

ABA-mediated signaling is one of the signal transduction pathways that is known to regulate the salt-stress-responsive genes (Narusaka et al. 2003; Zhu et al. 2005), and accumulation of ABA has been found after salinity stress in several plant species (e.g. Chen et al. 2006; Hong et al. 2007; Yang and Guo 2007; Szepesi et al. 2009). In water-stressed leaves, ABA triggers a signaling cascade in guard cells that leads to a very rapid closure of stomata within minutes of perception (Schroeder et al. 2001). ABA has also been shown to trigger changes in the amplitude and the oscillation of the free cytosolic Ca^{2+} level and these oscillations induced by ABA together with Ca^{2+}/CaM are involved in the stomatal movement of maize seedlings, where it was also shown that CaM participated in the ABA signal transduction process (Guo et al. 2008). Pretreatment with CaM antagonists almost completely inhibited the ABA-induced H_2O_2 production throughout the ABA treatment period, which suggests that Ca^{2+}/CaM is involved in the ABA-induced antioxidant defense system (Hu et al. 2007; Hu et al. 2008). In *Nicotiana glauca*, CaM antagonists prevented the methyl jasmonate (MJ)-induced stomatal closure and had a partial effect on the ABA-mediated stomatal closure (Suhita et al. 2003), suggesting that the MJ action on stomata closure is dependent on CaM, while the ABA effect was partially directed via CaM.

In order to evaluate the relationship between salt resistance and the level of stress signal molecules in rice during salinity stress, two rice cultivars/lines with a close genetic background but a difference in their salt-tolerance were used. Khao Dawk Mali105 (KDML105), the popular aromatic rice genotype from Thailand, and the FL530-IL rice line, which is a salt-tolerant line that originated from the cross between FL530 (Suriya-arunroj et al. 2004) and KDML105. The KDML105 cultivar shows a salt tolerance score of 7.7, which is classified as susceptible to salinity stress, while the salt tolerant line, FL530, has a significantly lower salt tolerance score of 3.7 (Suriya-arunroj et al. 2004). FL530-IL is the salt tolerant KDML105 introgression line derived by backcrossing FL530 with KDML105 for generations to select for the salt tolerant ability within a KDML105 genetic background.

Here, the levels of ABA and *OsCam1-1* transcripts were measured in response to salt stress in both rice cultivar/lines. To determine if the *OsCam1-1* expression level affects the endogenous ABA level, *OsCam1-1* over-expressing transgenic rice plants were generated and their ABA content and salt resistance was evaluated.

Materials and Methods

Plant Materials, Growing Conditions and Growth Determination

Rice (*Oryza sativa* L.) seeds of the KDML105 cultivar and FL530-IL line were kindly provided by the Rice Gene Recovery Unit, National Center for Genetic Engineering and Biotechnology, Thailand. Seeds of the T_1 transgenic rice with over-expression of *OsCam1-1*, and the T_1 control transgenic lines, were generated as described below.

Seeds were germinated on sand for one week, whereupon the germinated seeds were transferred to modified WP (mWP) nutrient solution (Vajrabhaya and Vajrabhaya 1991) for one week in a greenhouse under natural light (93 to 99 μmole photons $m^{-2}s^{-1}$) and a relative humidity of between 74 to 81%. The level of mWP nutrient solution was controlled via daily addition of carbon-filtered water to replace the water that had evaporated. The salt stress treatment was performed by the addition of 0.5% (w/v) NaCl to the mWP nutrient solution. Shoot and root dry weights were determined on d 0, 1, 3, 5, 7, 9 and 11 after initiation of the treatment. The experiment was performed in triplicate, and three independent seedlings were representative of each replicate. Seedlings grown in the mWP nutrient solution (without NaCl) were used as controls.

ABA Determination

The methods for extraction and purification of abscisic acid [natural(s)-ABA] were modified from those described by Walker-Simmons (1987). For ABA extraction, leaves were ground to a fine powder with liquid nitrogen and the extraction solution (4:1 (v/v) ratio of methanol: water, containing 0.1 mg mL^{-1} butylated hydroxyl-toluene and 0.5 mg mL^{-1} citric acid monohydrate) was added at the ratio of 1 g fresh weight tissue: 10 mL extraction solution. The extract was shaken in the dark for 16 h and then centrifuged at 4800 × g at 4°C for 15 min. The supernatant was harvested, evaporated to dryness and the pellet then resuspended in 1 mL of 100% (v/v) methanol from which the ABA content

was quantified using HPLC (Agilent Technologies Series 1100) equipped with a 250 mm × 4 mm column packed with 5 μm ODS Hypersil (Shandon Runcorn) and eluted with a gradient solvent system of methanol and water with 0.05 M acetic acid at a flow rate of 1.0 mL min^{-1}. Commercial (±) Cis-trans ABA (Sigma) was used as the standard. Data are reported as the mean ±1 standard deviation (SD) and are derived from three repetitions.

Construction of the *OsCam1-1* Over-Expression Vector

Since the *OsCam1-1* gene in japonica rice encodes exactly the same 149 amino acid-long polypeptide sequence as the *OsCam1-1* gene in the KDML105 rice cultivar (Phean-o-pas et al. 2008), the *OsCam1-1* cDNA clone (AU081299) from japonica rice, provided by the DNA Bank of the National Institute of Agrobiological Science (Ibaraki, Japan), was used as a template for construction of the *OsCam1-1* over-expression vector. The *35SCaMV-gus-nos* polyA cassette from pCAMBIA1301 was cloned into the pGEM®-T Easy vector (Promega), resulting in a *p35SCaMV-gus* recombinant plasmid. Based on the sequence of the *OsCam1-1* cDNA clone (AU081299), a pair of primers ((F: 5' CCATGGC-GGACCAGCTCACC 3'; R: 5' GTGCTAGCCTTGGCC-ATCATGA 3') for amplifying the coding region of *OsCam1-1* was designed with *Nco*I and *Nhe*I restriction sites engineered at the 5' and 3' ends (underlined above), respectively. The PCR product derived from amplification of the cDNA as template was then cloned into the pGEM®-T Easy vector. The purified recombinant plasmid, after clonal growth of ampicillin selected transformed JM109 *E. coli* (K12) cells, was digested with *Nco*I and *Nhe*I restriction enzymes and subcloned to substitute the *gus* coding region in the *p35SCaMV-gus* recombinant plasmid, resulting in a *35SCaMV-OsCam1-1-nos* polyA cassette. The *35SCaMV-OsCam1-1-nos* polyA cassette and flanking *Hind*III restriction sites was then inserted back into pCAMBIA1301 at the *Hind*III site in the multi-cloning site. The resulting recombinant vector was used for transformation to generate transgenic rice with the *OsCam1-1* over-expression. The pCAMBIA1301 containing only the *35SCaMV-gus-nos* polyA cassette was used to generate transgenic rice control lines that do not over-express *OsCam1-1*.

Rice Transformation

KDML105 rice seeds were dehusked and surface sterilized in 70% (v/v) ethanol for 2 min followed by 1.8% (v/v) sodium hypochlorite for 30 min and several rinses in sterile water. The seeds were then placed on nutrient broth (NB) medium (Li et al. 1993) containing 2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated in the dark at 28°C for two weeks to obtain the embryonic calli. The compact, yellowish embryonic calli were separated and subcultured on fresh medium before use for transformation.

The recombinant vector for *OsCam1-1* over-expression was introduced into *Agrobacterium* strain EHA105 by electroporation using the GENE pulser apparatus (Bio-Rad) at 2.5 kV, 25 μF and 200 Ω pulse. When rice calli were ready for transformation, *A. tumefaciens* were streaked on solid AB medium (Jefferson et al., 1986), containing 25 μg mL^{-1} rifampicin and 50 μg/mL^{-1} kanamycin. The cells were incubated at 28°C for 2 to 3 d, collected by scraping with a loop and resuspended in AAM medium (Toriyama and Hinata 1985) supplemented with 300 μM acetosyringone. The optical density (1 cm light path) at 600 nm of the bacterial suspension was adjusted to 1.0 with fresh medium. Embryonic calli were immersed in the bacterial suspension for 30 min with occasional shaking and blotted dry on sterile filter paper. The calli were then transferred to NB medium supplemented with 10 g L^{-1} glucose, 2 mg L^{-1} 2,4-D and 100 μM acetosyringone and incubated at 25°C for three d. To generate control plants, in a separate suspension, calli were co-cultivated with *Agrobacterium* carrying pCAMBIA1301.

Calli were removed from the co-cultivation medium, washed with sterile 250 mg mL^{-1} cefotaxime to remove the excess *Agrobacterium*, followed by several sterile water rinses and then blotted dry on sterile filter paper. The calli were then transferred to NB medium containing 250 μg mL^{-1} cefotaxime and 50 μg mL^{-1} hygromycin and incubated at 28°C for four weeks. The hygromycin-resistant calli were subjected to another round of hygromycin selection, as detailed above, and then transferred to regeneration medium containing 4 mg L^{-1} of 6-benzylaminopurine. The culture was incubated at 28°C under 16:8 h of light: dark period for 3 to 4 weeks. When green shoots developed, they were transferred to the hormone-free medium for stimulation of rooting and stem elongation for four weeks.

Verification of *35SCaMV-gus-nos* polyA Expression in the Transgenic Rice.

Total RNA, extracted from the leaves of the putative transgenic lines using Tri-Reagent® (Molecular Research Center, Inc.) according to the instructions from the manufacturer, was used for reverse transcription PCR (RT-PCR). To synthesize the first strand cDNA, 1 μg of total RNA was reversed transcribed in a 20 μL final volume reaction consisting of 1× RT-PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 mM oligo$(dT)_{15}$ and 10 U μL^{-1} M-MLV reverse transcriptase (Promega). Then, PCR was conducted with the forward primer specific to the *OsCam1-1* coding sequence: OsCam1nosF

5' CCATGGCGGACCAGCTCACC 3' and the reverse primer specific to the *nos* region: OsCam1nosR 5' TAATCATCGCA-AGACCGGCAACAGG 3' to give an expected amplicon of 574 bp. The cDNA amplification by *Taq* DNA polymerase (Fermentas) consisted of an initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 58 °C for 30 sec and 72 °C for 1 min. A control RT-PCR reaction without adding the reverse transcriptase was done in parallel.

Determination of the *OsCam1-1* Gene Expression Level

For the *OsCam1-1* transcript measurement by quantitative real-time RT-PCR (qrtRT-PCR), the first cDNA synthesis was synthesized by RT-PCR as described above, including the control with no added reverse transcriptase, and then PCR was performed in a final volume of 20 μL, which contained a 1 μL aliquot of the first strand cDNA reaction, 0.05 μM of each of the gene-specific primers and 1x iQTM SYBR® Green Super Mix (Bio-Rad). The reaction included an initial 8 min denaturation at 95 °C, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec. The specific primers for *OsCam1-1*, designed from the 3'UTR region of the *OsCam1-1* cDNA, were: OsCam1F 5'ACCG-TGCATTGCCGTATTAG 3' and OsCam1R 5'GCAAGCCT-TAACAGATTCAC 3', yielding an expected amplicon size of 177 bp.

The *OsActin* (AK101613) gene was used as an internal control and was amplified via the specific primers OsActinF 5'AGCTATCGTCCACAGGAA 3' and OsActinR 5'ACC-GGAGCTAATCAGAGT 3', yielding an expected amplicon of 155 bp. The qrtRT-PCR reactions were performed as above except that the annealing temperature was reduced to 49°C. The level of *OsCam1-1* gene expression was determined in comparison with that for the *OsActin* gene expression with reference to the expression on d 0 of the treatment. At least three independent qrtRT-PCR reactions were performed on the same cDNA preparation.

For determination of the *OsCam1-1* transcript levels in the transgenic rice, total RNA was separated by electrophoresis in a formaldehyde agarose gel and transferred onto a positively charged nylon membrane. The coding region of the *OsCam1-1* gene was radiolabelled with [α-^{32}P]dATP by random priming according to Hodgson and Fisk (1987) and used as a probe for hybridization. The membrane was hybridized at 40°C in 50% (v/v) deionized formamide, 5 × SSPE/NaOH (pH 7.4), 20 mM Na_2EDTA, 1 × Denhardt's solution and 0.2% (w/v) SDS. When hybridization was complete, the membrane was washed twice in 2 × SSPE, 0.1% (w/v) SDS and then once in 1 × SSPE, 0.1% (w/v) SDS at room temperature. Hybridizing bands were visualized by autoradiography.

Detection of Rice *ABA Aldehyde Oxidase* (*OsAAO*) and *9-Cis-Epoxycarotenoid Dioxygenase 3* (*OsNCED3*) Gene Expression Levels

Total RNA was extracted according to the method reported by Thikart et al. (2005). Ten micrograms of total RNA was treated with DNaseI (Takara) and then subjected to reverse transcription using M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol. The qrtRT-PCR was performed in a 20 μL solution containing a 2 μL aliquot of the first strand cDNA reaction, 0.25 μM of each of the gene-specific primers and 10 μL of iQSYBR Green Super mix (Bio-Rad Laboratories). The reaction included an initial 10 min denaturation at 95 °C, followed by 40 cycles of 95°C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and then followed by 72 °C for 10 min. The primers, designed from the *OsAAO* cDNA sequence (AK122123), were: OsAAOF 5' AGAGTTGGTGGTGG-CTTTGG 3' and OsAAOR 5' GGACAGGACGCTGCA-ACTTA 3'. To quantify the *OsNCED3* gene expression level the primers, designed from the *OsNCED3* cDNA sequence (AY838899), were: OsNCED3F 5' GAGAAGACGTCGCG-GTTC3' and OsNCED3R 5' AGAGGTGGAAGCAGAAG-CAG3'. *OsEF1-a* (AK105030) gene was used as the internal control with the primers OsEF1a F 5' ATGGTTGTGGA-GACCTTC 3' and OsEF1a R 5' TCACCTTGGCACCGG-TTG 3'. The qrtRT-PCR reactions were performed as above.

Rice seedlings of each cultivar or line were grown in a completely randomized design (CRD) with three replicates, and with four seedlings for each replicate being pooled for RNA extraction. The qrtRT-PCR reaction was performed in duplicate for technical replication. The amplification efficiency for the *EF-1α*, *OsAAO* and *OsNCED* genes were found to be 78.9% (r^2 = 99.9), 106.5% (r^2 = 98.4) and 91.5% (r^2 = 98.4), respectively.

Statistical Analysis

Rice seedlings were grown in a CRD. The statistical analysis of the ABA content was performed using analysis of variance (ANOVA) with three replicates, each of which contains three plants. The mean comparison was performed with Duncan's multiple range test (DMRT).

To compare the expression levels of the genes involved in ABA synthesis and the growth analysis of the transgenic lines and wild type, three independent transgenic lines with the over-expression of *OsCam1-1*, the transgenic line containing only the antibiotic resistant gene (T1), and the wild type KDML105 cultivar were grown in a CRD. The statistical analysis of gene expression level, tissue dry weights, and the relative growth rate (RGR) was performed using ANOVA with three replicates, each of which consisted of data from four seedlings. The mean

comparisons were performed using DMRT.

Results

The Salt-Tolerant FL530-IL Rice Line Has a Higher Salt-Resistance and ABA Content than the Salt-Sensitive KDML105 Cultivar.

When subjected to salinity stress, the 14-d old FL530-IL seedlings showed a more salt-resistant phenotype than the original cultivar, KDML105. Over the 13 d of salinity stress, a numerically slight but statistically insignificant gain in the shoot dry weight was detected over the first five d and then decreased slightly over the remaining eight d in both the FL530-IL and KDML105 seedlings, with a larger d shoot mass being observed in the FL530-IL line than the KDML105 cultivar at all time points (Fig. 1). In contrast, under the normal culture conditions a steady increase in the shoot dry weight was noted over the 13 d growth period leading to a significantly larger dry shoot weight in the non-salt stressed plants (both KDML105 and FL530-IL), compared to the salt-stressed plants from seven d onwards, having some two-fold larger dry mass by 13 d post-treatment.

The dry root mass showed a less clear trend, compounded by the large amount of variation within each treatment over time, but the broad trends of an essentially similar root dry mass between the two cultivars/lines under normal cultivation conditions, and a higher root dry mass in the resistant FL530-IL line than the salt-susceptible KDML105 cultivar when grown under the salt-stress condition, were numerically observed, although it was not statistically significant (Fig. 1B). Although a larger dry shoot mass was observed in the FL530-IL line than the KDML105 cultivar at all time points, they did not start with the same mass and so the data was reanalyzed in terms of the growth reduction percentage (GRP) to standardize for the initial differences in their starting dry masses. In this analysis a significantly lower GRP of the FL530-IL line, when compared to that for the KDML105 cultivar, indicated the higher salt tolerant ability of the FL530-IL line (Fig. 1C).

Changes in the total leaf ABA content were determined in leaf tissues during growth under normal and salt-stress conditions over 13 d. In contrast to when grown under normal conditions, in which the ABA content in both the

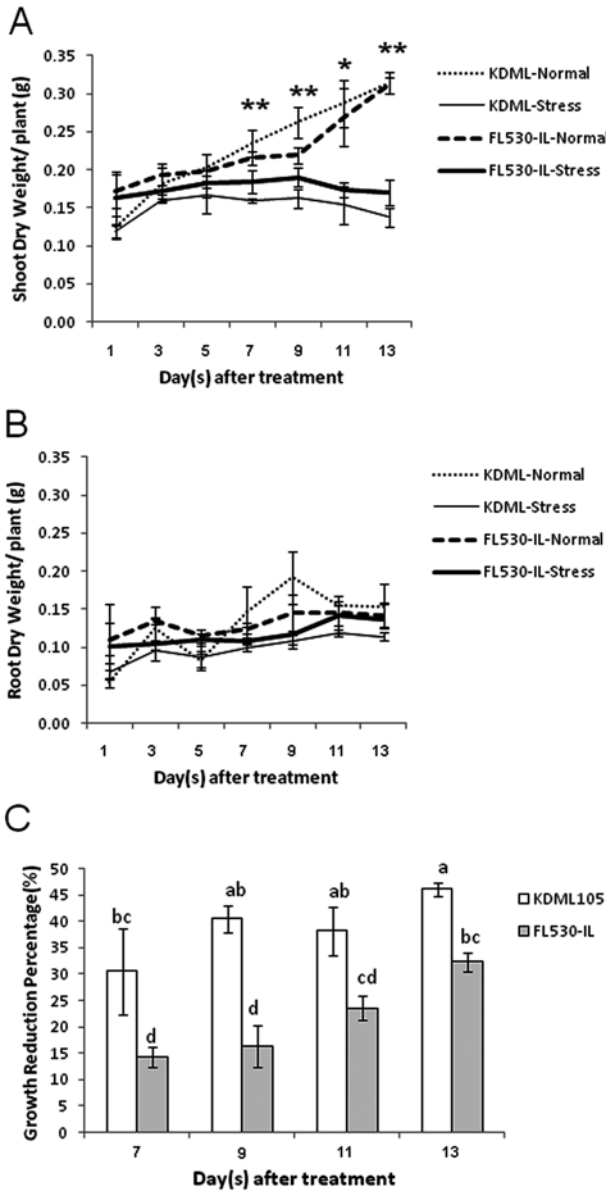

Fig. 1. (A) Shoot and (B) root dry weight of the KDML105 rice cultivar and FL530-IL rice line when grown under normal and salt-stress conditions, and (C) the growth reduction percentage (GRP) during salt stress of both rice cultivar/lines. In (A), * and ** represent significant difference of shoot dry weight found at the indicated time point, whilst in (C), means of the GRP with a different lowercase letter differ significantly ($P < 0.01$; DMRT).

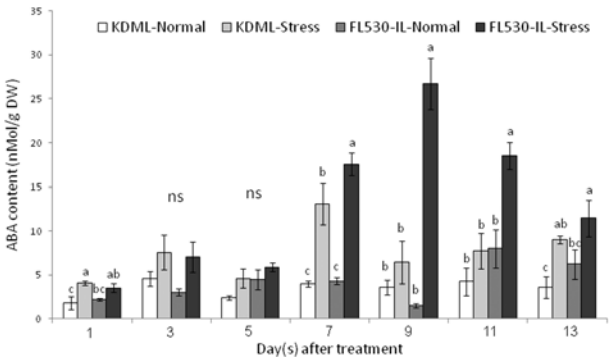

Fig. 2. Abscisic acid (ABA) content in the leaf tissues of the KDML105 cultivar and FL530-IL line when grown under normal and salt-stress conditions. Means with a different lowercase letter differ significantly ($P < 0.01$; DMRT), when the levels of ABA content were compared on the same d, and ns = no significant difference.

KDML105 cultivar and FL530-IL line remained relatively unchanged, an increase in the leaf tissue ABA content under the salt-stress conditions was observed, and this was more marked in the salt-tolerant FL530-IL line than in the susceptible KDML105 cultivar. The highest level of total leaf ABA content in the KDML105 cultivar was found after seven d (~3.3-fold higher) of salt treatment, and this then declined thereafter to almost its normal level by d nine onwards (Fig. 2). In contrast, the ABA content in the salt-tolerant FL530-IL line only began to significantly increase after seven d and reached the highest (~7.7-fold higher) level nine d after initiation of the salt stress, declining thereafter still remains significantly elevated above that seen in the normal conditions at 13 d, the longest time point assayed (Fig. 2).

OsCam1-1 Transcript Levels During Salt-Stress in Rice Seedlings with Different Salt-Resistant Abilities

A higher and earlier enhanced level of *OsCam1-1* transcript in response to salt stress was detected in the salt-resistant FL530-IL line compared to the salt-susceptible KDML105 cultivar (Fig. 3). Thus, for the salt sensitive KDML105 cultivar, the *OsCam1-1* transcript levels increased from 30 min up to 4 h after salt stress, but these were only significantly higher than that observed when cultured under normal conditions after 2 h of salt stress (~eight-fold higher) remaining at this level for at least another 2 h, but with the observed increase in the transcript levels in the control at 4 h after salt-stress this was effectively reduced to an ~1.2-fold higher transcript level in the salt-stressed plants at 4 h.

In contrast, in the salt resistant FL530-IL line, the *OsCam1-1* transcript levels also increased from 30 min after salt stress onwards, but were significantly higher from 30 min onwards than that seen in those plants grown under normal cultivation conditions, reaching 1.7-, 1.8-, 2.1- and 3.9- fold higher transcript levels after 0.5, 1, 2 and 4 h of salt treatment, respectively (Fig. 3). An almost comparable increased level of *OsCam1-1* transcript, up to 21 times higher than its normal level, was also detected in the KDML105 leaves, but only after two to four hr of salt treatment. We note that the actual *OsCam1-1* transcript levels increased more in the salt-stressed plants relative to that at the initial time points, but that these transcript levels also increased (and so lowered the fold level differences reported between salt-stressed and control plants) over the first 30 min and after 4 hr in the no salt-stress control for KDML105, and at all time points especially after 30 min and 2 h for FL530-IL. This is probably due to the mechanical response of the *OsCam1-1* gene in rice. In Arabidopsis, it was shown that calmodulin and calmodulin related genes are induced by touch, which can be caused by indirect (rain or wind) or direct contact (Braam and Davis, 1990). Nevertheless, the elevated *OsCam1-1* transcript levels in response to the salt stress are larger and longer lasting or with delayed kinetics to that seen in the controls.

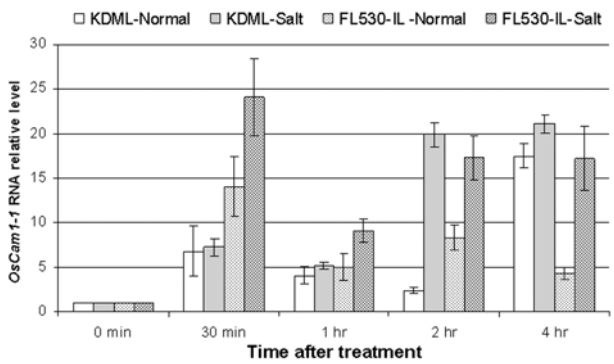

Fig. 3. *OsCam1-1* (calmodulin) gene expression pattern in KDML105 and FL530-IL rice cultivar/line grown under normal and salt-stress conditions.

Over-Expression of the *OsCam1-1* Gene in Rice Affects ABA Biosynthesis Gene Expression During Salt Stress.

The *OsCam1-1* gene construct, regulated by the *35SCaMV* promoter, together with the GUS coding sequence as a reporter gene and the hygromycin resistance gene as a selectable marker (Fig. 4A), was introduced to the KDML105

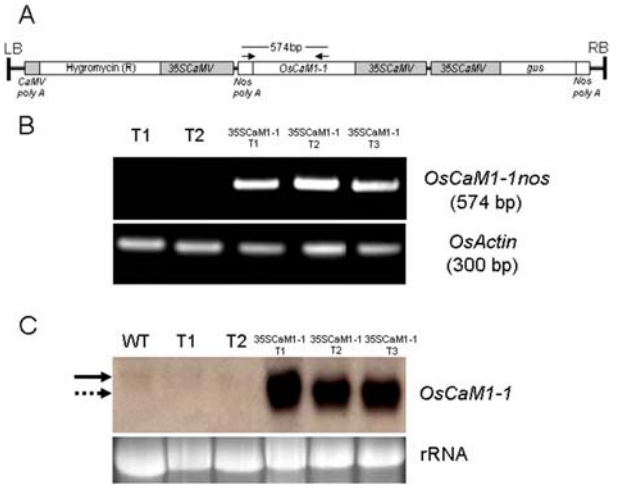

Fig. 4. The (A) *OsCam1-1* over-expressing gene construct that was used to create the transgenic rice lines, 35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3, showing the location of the OsCamNosF/R primers (arrows) and the 574 bp expected amplicon size. (B) Transcript expression of the *OsCam1-1* gene construct in the transgenic rice of the *OsCam1-1* gene construct detected by RT-PCR. (C) The northern blot analysis of the non-expressing transgenic (T1, T2) and wild type (WT) lines, and the three *OsCam1-1* over-expressing transgenic rice lines (35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3), using the *OsCam1-1* gene as the probe.

rice cultivar. Three independent transgenic lines were obtained, named 35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3 hereafter. Two transgenic lines, containing only the GUS coding sequence and the antibiotic resistant marker from pCAMBIA1301, called T1 and T2 hereafter, were also generated as controls for the analysis of the *OsCam1-1* over-expression phenotype.

To confirm that the inserted *OsCam1-1* gene was over-expressed at the mRNA level in all three *OsCam1-1* transgenic lines, RT-PCR was performed using the OsCamnosF/R primers (Fig. 4A). Positive amplicon bands were only detected in the three transgenic lines, 35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3, and not in the two control lines, T1 and T2 (Fig. 4B). To compare the *OsCam1-1* expression level among the *OsCam1-1* over-expressing transgenic lines, as well as in the T1 and T2 transgenic control lines and the wild type KDML105 rice plants, RNA-DNA hybridization was performed using the *OsCam1-1* cDNA labeled with [α^{32}P] dCTP as a probe. A much higher level of the *OsCam1-1* transcript was detected in the *OsCam1-1* over-expressing transgenic lines (Figure 4C), as expected. However, that the positive bands found in the three transgenic lines (35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3) (dashed line, Fig. 4C) were slightly smaller than those in the other samples (solid line, Fig. 4C) is expected because the over-expression construct lacks the 5'UTR and the 3'UTR of the endogenous gene transcripts.

To determine if the over-expression of the *OsCam1-1* gene affects the expression of other genes in the ABA biosynthesis pathway, the expression of *ABA aldehyde oxidase* (*AAO*), whose expression was previously shown to be transcriptionally induced by salt stress (Seo et al. 2000), and 9-cis-epoxycarotenoid dioxygenase (NCED), the enzyme involved in the cleavage of the carotenoid precursor in ABA biosynthesis (Nambara and Marion-Poll 2005), were investigated in growth under normal and salt stress conditions.

When grown under normal cultivation conditions, *AAO* gene expression in the *OsCam1-1* over-expressing transgenic lines was 1.2 to 3.0 times higher than that in the control lines (Fig. 5A). After three d of salt stress, up-regulation of *AAO*

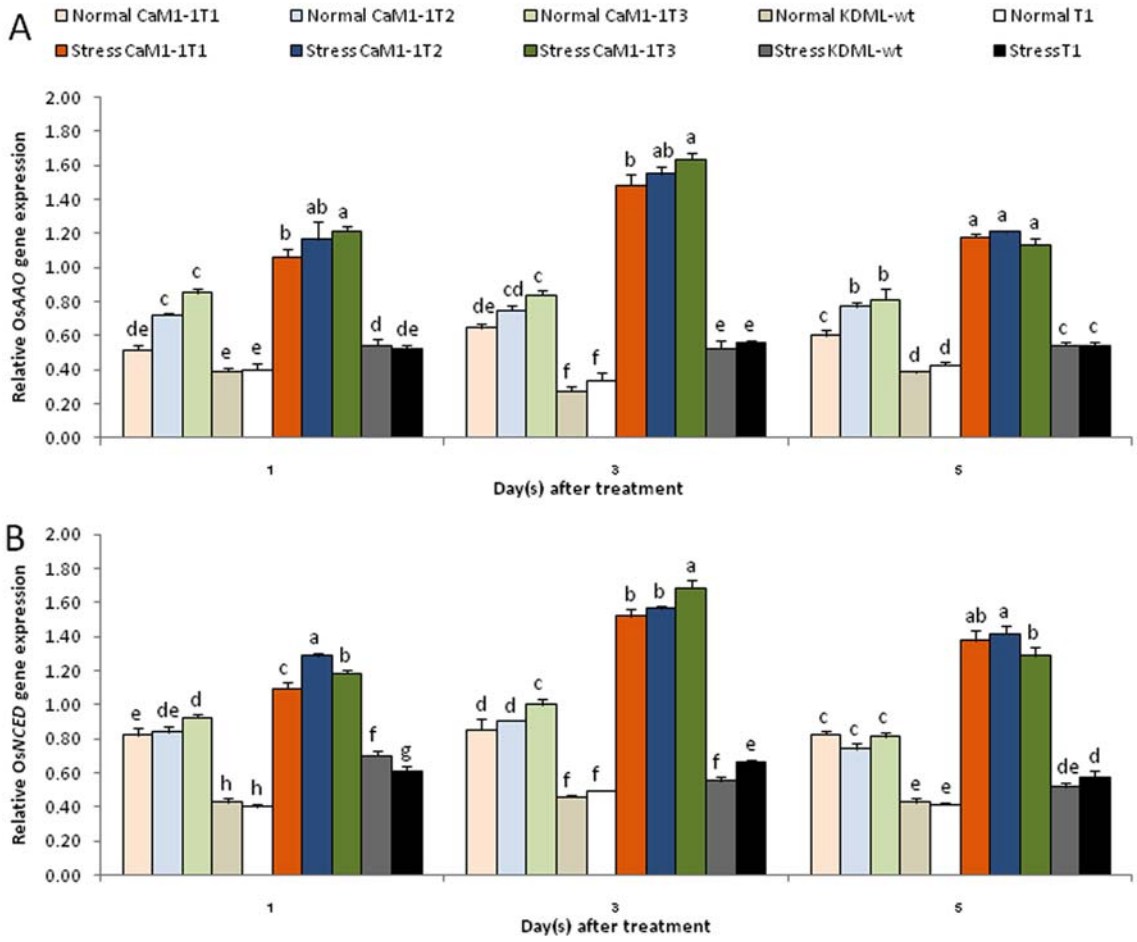

Fig. 5. Relative gene expression levels of (A) *AAO* and (B) NCED in the over-expressing *OsCam1-1* transgenic lines grown under normal or salt-stress conditions. Means with a different lowercase letter differ significantly ($P < 0.01$; DMRT), when the levels of expression were compared on the same d.

transcripts was found in all rice lines compared to those grown without salt stress, but whilst this was at a 1.9- or 1.6-fold increase in the wild type and transgenic control line (T1), respectively, it was much higher, at 2.0- to 2.3- fold higher, in the *OsCam1-1* over-expressing transgenic lines, 35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3. However, after five d of salt stress conditions, the *AAO* gene expression level in every line decreased, but was still higher than that for each line grown under normal conditions (Fig. 5A).

In both normal and salt stress conditions, a higher *NCED3* gene expression level was also found in all three *OsCam1-1* over-expressing transgenic lines compared to that in the T1 control and the wild type lines. In the normal cultivation condition, the three transgenic lines with *OsCam1-1* over-expression showed a 1.8 to 2.2 times higher *NCED3* gene expression than the wild type and the transgenic T1 control line. In salt stress conditions, the three transgenic lines (35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3) showed the highest induction of *NCED3* gene expression after 3 d of salt treatment, revealing a 1.7- to 1.8- fold induction of *NCED* transcripts, while in the wild type and control T1 lines, the highest salt-induced *NCED3* gene expression was found after one d of the salt stress treatment, reaching 1.5- to 1.6- fold higher transcript levels. After 5 d, the relative gene expression level decreased in every line, but was still higher than in those without salt-stress (Fig. 5B).

Based on the up-regulation of *AAO* and *NCED* gene expression, it appears that the over-expression of *OsCam1-1* in the transgenic rice affects the ABA biosynthesis pathway via these genes during salt stress conditions.

OsCam1-1 Over-Expressing Rice Lines Exhibit a Better Salt Stress Adaptation.

To determine if the up-regulation of the genes involved in the ABA biosynthesis pathway would lead to a higher accumulation of ABA in the *OsCam1-1* over-expressing transgenic lines, the 35SCaM1-1T1 line was used as the representative transgenic line, since all three transgenic lines expressed similar levels of *AAO* and *NCED3* gene expression, and in some cases, 35SCaM1-1T1 showed the lowest level of *AAO* and *NCED3* gene expression (Fig. 5).

The ABA content in the leaves was found to increase in the wild type, T1 control and the *OsCam1-1* over-expressing transgenic plants, reaching a maximum level at 7 to 9 d of salt-stress and thereafter started to decline. However, significantly higher ABA levels were found in the leaves of the *OsCam1-1* over-expressing transgenic line (35SCaM1-1T1) grown under normal conditions (0 d after salt stress), when compared to that in the non-expressing transgenic control (T1) and the wild type seedlings (Fig. 6). The ABA

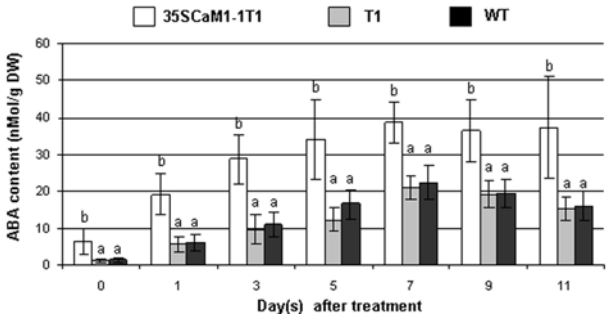

Fig. 6. Comparison of the total leaf ABA content with time in the over-expressing *OsCam1-1* transgenic line (35SCaM1-1T1), the control transgenic line (T1) and the wild type (WT) rice plants grown under salt-stress conditions. Means with a different lowercase letter differ significantly ($P < 0.05$; DMRT), when the levels of ABA content were compared on the same d.

content in all plants was clearly up-regulated in response to salt stress, whilst levels in the 35SCaM1-1T1 line were 1.7- to 1.8- fold higher than those found in the T1 control and wild type plants (Fig. 6).

To determine if the up-regulation of the genes involved in ABA biosynthesis would lead to a higher salt stress adaptation, the three *OsCam1-1* over expressing transgenic lines, plus the control T1 and wild type plants, were subjected to salt stress for 15 d and the dry weight of the whole plant and the RGR of each rice line were determined. The plants likewise grown in normal media (salt-stress free) were used as the controls.

When cultivated under salt-stress free conditions, 24 d-old seedlings of the three transgenic lines (35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3) showed a significantly higher RGR than that of the transgenic control line (T1) or the wild type (KDML-wt) cultivar after 11 to 15 d growth (Fig. 7A), which resulted in a significantly higher plant d weight after 10 and 15 d growth (Fig. 7B).

When grown under the salt stress condition, 35SCaM1-1T3 showed a significantly higher RGR during the first five d of the salt treatment, when compared to the other lines, but then declined to a similar level to those of the wild type and the control T1 line. However, the 35SCaM1-1T1 and 35SCaM1-1T2 lines showed a significantly higher RGR than the other lines during d 6 to 10 of the treatment, and for the 35SCaM1-1T1 line this was also true at d 11 to 15 (Fig. 7C), which resulted in significantly higher average plant dry weights for the *OsCam1-1* gene over-expressing transgenic lines compared to the control lines, from d 5 (35SCaM1-1T3) or d 10 (35SCaM1-1T1 and 35SCaM1-1T2) onwards (Fig. 7D).

Overall, over-expression of *OsCam1-1* gene in the KDML105 rice cultivar was found to enhance the plant adaptation to salt stress (Fig. 8).

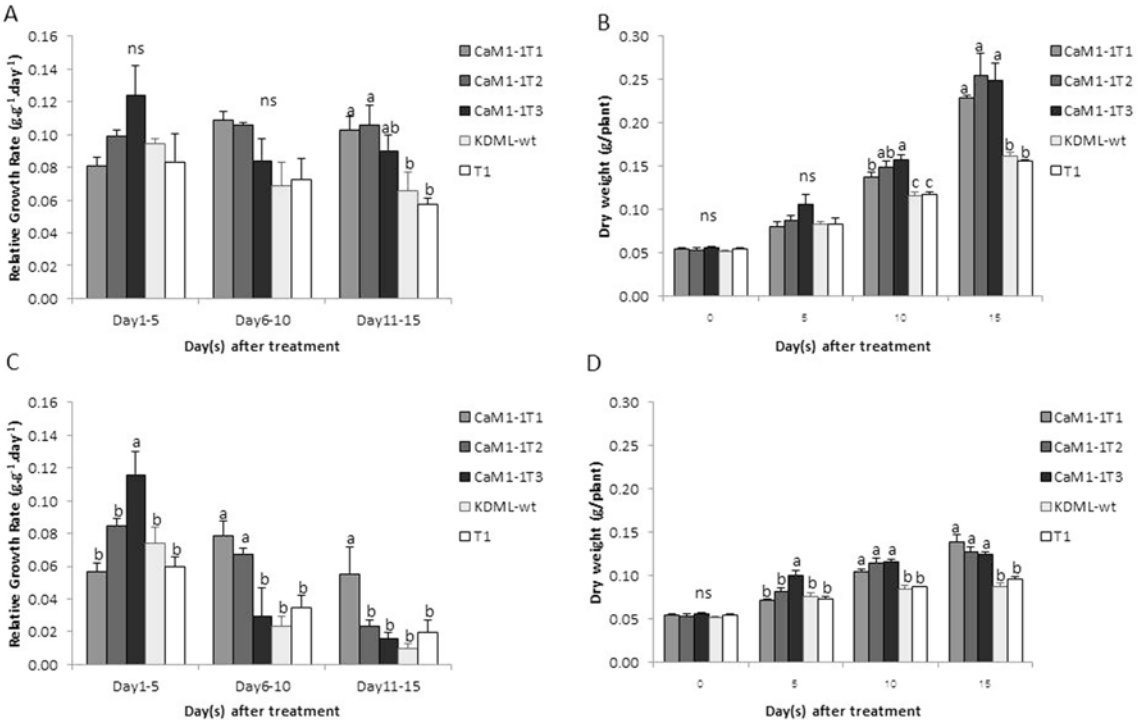

Fig. 7. The (A and C) relative growth rate and (B and D) dry weight of the over-expressing *OsCam1-1* transgenic lines (35SCaM1-1T1, 35SCaM1-1T2 and 35SCaM1-1T3), the control transgenic line (T1; KDML-vector) and the wild type KDML105 rice cultivar, plants grown under (A and B) normal and (C and D) salt-stress conditions. Means with a different lowercase letter differ significantly ($P < 0.05$; DMRT), when the comparison was performed with the data at the same period of time.

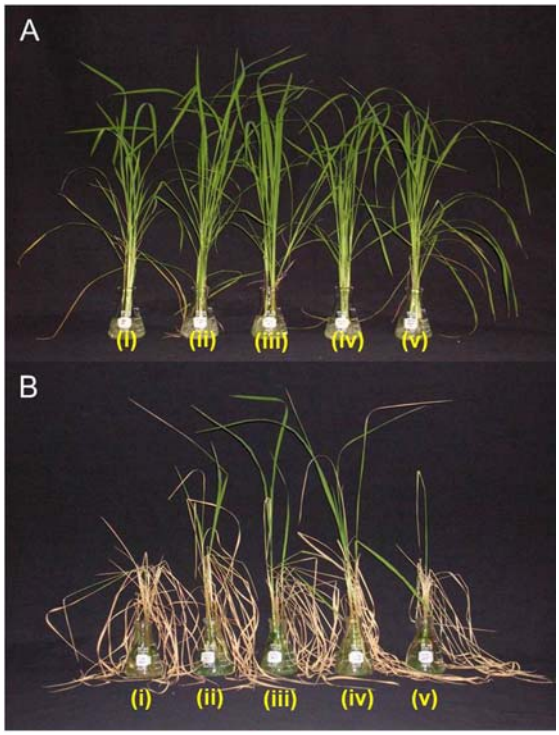

Fig. 8. The phenotype comparison of (i) wild type (KDML-wt) rice cultivar, the three transgenic rice lines, (ii) CaM1-1T1, (iii) CaM1-1T2 and (iv) CaM1-1T3, and (v) the control KDML-vector (T1) line, all grown under (A) normal or (B) salt-stress conditions for 15 d.

Discussion

The higher ABA level and the prolonged period of ABA accumulation in tissues may be one of the phenotypes that contribute to salt resistance in rice. The salt tolerant rice cell line, when grown under salt stress conditions, accumulated a higher level of endogenous ABA than the salt-susceptible one (Perales et al. 2005). A similar phenomenon was also found in the naturally selected populations of Blue Panicgrass (*Panicum antidotale* Retz.), where a higher accumulation of the free ABA content was found to strongly correlate with the degree of adaptability to the saline environment (Ahmad et al. 2009), suggesting a role for ABA in salt resistance in rice.

OsCam1-1 was previously shown to be up-regulated by salt stress during the first four-h of salt treatment (Phean-o-pas et al. 2005), consistent with the results of this report. In *Arabidopsis*, the over-expression of one of the CaM isoforms that binds to the transcription factor MYB2 resulted in enhancing its DNA binding activity and salt tolerance (Jae et al. 2005). CaM was also shown to be involved in the ABA-induced antioxidant defense in maize during water stress (Hu et al. 2008). With the consistency in the level and the sensitivity of the *OsCam1-1* response, and of the salt resistance, of the rice lines reported here, it suggests that *OsCam1-1* may play a role in the salt-stress signaling

cascade and its level and sensitivity of expression may contribute to salt resistance in rice.

It has previously been shown that the increase in ABA levels was at least partially the result of transcriptional regulation of the enzymes in the ABA biosynthesis pathway (Xiong et al. 2001b). The up-regulation of *AAO* and *NCED* genes after salt stress in this study is consistent with previous reports that have shown the transcriptional up-regulation of several genes in the ABA biosynthesis pathway by salt stress (Audran et al. 1998; Iuchi et al. 2000; Seo et al. 2000; Xiong et al. 2001a, 2002). The *OsCam1-1* over-expressing rice line, 35SCaM1-1T1, which had a higher level of *AAO* and *NCED* gene expression than the transgenic control lines, showed a significantly higher level of ABA accumulation after salt stress than the control lines.

Populus euphratica, the salt-resistant poplar tree species, has been reported to have relatively higher ABA and CaM concentrations when exposed to a high salinity condition, compared to the susceptible cultivar/species, *P. nigra* cv. *Italica* and *P. popularis* (Chang et al. 2006). Similarly, in this report here, the higher average total leaf concentration of ABA during salt stress in the 35SCaM1-1T1 transgenic line was consistent with the ability to maintain the plant dry weight during salt stress compared to the transgenic control line (Fig. 7). The other *OsCaM1-1* over-expressing lines also showed significant RGRs during salt stress. Taken together, these results suggest that *OsCam1-1* over-expression confers a better salt-resistant ability to the transgenic plants, and that this is possibly mediated via ABA accumulation. Recently, it was shown that the overexpression of the rice calmodulin-like gene, *OsMSR2*, could enhance drought and salt tolerance in Arabidopsis. Moreover, it also increased ABA sensitivity during seed germination and post-germination stages (Xu et al. 2011). Taken together, this suggests that calmodulin and calmodulin-like genes are involved in abiotic stress tolerance via the ABA-mediated pathway.

These data support the conclusion that the higher capacity to synthesize stress signals, such as ABA and CaM, contributes to a better salt resistance. The effects of *OsCam1-1* over-expression on the up-regulation of *AAO* and *NCED3* gene expression and the increased total leaf ABA content suggests the function of the calcium-dependent OsCaM1-1 protein in the signaling cascade for ABA biosynthesis during salt-stress conditions. Together, the modulation of calmodulin (*OsCam1-1*) and accumulation of ABA in tissues ultimately contributes to the salt resistance in rice.

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