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***OsCaM1-1* overexpression in the transgenic rice mitigated salt-induced oxidative damage**

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Abstract

Various physiological and biochemical parameters associated with improved salinity tolerance in the transgenic rice lines overexpressing *OsCaM1-1* gene and wild-type KDML105 were compared 3 d after exposure to 150 mM NaCl. The results showed higher relative water content, relative growth rate, content of photosynthetic pigments (chlorophylls *a*, *b*, and carotenoids), DPPH scavenging activity, and activities of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase in the transgenic plants when compared with the wild-type and control, KDML105 transformed with blank vector, whereas H₂O₂ content, Na/K and Na/Ca ratio, lipid peroxidation, and electrolytic leakage were lower. Taken together, the *OsCaM1-1* gene overexpression probably reduced salt-induced oxidative damage in the transgenic plants by enhancing the activities of antioxidant enzymes.

Additional key words: antioxidant activity, calmodulin, carotenoids, chlorophyll, NaCl, reactive oxygen species.

Introduction

Various abiotic stresses such as salinity, drought, or extreme temperatures adversely affect growth, development, and the yield of crop plants. Exposure of plants to these stresses may provoke osmotic stress, oxidative stress, and protein denaturation. Simultaneously, plants defend themselves by accumulation of compatible solutes, stress proteins, and antioxidants (Zhu 2002, Apel and Hirt 2004). Reactive oxygen species (ROS) can cause damage to DNA and almost every organic constituent of the living cell (Fridovich 1986, Davies 1987, Imlay and Linn 1988). Thus, a highly efficient antioxidant system is present in all plants to detoxify ROS. Plants have developed a series of enzymatic and non-enzymatic antioxidants (Sairam and Tyagi 2004, Khan *et al.* 2012, 2014, Per *et al.* 2017). Major ROS scavenging enzymes include superoxide dismutase (SOD: EC 1.15.1.1), ascorbate peroxidase (APX: EC 1.11.1.11), catalase (CAT: EC 1.11.1.6),

glutathione reductase (GR: EC 1.8.1.7) and non-enzymatic antioxidants include ascorbic acid and glutathione.

Eukaryotes utilize changes in the cytosolic Ca²⁺ as a second messenger to generate cellular responses to a wide variety of extracellular stimuli including abiotic and biotic stresses and also plant hormones, *e.g.*, abscisic acid (ABA). The Ca²⁺ transmits the stress signal downstream by several Ca²⁺ sensors or Ca²⁺-binding proteins such as calmodulin (CaM), calcium-dependent protein kinases (CDPK), and calcineurin B-like proteins (CBLs), which can regulate intracellular physiological responses (Yang and Poovaiah 2002, Bouche *et al.* 2005). The CaM is a primary calcium sensor in all eukaryotes. It binds calcium and regulates the activity of a wide range of effector proteins in response to calcium signals. *Cam* genes families have been identified in several plants including rice which has five *OsCam* genes. *OsCam1-1*, *OsCam1-2*,

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Abbreviations: APX - ascorbate peroxidase; CaM - calmodulin; CAT - catalase; GR - glutathione reductase; KDML105 - Khao Dawk Ma Li 105; MDA - malondialdehyde; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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and *OsCam1-3* encode identical protein, *OsCaM1*, whereas *OsCam2* and *OsCam3* encode a protein with only two amino acid differences from typical CaM, and share 98.7 % amino acid sequence identity with *OsCaM1* (Phean-O-Pas *et al.* 2005, Booburapong and Buaboocha 2007). *OsCaM1-1* encodes a functional Ca^{2+} -binding CaM and is highly expressed in vascular tissues and the emerged lateral roots (Phean-O-Pas *et al.* 2008). The expression of *OsCaM1-1* was induced under osmotic stress and salt stress (Chinpongpanich *et al.* 2012). In addition, the transgenic rice plants overexpressing

OsCam1-1 exhibited increased ABA biosynthesis and better resistance to salinity than the control plants (Saeng-ngam *et al.* 2012). Chaicherdasakul *et al.* (2017) also suggested a possible CaM-regulated salinity tolerance mechanism *via* redox signaling and homeostasis. Taken together, *OsCam1-1* may contribute to salt resistance in rice. Therefore, we investigated antioxidant systems and other physiological changes in the transgenic plants overexpressing *OsCam1-1* induced by salt stress and compared them with those in the wild-type and control transgenic lines without *OsCam1-1* overexpression.

Materials and methods

Growth conditions and treatments: Seeds of *Oryza sativa* L. var. *indica* cv. Khao Dawk Ma Li 105 (KDML105) were obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The transgenic rice plants containing *OsCaM1-1* gene under the control of 35SCaMV promoter (35SCaMV-*OsCaM1-1*) were previously constructed by *Agrobacterium*-mediated transformation *via* pCAMBIA1301 plasmid (Saeng-ngam *et al.* 2012).

Seeds of three transgenic rice plants overexpressing *OsCaM1-1*, the transgenic line harboring the T-DNA from pCAMBIA1301 alone as the negative control, and wild-type KDML105 were dehusked and sterilized with 70 % (v/v) ethanol for 2 min and then with 5 % (m/v) sodium hypochlorite for 20 min. The seeds were rinsed three times with sterile water and germinated in a nutrient broth medium (NB) (Li *et al.* 1993) containing 0.8 % (m/v) agar at a temperature of 25 - 28 °C, a 16-h photoperiod, and an irradiance of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 7 d, germinated seeds were transferred to a Limpinuntana's nutrient solution (Wutipraditkul 2015) and grown under the previously mentioned conditions for 14 d. Three-week-old seedlings were exposed to a nutrient solution supplemented with 0, 50, 100, or 150 mM NaCl for 3 d. After 3 d of the salt stress, the leaves were harvested and were randomly selected and oven-dried at 60 °C for 72 h for determination of dry mass. Relative growth rate (RGR) was determined following the method of Beadle (1993) and it was calculated from the increase in dry mass from the beginning to the end of NaCl treatment using an equation: $\text{RGR} = (\ln M_f - \ln M_i) / (t_f - t_i)$, where M is the shoot or root dry mass, t is the time and subscripts denote the initial (day 0) and final samplings (day 3).

Content of Na^+ , K^+ , and Ca^{2+} : Leaves (0.02 g) were dried in an electric oven at 60 °C for 3 d. Dry samples were homogenized in 0.5 M HNO_3 (65 %, v/v) and rested in normal condition for 2 d. Then, the sample was incubated at 80 °C for 1 h in an electric oven. The content of Na^+ , K^+ , and Ca^{2+} was measured by atomic absorption spectrophotometer (AA280FS, Agilent Technologies, USA) according to the method of Munns *et al.* (2010).

H_2O_2 content was measured by the modified method of Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 100 mg leaf tissue with liquid nitrogen followed by grinding in 3 cm^3 of 50 mM phosphate buffer (pH 7). The homogenate was filtered and centrifuged (6 000 g, 4 °C, 25 min), and 0.9 cm^3 of the supernatant was mixed with 0.3 cm^3 of 1 % (v/v) TiCl_4 in conc. HCl and centrifuged (6 000 g, 4 °C, 15 min). The absorbance of the yellow supernatant was measured at 410 nm. H_2O_2 content was then calculated according to a standard curve drawn using different known concentrations of H_2O_2 .

MDA content: Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA), a breakdown product of lipid peroxidation, according to De Vos *et al.* (1989). Leaves (0.1 g) were ground to fine powder by mixer mill (MM400, Retsch, Germany) at a frequency of 35 Hz per min twice. Then, 1 cm^3 of 0.1 % trichloroacetic acid (TCA) was added and the mixture was centrifuged (5 000 g, 25 °C, 15 min). After centrifugation, 0.3 cm^3 of the supernatant was mixed with 0.75 cm^3 of 0.25 % 2-thiobarbituric acid (TBA) in 10 % TCA. The absorbance of resulting supernatant was taken at 532 and 600 nm. MDA content was determined by subtracting absorbance of supernatant at 600 nm from that of 532 nm using absorbance coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$.

Membrane permeability (electrolytic leakage): Leaves were harvested 3 d after salt stress imposition and cut into 1-cm segments. Leaf segments were placed into 5 cm^3 of deionized water in a test tube to remove solutes from leaf surfaces. Then the sample was submerged in 5 cm^3 of deionized water in test tube for 2 h and electrical conductivity (EC) was measured before and after autoclaving at 121 °C for 20 min. The cell membrane stability [%] was calculated as $100 - [(EC_1/EC_2) \times 100]$, where EC_1 is electrical conductivity after dipped in deionized water for 2 h and EC_2 is electrical conductivity after autoclaved for 20 min (Blum and Ebercon 1981).

Relative water content: Plants from each treatment were randomly selected and the method described by Barrs and Whetterley (1962) was followed. About 0.1 g leaf sample was cut into smaller pieces and weighed to determine initial mass (M_i). The leaf samples were then floated in freshly de-ionized water for 12 h and weighed thereafter to determine fully water saturated mass (M_f). The sample was oven-dried at 60 °C for 3 d and the dry mass was obtained (M_d). The RWC [%] was determined as $[(M_i - M_d)/(M_f - M_d)] \times 100$.

Photosynthetic pigments: Chlorophylls and carotenoids were extracted in 2 cm³ *N, N*-dimethyl formamide (DMF) by grinding 0.05 g of rice leaves in mixer mill MM400 at a frequency of 35 Hz per min twice. The homogenate was centrifuged (15 000 g, 10 min, 4 °C) and the absorbance of the resulting supernatant was taken at 461, 625, and 664 nm. Total chlorophyll and carotenoid content was calculated according to modified method of Arnon (1949).

DPPH radical scavenging activity: Leaves (0.1 g) were ground to fine powder by mixer mill MM400. Then, 1.0 cm³ of absolute methanol was added and evaporated by *Centrivap* (Labconco, Canada) concentrator. The samples were reacted with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) in an ethanol solution by mixing 0.5 cm³ of sample, 3 cm³ of absolute ethanol, and 0.3 cm³ of 0.5 mM DPPH radical solution in ethanol. The change in colour (from deep violet to light yellow) was read at

517 nm after 30 min of reaction. The mixture of ethanol and sample (without DPPH radical solution) served as blank. The control solution was prepared by mixing ethanol and DPPH radical solution. The scavenging activity percentage AA [%] was calculated as $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$ according to Brand-Williams *et al.* (1995).

Enzyme extractions and assay: Enzyme extracts for evaluating SOD, CAT, APX, and GR activities were prepared by freezing leaf samples (0.5 g) in liquid nitrogen followed by grinding with 5 cm³ of cold extraction buffer [0.1 M phosphate buffer (pH 7.5) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM dithiothreitol], except that in the case of APX activity, 5 mM ascorbic acid was added to the extraction buffer. Homogenates were filtered and centrifuged (15 000 g, 4 °C, 20 min) prior to harvesting the supernatant as the enzyme extract. Activities of SOD, CAT, APX, and GR were assayed as described previously (Wutipraditkul *et al.* 2015).

Protein content in the enzyme extract was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis: All analyses were completely randomized with five to ten replicates per treatment. Statistical analysis was performed using one-way ANOVA and differences between the mean values were compared using Duncan's test at $P < 0.05$.

Results and discussion

The Na⁺ content in leaves of all the three independent transgenic lines overexpressing *OsCaM1-1*, the control transgenic rice line (with T-DNA from pCambia1301), and wild-type (WT) plants significantly increased after subjecting them to 150 mM NaCl stress for 3 d. However, no significant difference of K⁺ content was observed, while Ca²⁺ content of the control and WT decreased significantly. It is widely known that K⁺ is required for maintaining the osmotic balance, affects opening and closing of stomata, and it is also an essential cofactor for many enzymes (Mahajan and Tuteja 2005). The Ca²⁺, which is an important determinant for plant salt tolerance, confers protective effects on plants under abiotic stress, plays an essential role for maintaining the structural and functional integrity of the plant cell membranes, regulates ion transport (Hadi and Karimi 2012) and acts as a second messenger in stress signaling. In this study, the increase of Na/K and Na/Ca ratios under salinity in leaves of the control and WT were significantly higher than in the three transgenic lines (Fig. 1A,B). It has been known that due to high content of Na⁺, which is toxic to cell metabolism, ROS are produced and causes deleterious

effect on the functions of some enzymes (Niu *et al.* 1995). There are reports showing that Na⁺ in plant compete with Ca²⁺ by entering the cell through channels resulting in increased Na/Ca ratio under salinity (Hasegawa *et al.* 2000), which is consistent with our study. Similar to the transgenic rice, salt tolerant genotypes of *Aloe vera* maintained significantly lower Na/Ca ratio and suffered less membrane damage (Jin *et al.* 2007).

The H₂O₂ contents in the leaves of all three transgenic lines, control and WT increased and reached its maximum after 3 d (data not shown). H₂O₂ content in all the plants increased in a concentration-dependent manner and reached its maximum at 150 mM NaCl but it reduced rapidly at 200 mM NaCl (data not shown). These results indicated that exposure time and NaCl concentration were important factors that affected rice oxidative stress. All parameters in subsequent experiments were examined after 3-d exposure to 150 mM NaCl. The H₂O₂ content in all the transgenic lines was lower than that of the control and WT (Fig. 1C). The damage to cellular membranes due to lipid peroxidation can be indicated by the

accumulation of the MDA. Here, MDA content was increased under 150 mM NaCl in all the plants (Fig. 1D), which is consistent with the previous reports in cotton and tobacco (Meloni *et al.* 2003, Savouré *et al.* 1999). However, the increase of MDA was less pronounced in the transgenic plants as compared with the control and WT. In many reports, production of ROS increases under salt stress (Hasegawa *et al.* 2000) and ROS-mediated membrane damage was a major cause of the NaCl toxicity in different plants such as rice, tomato, citrus, and cotton (Gueta-Dahan *et al.* 1997, Dionisio-Sese and Tobita 1998, Mittova *et al.* 2004, Ahmad *et al.* 2009, Pérez-Clemente *et al.* 2012). Salt stress induced ion leakage, indicating injury to membranes, which could be affected by ROS enhancing membrane lipid oxidation (Lechno *et al.* 1997, Savouré *et al.* 1999). The degree of cell membrane injury can be easily estimated by measurements of electrolytic leakage from the cells. Cell membranes are one of the first targets of many plant stresses and there are many reports showing that ion

removal from leaf samples under abiotic stresses is higher than under normal conditions, *e.g.*, in durum wheat under water stress, coffee under chilling stress, and *NTHK1*-transgenic *Arabidopsis* under salinity (Bajji *et al.* 2001, Campos *et al.* 2003, Cao *et al.* 2007). Our results revealed a significant increase in electrolyte leakage in the control and WT plants (but not in the transgenic plants) (Fig. 1E), which correlated with lipid peroxidation (Fig. 1D). These results suggested that the overexpression of *OsCaM1-1* protected membrane to oxidative stress. It was observed that growth and adaptation of all transgenic lines were better than that of the control and WT. Consequently, the control and WT seedlings wilted and leaves turned yellow under salinity while the transgenic plants showed less wilting (Fig. 1 Suppl.).

RWC in the leaves of all the plants grown under salinity stress decreased significantly when compared to normal conditions (Fig. 2A). The reduction of RWC in stressed plants may be associated with a decrease in plant vigor, which was observed in many plant species such as

Fig. 1. Effect of 150 mM NaCl for 3 d on Na/K ratio (A), Na/Ca ratio (B), H_2O_2 content (C), MDA content (D), and electrolyte leakage (E) of the transgenic rice plants overexpressing *OsCaM1-1* gene (CaM1-1T1, 2, and 3), the negative control transgenic line, and the wild-type. Means $\pm$ SEs, $n = 5$. Different letters indicate significant differences at $P < 0.05$.

rice, kidney bean, and maize or may result from the damage of cell membranes (Halder and Burrage 2003, Lopez *et al.* 2002, Valentovič *et al.* 2006). The higher reduction of RWC in the control and WT plants than the transgenic plants suggested that the overexpression of *OsCaM1-1* helps the plants to maintain their ability for preserving more water in tissues. The relative growth rate

(RGR) of shoots (Fig. 2B) and roots (Fig. 2C) in the control and WT was reduced significantly but the RGR of shoots in the transgenic plants decreased only slightly. Consequently, the transgenic plants could recover and grow faster than the control and WT under salt stress condition (Fig. 1 Suppl.).

Our results also showed that salinity had no

Fig. 2. Effect of 150 mM NaCl for 3 d on RWC (A), RGR of shoot (B), RGR of root (C), total chlorophyll content (D), and carotenoid content (E) in the three transgenic plants overexpressing *OsCaM1-1* gene (CaM1-1T1, 2, and 3), the negative control transgenic line, and the wild-type. Means $\pm$ SEs, $n = 10$ for RGR and 5 for others). Different letters indicate significant differences at $P < 0.05$.

significant effect on photosynthetic pigments (total chlorophyll and carotenoids) in the transgenic plants but had a significant negative effect on them in the control and WT (Fig. 2D-E). The decreased content of photosynthetic pigments under salinity may be due to the increasing activity of the chlorophyll-degrading enzyme, chlorophyllase (Reddy and Vora 1986), degradation of β -carotene, and formation of zeaxanthins, which are apparently involved in protection against photoinhibition (Sharma and Hall 1991). Carotenoids are main antioxidants in plant leaf cells and play an important role against oxidative stress (Verma and Mishra 2005, Gill

and Tuteja, 2010). Their content is often increased in the plants under abiotic stress (Kholová *et al.* 2011, Peng *et al.* 2015), but our result showed that their content in the transgenic plants decreased slightly under salinity when compared to the control and WT. These results suggested that the transgenic lines overexpressing *OsCaM1-1* could protect the photosynthetic systems from salt-induced injury better than the control and WT.

The DPPH assay is rapid and sensitive way to analyze the antioxidant activity of plant extracts. The salinity stress caused a significant increase in DPPH radical scavenging activity in the transgenic plants, which was

about 2-fold higher than that of control and WT (Fig. 3A). Various environmental stresses lead to excessive production of ROS such as H_2O_2 , a product of SOD reaction, a strong oxidant, however, it has an important role in redox signaling (Rhee 2006). ROS can be eliminated by the action of several non-enzymatic and enzymatic processes in cells. In enzymatic processes, excessive content of H_2O_2 could be minimized through

the activities of CAT and different peroxidases, which help maintain its level and prevent uncontrolled export of this toxic species from organelles to cytosol. While GR, one of the important enzymes in the ascorbate-glutathione cycle, catalyzes the NADPH-dependent reduction of oxidized glutathione and is important in protecting many plants from oxidative stress (Foyer *et al.* 1991). Induction of antioxidant enzymes is one of the earlier response

Fig. 3. Effect of 150 mM NaCl for 3 d on DPPH radical scavenging activity (A), and SOD (B), CAT (C), APX (D), and GR (E) activities of the transgenic rice plants overexpressing *OsCaM1-1* gene (CaM1-1T1, 2, and 3), the negative control transgenic line, and the wild-type. Means $\pm$ SEs, $n = 5$. Different letters indicate significant differences at $P < 0.05$.

mechanisms against environmental stresses. To determine the response of the transgenic plants to salt-induced oxidative stress, antioxidant enzymes activities in leaves of seedlings grown under salinity were measured in comparison with the control and WT plants. The results showed that salinity caused an increase in SOD, APX, and GR activities (Fig. 3B,D,E) in all plants, but especially in the transgenic plants. However, salinity led to a significant decrease in CAT activities in the control and WT, whereas in the transgenic plants, a significant increase in CAT activity was observed (Fig. 3C). The

increased activities SOD, GR, and APX under salinity are consistent with the previous reports in moderately salinity tolerant wheat genotype and in pea leaves (Hernandez and Alamansa 2002, Sairam *et al.* 2005), whereas in tobacco salinity has no effect on SOD activity (Hoque *et al.* 2007). A decreased CAT activity under salinity was observed in the rice cv. Dongjin seedlings, mung bean, tobacco suspension, and rapeseed under salinity (Lee *et al.* 2001, Hossain *et al.* 2011, Hoque *et al.* 2006, Hasanuzzaman *et al.* 2011), which is similar to our result in the control and WT plants. However, other reports

showed that salinity results in an increase in the CAT activity, such as in the *Lycopersicon pennellii* and *Plantago maritima* (Mittova *et al.* 2003, Sekmen *et al.* 2007), which is consistent with our report in the transgenic plants. An increase in H₂O₂ generation in the leaves of salt-stressed rice plants may function in the signaling of oxidative stress, which leads to induction of

some enzyme activities.

These results suggested that the overexpression of *OsCaM1-1* gene may confer the transgenic plants with a more active antioxidative capacity, thus providing protection against ROS under salinity. This is likely through induced cooperation of antioxidants to eliminate excessive ROS under salinity.

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