จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

ผลการแสดงออกในข้าวทรานสเจนิกส์ของยีนสร้างคัลโมดูลินต่อการตอบสนองต่อความเค็ม

โดย

1. ธีรพงษ์ บัวบูชา
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ตุลาคม 2552

กิตติกรรมประกาศ

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บทคัดย่อภาษาไทย

ชื่อโครงการวิจัย ผลการแสดงออกในข้าวทรานสเจนิกส์ของยีนสร้างคัลโมดูลินต่อการตอบสนองต่อความเค็ม

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บทคัดย่อ

พืชใช้แคลเซียมในการส่งผ่านสัญญาณการเปลี่ยนแปลงจากสิ่งแวดล้อมเพื่อกระตุ้นให้เกิดการตอบสนองที่เหมาะสม คัลโมดูลิน (CaM) เป็นสมาชิกในกลุ่มครอบครัวโปรตีนที่มี EF hand ซึ่งมีความสำคัญในการส่งผ่านสัญญาณแคลเซียมในพืช ในงานวิจัยนี้ผู้วิจัยสร้างข้าวทรานส์เจนิกที่ได้รับยีน *OsCam1-1* ที่ควบคุมด้วยโปรโมเตอร์ 35SCaMV (*35SCaMV-OsCam1-1*) ด้วยการทรานส์ฟอร์มโดยอาศัยอะโกรแบคทีเรียผ่าน pCAMBIA1301 ซึ่งสามารถสร้างข้าวทรานส์เจนิกที่ได้รับยีน *OsCam1-1* ทั้งหมดสามสายพันธุ์ และข้าวทรานส์เจนิกที่ได้รับ T-DNA จาก pCAMBIA เพื่อเป็นชุดควบคุมสองสายพันธุ์ การวิเคราะห์ด้วยพีซีอาร์ยืนยันว่าสายพันธุ์ที่ผ่านการทรานส์ฟอร์มด้วยยีน *OsCam1-1* ซึ่งย้อมติดสีน้ำเงินเจากแอกติวิตีของยีนกัสมีการแทรกของ *35SCaMV-OsCam1-1* ภายในจีโนม และเมื่อวิเคราะห์ด้วย northern blot พบว่าการแสดงออกของยีน *OsCam1-1* ในข้าวทรานส์เจนิกที่ได้รับยีน *OsCam1-1* เพิ่มสูงขึ้นอย่างมาก จากการศึกษาข้าวทรานส์เจนิกที่ได้รับยีน *OsCam1-1* พบว่ามีอัตราการเติบโตและความสามารถในการทนเค็มสูงกว่าข้าวชุดควบคุม นอกจากนี้ข้าวทรานส์เจนิกที่ได้รับยีน *OsCam1-1* ยังมีปริมาณ ABA สูงกว่าพืชชุดควบคุมทั้งในข้าวที่ปลูกในภาวะปกติและที่ได้รับความเครียดจากความเค็ม จากผลการทดลองเหล่านี้แสดงให้เห็นว่าผลิตภัณฑ์จากยีน *OsCam1-1* น่าจะทำหน้าที่เป็นตัวรับสัญญาณแคลเซียมของความเครียดจากความเค็มโดยอาจไปควบคุมการสังเคราะห์ ABA และช่วยทำให้ข้าวตอบสนองต่อความเครียดจากความเค็มได้ดีขึ้น

Project Title	Effect of transgenic expression in rice of a calmodulin on salt stress response
Name of the Investigators	Assistant Professor Dr. Teerapong Buaboocha Associate Professor Dr. Supachitra Chadchawan
Year	October 2009

Abstract

Calcium signaling has been implicated in transducing signals from environmental changes into adaptive responses in plants. Calmodulin (CaM), members of the EF-hand family of Ca^{2+}-binding proteins, represent important relays in plant calcium signals. Here, transgenic rice plants containing *OsCam1-1* gene under the control of *35SCaMV* promoter (*35SCaMV-OsCam1-1*) were constructed by *Agrobacterium*-mediated transformation via pCAMBIA1301 plasmid**.** Three putative transgenic rice lines harboring the *35SCaMV-OsCam1-1* and two putative transgenic lines harboring the T-DNA from pCAMBIA1301 alone as negative controls have been produced. PCR analyses confirm that all transgenic plants harboring the *35SCaMV-OsCam1-1* with positive histochemical assay of the GUS reporter gene contain the *OsCam1-1* transgene in their genomes. Northern blot analysis indicates that expression of the *OsCam1-1* gene was highly increased in these transgenic lines. As a result, transgenic plants over-expressing this gene exhibited higher growth rate and better resistance to salt stress than the control plants. In addition, *OsCam1-1*-over-expressing plants were shown to contain higher levels of ABA than the control plants when grown in normal conditions or under salt stress. These results suggest that the *OsCam1-1* gene product functions as a sensor for salt stress-induced calcium signals that lead to ABA biosynthesis, which in turn helps the plant to cope with salt stress.

สารบัญ

รายการรูปประกอบ

รายการสัญลักษณ์

°C	degree Celsius
Ca^{2+}	calcium ion
cm	centimeter
cDNA	complementary deoxyribonucleic acid
dATP	2′-deoxyadenosine 5′-triphosphate
DNA	deoxyribonucleic acid
EST	expressed sequence tag
g	gram
HPLC	high performance liquid chromatography
hr	hour
kb	kilobase
KDML105	Khao Dok Ma Li 105
l	liter
μg	microgram
μm	micrometer
μmol	micromole
M	molar
m	meter
mM	millimolar
min	minute
mg	milligram
ml	milliliter
mm	millimeter
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate

nm	nanometer
nmol	nanomole
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
s	second
SDS	sodium dodecyl sulfate
UV	ultraviolet
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

บทที่ 1 บทนำ (Introduction)

Plants have evolved complex defense mechanisms as a series of cellular events to perceive and transmit stress signals from both biotic and abiotic stimuli. Osmotic stress caused by salinity and drought is a major problem in agriculture worldwide. A number of evidence suggests the involvement in osmotic stress signaling of common relay processes such as receptor-coupled phosphorelay, phosphoinositol-induced Ca^{2+} changes, and mitogen-activated protein kinase cascades. These processes sense stress signals and transmit them to cellular machineries to generate adaptive responses. Tolerance response to osmotic stress is mediated by complex mechanisms involving multiple effectors; therefore, manipulating one enzyme or pathway is unlikely sufficient to improve salt tolerance in plants as a whole. However, altering coordinate regulation of several effectors through the manipulation of signal transduction pathways or transcriptional activation of stress-regulated genes may have greater effects on salt tolerance at the physiological level.

Many responses to abiotic stresses including osmotic stress caused by salinity and drought involve Ca^{2+} signaling. Ca^{2+}, an important signaling messenger in eukaryotic cells, mediates a large number of signals including stress signals. Stress signals evoke transient increases in cytosolic Ca^{2+} concentrations, which are typically transmitted by protein sensors that bind Ca^{2+} and change their conformation and activity. Current research focuses on understanding Ca^{2+} signaling mechanisms of perceiving and transmitting stress signals to initiate responses and involves calcium sensors and their target proteins. Our preliminary result has shown that expression of a calmodulin gene, *OsCam1-1* was rapidly (1 hour after stress) and highly increased in response to osmotic stress. This result indicates that OsCaM1-1 probably plays an important role in mediating osmotic stress response. If the product of this gene functions to transduce the signal from osmotic stress into adaptive responses, constitutively expressing this gene in transgenic plants may improve tolerance to osmotic stress at the physiological level. Here, transgenic rice plants containing *OsCam1-1* gene under the control of *35SCaMV* promoter (*35SCaMV-OsCam1-1*) were constructed by *Agrobacterium*-mediated transformation via pCAMBIA1301 plasmid and their characteristics were examined.

บทที่ 2 การสำรวจแนวความคิดและการวิจัยที่เกี่ยวข้อง (Survey of Related Literature)

Ca^{2+} ions play an essential role as a second messenger in eukaryotic cells. A large number of stimuli whose responses are mediated by Ca^{2+}-regulated signaling pathways have been reported. These stimuli include environmental signals produced by light, temperature shocks, mechanical perturbation and stress signals such as wounding, drought, cold, and salinity (Gilroy & Trewavas, 2001). These stimuli evoke transient increases in cytosolic Ca^{2+} concentrations, which are typically transmitted by protein sensors that bind Ca^{2+}. Three groups of signaling components that have been characterized as Ca^{2+} sensors in plants are calmodulin (CaM), calcineurin B-like protein (CBL), and Ca^{2+}-dependent protein kinase (CDPK). CaM is probably the most well characterized Ca^{2+} sensor among these groups of proteins. In the presence of Ca^{2+}, CaM functions by binding to and altering the activities of a variety of proteins. The activities of these proteins affect physiological responses to the vast array of specific stimuli received by plants (Zielinski, 1998).

CaM is a small (148 residues) multifunctional protein, of which primary structures are generally conserved throughout evolution. It possesses four EF-hand Ca^{2+}-binding motifs that are paired to form two globular domains. In the crystal structure, there is an unusual structure; a long, solvent exposed central α-helix acting as a flexible linker between the two globular domains (Babu et al, 1988). This flexible structure allows CaM to interact with peptides of different lengths and is the key to the mechanism of action of CaM on various target proteins (Ikura et al, 1991). In plants, the defining characteristic of CaM is the expression of multiple CaM isoforms. The broad significance of multiple CaM isoforms is not understood. However, a frequently proposed hypothesis is that diverged CaM isoforms may activate selected subsets of target proteins involved in Ca^{2+}-mediated signal transduction (Heo et al, 1999; Liao et al, 1996).

A large family of genes encoding CaM isoforms and their related proteins from plants has been identified. In Arabidopsis, McCormack and Braam (2003) have characterized members of Groups IV and V from the 250 EF-hand encoding genes identified in the Arabidopsis genome (Day et al, 2002). Six loci are defined as *Cam* genes and 50 additional genes are *CaM-like* (*CML*) genes, encoding proteins composed mostly of EF-hand Ca^{2+}-binding motifs. Recently, we have identified a large family of five *Oryza sativa* L. *Cam* (*OsCam*) genes and 32 *Oryza sativa* L. *CaM*-like (*OsCML*)

genes (Boonburapong & Buaboocha, 2007). *OsCam* genes encode proteins of which sequences share the highest degrees of amino acid sequence identity ($\geq$ 97%) to known typical CaMs from other plant species. Because of these high degrees of amino acid identity, they were classified as "true" CaMs that probably function as typical CaMs. They were named *OsCam1-1*, *OsCam1-2*, *OsCam1-3*, *OsCam2* and *OsCam3*.

OsCam1-1; *OsCam1-2* and *OsCam1-3* encode identical proteins, which we call OsCaM1, whereas *OsCam2* and *OsCam3* encode a protein of only two amino acid differences and their sequences share 98.7% identity with those of OsCaM1 proteins. Multiple sequence alignment of the OsCaM amino acid sequences with those of typical CaMs from other species shown in Figure 1 indicates their high degree of sequence conservation. Figure 1 delineates four regions of CaM, each encompassing an EF-hand Ca^{2+}-binding motif. By RNA blot analysis, *OsCam1-1* gene was shown to be rapidly (1 hour after stress) and highly increased in response to salt stress (0.15 M) (Figure 2) (Phean-o-pas et al, 2005). The expression level then slowly decreased after 2 and 4 hours after the treatment. This result indicates that this CaM isoform probably plays an important role in mediating salt stress response.

In soybean, two CaM divergent isoforms called SCaM4 and SCaM5 were reported to be induced within 30 min by fungal elicitor or pathogen (Heo, 1999). Constitutive expression of these CaM isoforms in transgenic tobacco plants induced an array of systemic acquired resistance (SAR)-associated genes and the transgenic plants exhibit enhanced resistance to a wide spectrum of virulent and avirulent pathogens. These results demonstrated that constitutively expressing a *Cam* gene in transgenic plants can successfully improve tolerance to stress that is transduced by that particular CaM isoform. In addition, transgenic plants have helped define *in planta* CaM target proteins or CaM-regulated pathways in conjunction with cellular functions of interest.

This approach has been used to investigate NAD kinase, the potential target enzyme of CaM by making transgenic plants expressing a mutant CaM that is incapable of methylation, and as a result it hyperactivates NAD kinase (Harding et al, 1997). Transgenic tobacco cells overexpressing this mutant CaM exhibited a stronger active oxygen burst, which is part of plant defense system; and a higher enhanced level of NADPH, a product of NAD kinase, than that in normal cells challenged with the same stimuli. These results showed that CaM is involved in plant defense response pathway and

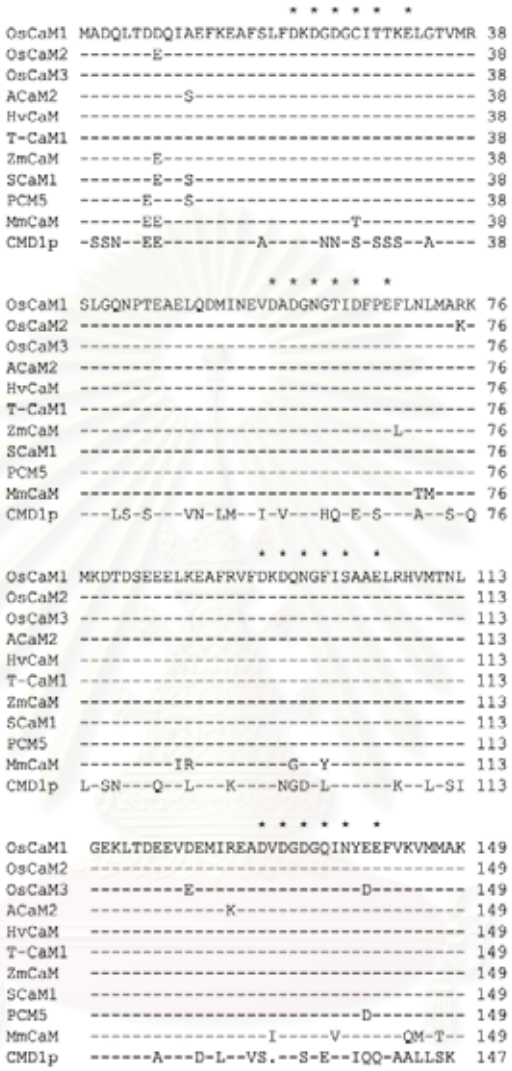

Figure 1 OsCaM protein sequence similarity with CaM from other species. Comparison of the deduced amino acid sequences of OsCaM1, 2, and 3 with those of CaMs from Arabidopsis (AtCaM2); barley (HvCaM); wheat (T-CaM); maize (ZmCaM), soybean (SCaM1); potato (PCM5); *Mus musculus* (MmCaM), and *Saccharomyces cerevisiae* (CMD1p). The sequences are compared with OsCaM1 as a standard; identical residues in other sequences are indicated by dash (-), and gaps introduced for alignment purposes are indicated by dot (.). Residues serving as Ca^{2+}-binding ligands are marked with asterisks (*).

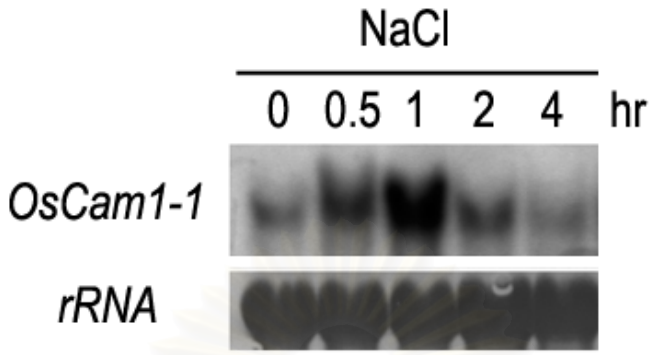

Figure 2 Expression pattern of *OsCam1-1* gene in response to salt stress. Five μg of total RNA isolated from seedlings treated with 0.15 M NaCl was separated by formaldehyde agarose gel electrophoresis and blotted onto positively charged nylon membranes. The blots were hybridized with probe made from an EST clone corresponding to the *Cam1-1* gene.

NAD kinase may be a target that modulates the ratio of NADH/NADPH and results in oxygen burst in response to environmental stresses (Harding et al, 1997). The effect of overexpression of CaM was also assessed in *Arabidopsis* upon the cold-induced expression of COR (Cold On Regulated) genes (Townley & Knight, 2002). Interestingly, the levels of three COR transcripts in the transgenic line were substantially reduced at 4 ^{o}C. This result suggested that CaM acts as a negative regulator with respect to COR gene expression in *planta*.

Recently, Bülow and colleagues investigated how calmodulin affects germination on media with high concentrations of NaCl by generating and characterizing transgenic tobacco plants expressing heterologous CaM (Olsson et al, 2004). Transgenic tobacco seeds showed significantly shortened germination times on media containing varying salt (120-160 mM) concentrations, compared to control seeds. In addition, the germinating transgenic seeds contained higher transient levels of γ–aminobutyric acid (GABA) compared to control seeds. It has been known that plant Ca^{2+}/calmodulin modulates the activity of glutamate decarboxylase, an enzyme catalyzing the conversion of glutamate to GABA, which has been reported to function as an osmolyte during early stages of water stress (Shelp et al, 1999). In transgenic seeds, the tobacco Ca^{2+}/calmodulin-regulated glutamate carboxylase may therefore be stimulated by the heterologous CaM. From all of the experiments mentioned above, transgenic plants overexpressing CaM have successfully been used to characterize downstream elements of transduction pathways mediating responses to plant stress as well as exhibit certain degree of stress tolerance.

บทที่ 3 วิธีการวิจัย (Procedure)

1. Construction of *Agrobacterium* vector

The *35SCaMV-GUS-Nos-poly A* cassette in pCAMBIA1301 (Figure 3) was subcloned into pGEM®-T Easy vector (Promega, USA) to use as a backbone for the overexpression cassette of *OsCam1-1*. cDNA clone of *OsCam1-1* (accession number AU081299) provided by DNA Bank of the National Institute of Agrobiological Science (Ibaraki, Japan) was sequenced at Bioservice Unit of the National Science and Technology Development Agency (Bangkok, Thailand). The nucleotide sequence and its deduced amino acid sequence are shown in Figure 4. Based on the cDNA sequence, a pair of primers 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, respectively. PCR product using the cDNA as a template was obtained and cloned into pGEM®-T Easy vector. The resulting recombinant plasmid was digested with *Nco*I and *Nhe*I restriction enzymes and the digested *OsCam1-1* coding sequence was subsequently subcloned to replace the *GUS*-coding sequence, previously cloned into pGEM®-T Easy vector. Then, the overexpression cassette, *35SCaMV- OsCam1-1 –Nos-poly A*, was transferred as a *Hin*dIII fragment into the polycloning site of pCAMBIA1301, digested with the same enzymes. The correct insertion of gene was clarified by agarose gel electrophoresis and DNA sequencing.

2. Rice callus induction

The indica rice (*Oryza sativa* L.) cultivar KDML105 seeds were obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). Rice seeds were dehusked and sterilized with 70 %(v/v) ethanol for two minutes and then with 2 %(w/v) sodium hypochlorite for 20 minutes. The seeds were rinsed three times with sterile water and placed on NB medium (Li et al, 1993) containing 2 mg/l 2,4-dinitrophenoxy acetic acid (2,4-D) and incubated in the dark at 28 °C for two weeks. Before transformation, the growing calli were subcultured on fresh medium and incubated in the same conditions for four days.

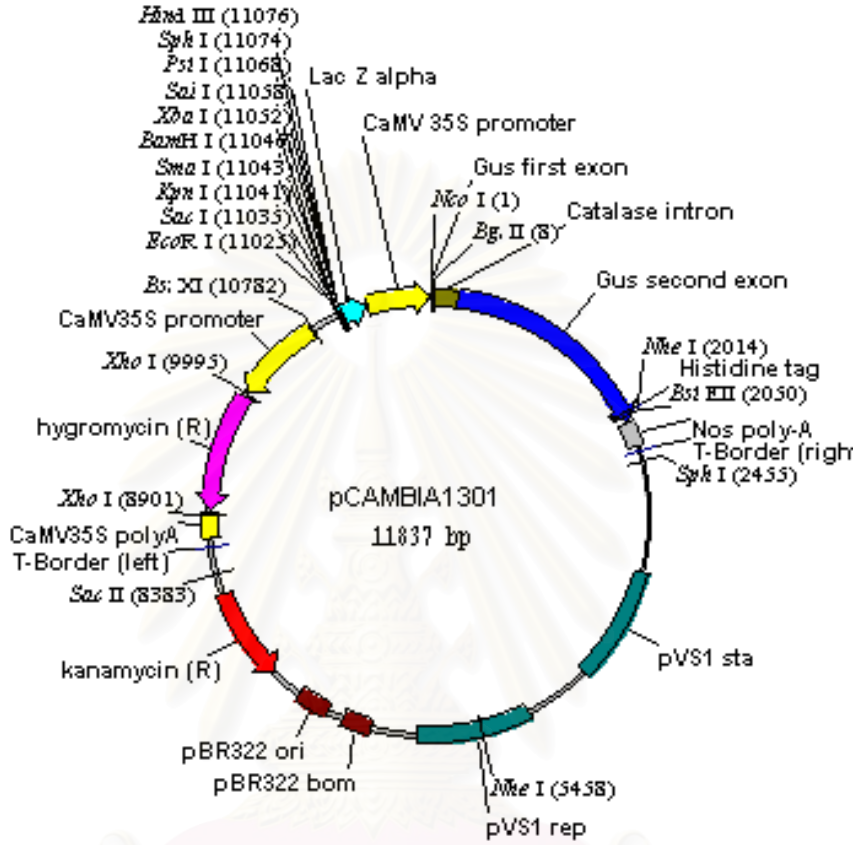

Figure 3 pCAMBIA1301 circle map (Center for the Application of Molecular Biology for International Agriculture, Canberra, Australia)

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5′
  CATTCTCTCCGCGACGGTCTCGTCTTCCCCACCCCTCGCCTCCTCGCGCGCTCGG
TGAGAGAAGCGAAGAAGAAGAAGAAGAGGAGGAGGAAGAAGCCAGGCTAAGCCCAGCGGC
ATGGCGGACCAGCTCACCGACGACCAGATCGCCGAGTTCAAGGAGGCCTTCAGCCTCTTC
 M  A  D  Q  L  T  D  D  Q  I  A  E  F  K  E  A  F  S  L  F
GACAAGGACGGCGATGGTTGCATCACAACCAAGGAGCTGGGAACCGTGATGCGTTCGCTG
 D  K  D  G  D  G  C  I  T  T  K  E  L  G  T  V  M  R  S  L
GGGCAGAACCCAACGGAGGCCGAGCTCCAGGACATGATCAACGAGGTCGACGCGGACGGC
 G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A  D  G
AACGGCACCATCGACTTCCCGGAGTTCCTCAACCTGATGGCACGCAAGATGAAGGACACC
 N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K  D  T
GACTCGGAGGAGGAGCTCAAGGAGGCGTTCAGGGTGTTCGACAAAGACCAGAACGGCTTC
 D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q  N  G  F
ATCTCCGCCGCCGAGCTCCGCCACGTCATGACCAACCTCGGCGAGAAGCTGACCGACGAG
 I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T  D  E
GAGGTCGACGAGATGATCCGCGAAGCCGACGTCGACGGTGACGGCCAGATCAACTACGAG
 E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q  I  N  Y  E
GAGTTCGTCAAGGTCATGATGGCCAAGTGAGGCACCACTTCCCCTGCCGATGATGGCATA
 E  F  V  K  V  M  M  A  K
GTACCCTGGGAGGAGGAAACCGTGCATTGCCGTATTAGTAAGGGGATGCAAACACTGGTT
TCAGTCGTCTTCCCTGATGAAGAAAACCGAACCGTACTAGTTGTAGTTGCTGAACATTTT
TCTATCTCTCCAGTCTCTCCGGTGTGCCATGGAACTTCTTGCTTGATTTTTCTGTGTGAA
TCTGTTAAGGCTTGCTCTGATCTCTCCGAAAAAAAAAAAAAAAAAAAA
                                                          3′
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Figure 4 cDNA and deduced amino acid sequences of *OsCam1-1*. The deduced amino acid sequence is shown with the Ca^{2+}-binding motifs underlined.

3. Preparation of *Agrobacterium* cells and co-cultivation of calli with *Agrobacterium*

The recombinant clone was introduced into *Agrobacterium* strain EHA105 by electroporation. When the embryonic calli were obtained, *A. tumefaciens* cells were streaked on solid AB medium (Jefferson et al, 1986) containing 25 μg/ml rifampicin and 50 μg/ml kanamycin. The cells were incubated at 28 °C for 2-3 days, collected by scraping with a loop and resuspended in AAM medium (Toriyama & Hinata, 1985) supplemented with 300 μM acetosyringone. The optical density at 600 nm of the bacterial suspension was adjusted to 1.0 by fresh medium. Embryonic calli were immersed in the bacterial suspension for 30 minutes with occasional shaking and blotted dry on sterile filter papers. The calli were then transferred to NB medium supplemented with 10 g/l glucose, 2 mg/l 2,4-dichlorophenyxy acetic acid (2,4-D) and 100 μM acetosyringone and incubated at 25°C for three days. To generate control plants, in a separate suspension, calli was also co-cultivated with *Agrobacterium* carrying pCAMBIA1301.

4. Selection and regeneration of transgenic plants

Calli were removed from the co-cultivation medium, washed with sterile 250 μg/ml cefotaxime to remove the excess *Agrobacterium*, followed by several sterile water rinses before blotted dry on sterile filter papers and then transferred to NB medium containing 250 μg/ml cefotaxime and 50 μg/ml hygromycin and incubated at 28 °C for 4 weeks. The hygromycin-resistant calli was subjected for another round of selection and then transferred to regeneration medium containing 4 mg/L of 6-benzylaminopurine (BAP). The culture was incubated at 28 °C under 16/8 hours of light/dark period for 3-4 weeks. When green shoots were developed, they were transferred to hormone-free regeneration medium to stimulate rooting and stem elongation for 4 weeks.

5. Analysis of putative transformants

5.1 Preliminary characterization of putative transformants

The binary plasmid pCAMBIA1301 carries the *β-glucuronidase* (*GUS*) (Chilton et al, 1974) reporter gene within the T-DNA, so all of the putative transgenic rice plants were examined for GUS activity using X-Gluc as a substrate. PCR amplification of the *35SCaMV-OsCam1-1* was used to confirm gene insertion in the putative transgenic lines.

A pair of primers was designed to include a part of the *35SCaMV* promoter so it would not amplify the endogenous *OsCam1-1* gene. PCR products were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The putative transgenic lines containing T-DNA without the *OsCam1-1* overexpression construct were confirmed for transformation with the same method as above except that amplification of the *GUS* gene under the control of the *35SCaMV* promoter (*35SCaMV-GUS*) was conducted rather than that of the *35SCaMV-OsCam1-1*.

5.2 *OsCam1-1* gene expression in the transgenic rice plants.

Comparison of *OsCam1-1* gene expression between the transgenic lines containing the *35SCaMV-OsCam1-1* and the control lines containing only the *35S CaMV-GUS* was done by RT-PCR and northern blot analysis. For RT-PCR, first strand cDNA was made from total RNA by M-MLV reverse transcriptase and used for PCR amplification using a pair of primers encompassing a region within the *OsCam1-1-Nos* terminator sequence. For northern blot analysis, 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 and used as a probe for hybridization. Hybridizing bands were visualized by autoradiography.

6. Growing conditions & growth determination

Germinated seeds were transferred to modified WP nutrient solution (Vajrabhaya & Vajrabhaya, 1991) for one week in a greenhouse under natural light (93-99 μmole photons $m^{-2}s^{-1}$) and a relative humidity of between 74-81%. The modified WP nutrient solution are composed of 580 mg/l KNO_3, 500 mg/l $CaSO_4$, 450 mg/l $MgSO_4.7H_2O$, 250 mg/l Triple super phosphate, 100 mg/l $(NH_4)_2SO_4$, 160 mg/l Na_2EDTA, 120 mg/l $FeSO_4.7H_2O$, 15 mg/l $MnSO_4.H_2O$, 5 mg/l H_3BO_3, 1.5 mg/l $ZnSO_4.7H_2O$, 1 mg/l KI, 0.1 mg/l $Na_2MoO_4.2H_2O$, 0.05 mg/l $CuSO_4.5H_2O$, and 0.05 mg/l $CoCl_2.6H_2O$. The level of nutrient solution was controlled via daily addition of carbon filtered water to replace that which had evaporated. The salt stress treatment was performed by addition of 0.5% NaCl to the WP nutrient solution. Shoot and root dry weights were determined

on day 0, 1, 3, 5, 7, 9, and 11 after treatment. Seedlings grown in the nutrient solution without NaCl was used as controls.

7. 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 in a mortar in extraction solution (80% methanol containing 0.1 mg ml^{-1} butylated hydroxyl-toluene and 0.5 mg ml^{-1} citric acid monohydrate) at the ratio of 1g fresh weight tissue: 10 ml extraction solution. The extract was shaken in dark for 16 hr and then centrifuged at 4800 x g at 4 ^{o}C for 15 min. The supernatant was evaporated to dryness using rotary evaporator. Then, the dried pellet was resuspended with 1 ml 100% methanol and ABA was quantified using HPLC (Agilent Technologies Series 1100). HPLC instrument equipped with a UV absorbance detector operating at 254 nm was used. An aliquot (50 µl) of each sample was loaded onto a 250 mm x 4 mm column packed with 5 µm ODS Hypersil (Shandon Runcorn, UK), and HPLC was performed using a gradient solvent system of methanol and water with 0.05 M acetic acid (30% methanol for 6 min, 30 to 50% linear gradient of methanol over 20 min, 50% methanol for 6 min and 50 to 100% methanol over 15 min) at a flow rate of 1.0 ml/min. ABA content is expressed on the basis of dry weight. (±) Cis-trans ABA (Sigma) was used as a standard. Data were reported as mean SD of three repetitions.

8. Statistical analysis

Rice seedlings were grown in completely randomized design (CRD). The comparison of growth by shoot and root dry weight, and 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).

บทที่ 4 ผลการทดลอง (Results)

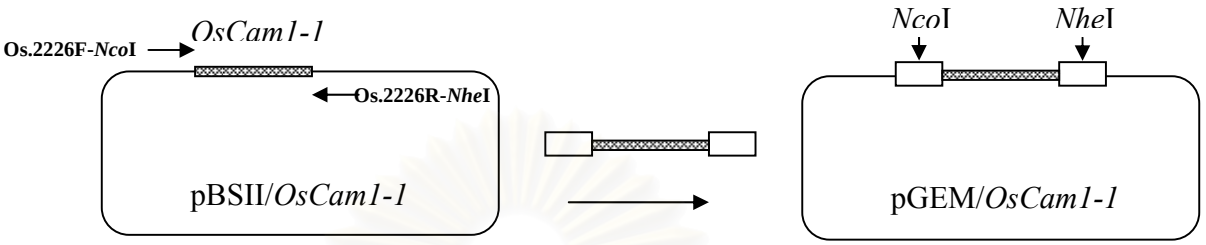
1. Construction of *Agrobacterium* vector

To construct an *Agrobacterium* plasmid containing *OsCam1-1* gene under the control of *35SCaMV* promoter, our strategy was to amplify the *35SCaMV*/*GUS* cassette using pCAMBIA1301 as template, subclone it into pGEM-T and replace the *GUS* gene with the *OsCam1-1* coding region. The *35SCaMV*/*OsCam1-1* cassette would then be subcloned back into pCAMBIA1301 plasmid at the multiple cloning site using *Hin*dIII. This strategy would allow us to use the *Nhe*I restriction site adjacent to the His tag sequence at the 3′ end to encode an OsCaM1-1/His tag fusion protein for easier detection in plants. As the first step, an *Nco*I restriction site was engineered at the start codon and an *Nhe*I restriction site next to the stop codon according to step I in the four-step strategy outlined in Figure 5.

In step II, the *35SCaMV* promoter taken from pCAMBIA1301 as a cassette of the *35SCaMV*/*GUS* sequence by PCR amplification was cloned into pGEM-T cloning vector. The construct referred as pGEM/*P35S*/*GUS* was confirmed by restriction digestion as shown in Figure 6. In step III, the *GUS* sequence was replaced with the *OsCam1-1* gene which the *Nco*I and *Nhe*I restriction sites were previously engineered into. However, pGEM-T has an *Nco*I restriction site, to cut out the *GUS* sequence pGEM/*P35S*/*GUS* needed to be partially digested with *Nco*I after a complete digestion with *Nhe*I. The resulting products are shown in Figure 7 in which the desired fragment was purified for further manipulation.

At the same time, pGEM/*OsCam1-1* was digested with the same enzymes *Nco*I and *Nhe*I. Both of the desired fragments purified from agarose gels were ligated and their products were transformed into *E. coli* XL1 Blue. Several clones referred as pGEM/*P35S*/*OsCam1-1* were obtained and verified by *Nco*I/*Nhe*I double digestion as shown in Figure 8. After the *35SCaMV*/ *OsCam1-1* cassette was constructed within pGEM-T, it was then transferred into pCAMBIA1301 (step IV) at the multiple cloning site using *Hin*dIII in which its sites were previously engineered at both ends of the *35SCaMV/GUS* cassette by PCR amplification. In this step, both pGEM/*P35S*/*OsCam1-1* and pCAMBIA1301 were digested with *Hin*dIII and the desired fragments purified from agarose

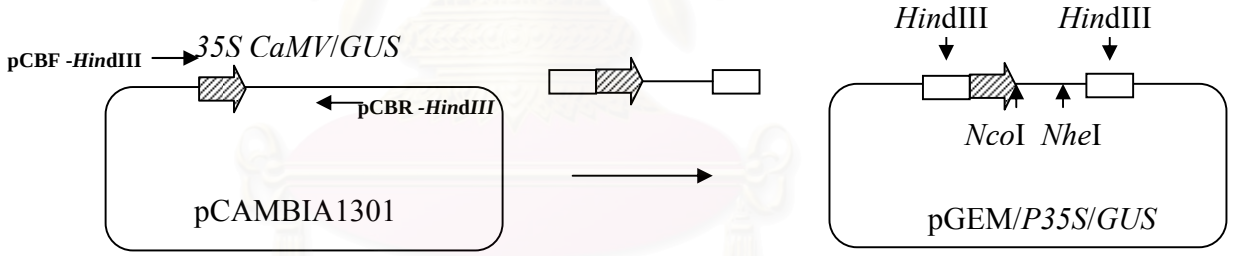
I. Engineering the *Nco*I and *Nhe*I restriction sites into the *OsCam1-1-1* coding region



PCR amplification of *OsCam1-1* gene using the *OsCam1-1* cDNA clone as a template

Cloning of the PCR product into pGEM-T

II. Cloning of the *35SCaMV*/*GUS* cassette into pGEM-T



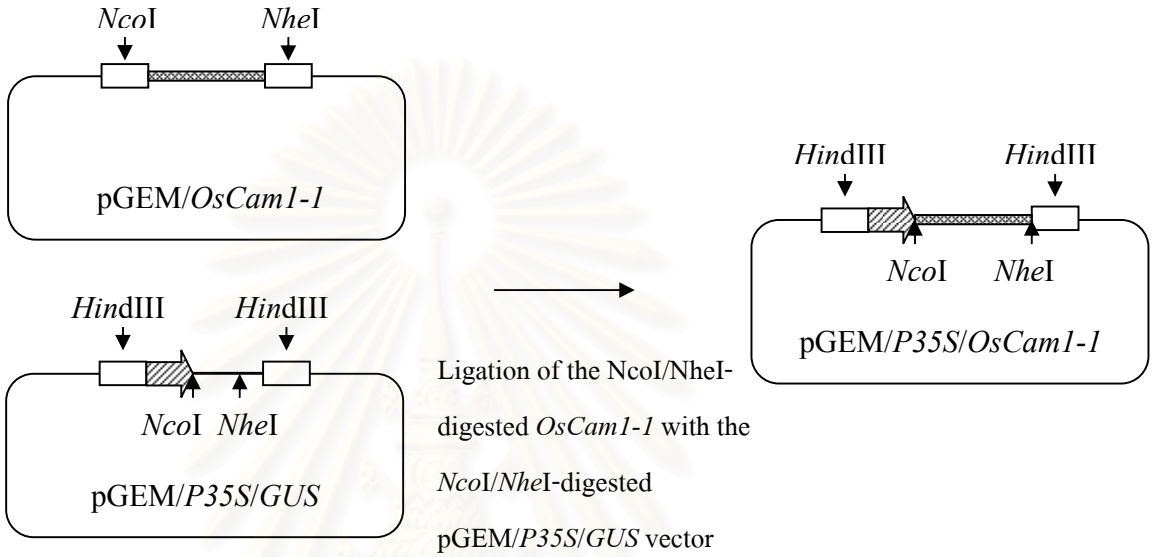
PCR amplification of the *35SCaMV*/*GUS* cassette using pCAMBIA1301 as a template

Cloning of the PCR product into pGEM-T

Figure 5 Four-step strategy for constructing a plant plasmid encoding OsCaM1-1/His tag fusion protein under the control of *35SCaMV* promoter**.**

Figure 5 (continued)

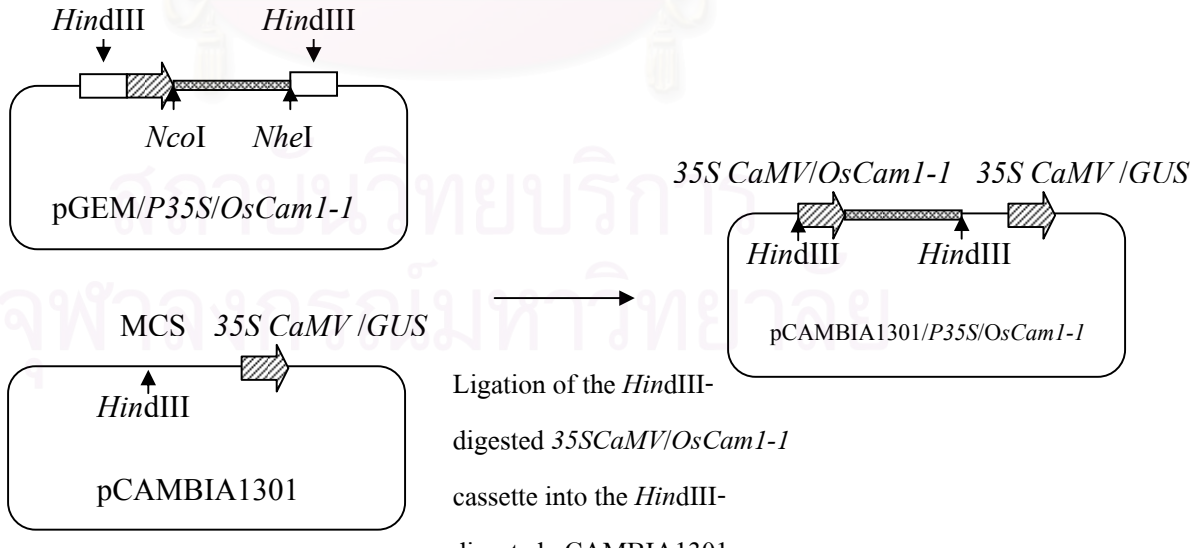
III. Replacing the *GUS* gene with the *OsCam1-1* gene



Digestion of pGEM/*OsCam1-1* and pGEM/*P35S*/*GUS* with *Nco*I and *Nhe*I

Ligation of the NcoI/NheI-digested *OsCam1-1* with the *Nco*I/*Nhe*I-digested pGEM/*P35S*/*GUS* vector

IV. Cloning the *35SCaMV*/*OsCam1-1* cassette into the multiple cloning site of pCAMBIA1301



Digestion of pGEM/*P35S*/*OsCam1* -1 and pCAMBIA1301 with *Hin*dIII

Ligation of the *Hin*dIII-digested *35SCaMV*/*OsCam1-1* cassette into the *Hin*dIII-digested pCAMBIA1301

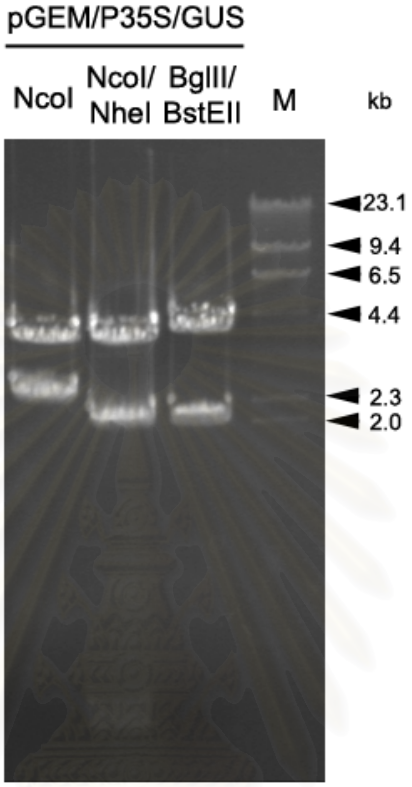


Figure 6 The *35SCaMV/GUS* cassette was cloned into pGEM-T. The construct was verified by digestion with restriction enzymes indicated. Lane M: λ/*Hin*dIII standard marker

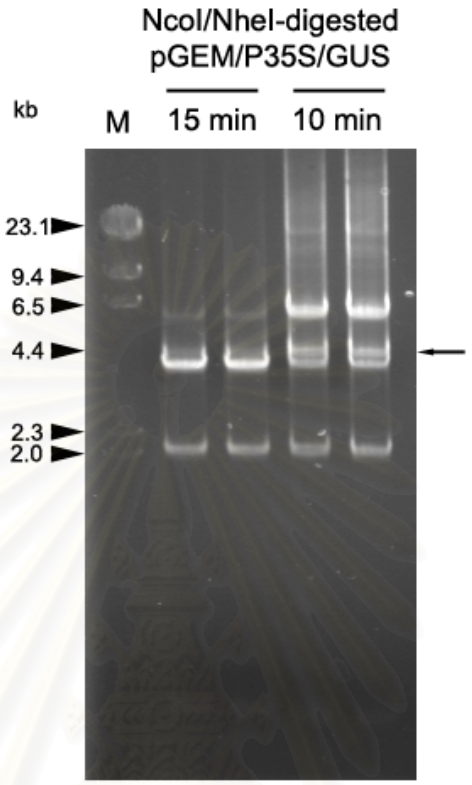


Figure 7 Preparation of pGEM/*35SCaMV* for constructing a *35SCaMV* /*OsCam1-1* cassette. The pGEM/*P35S*/*GUS* plasmid was digested with *Nhe*I and partially digested with *Nco*I. Times of partial digestion were indicated above the lanes. The bands indicated by the arrow were purified and used in step III ligation. Lane M: λ/*Hin*dIII standard marker.

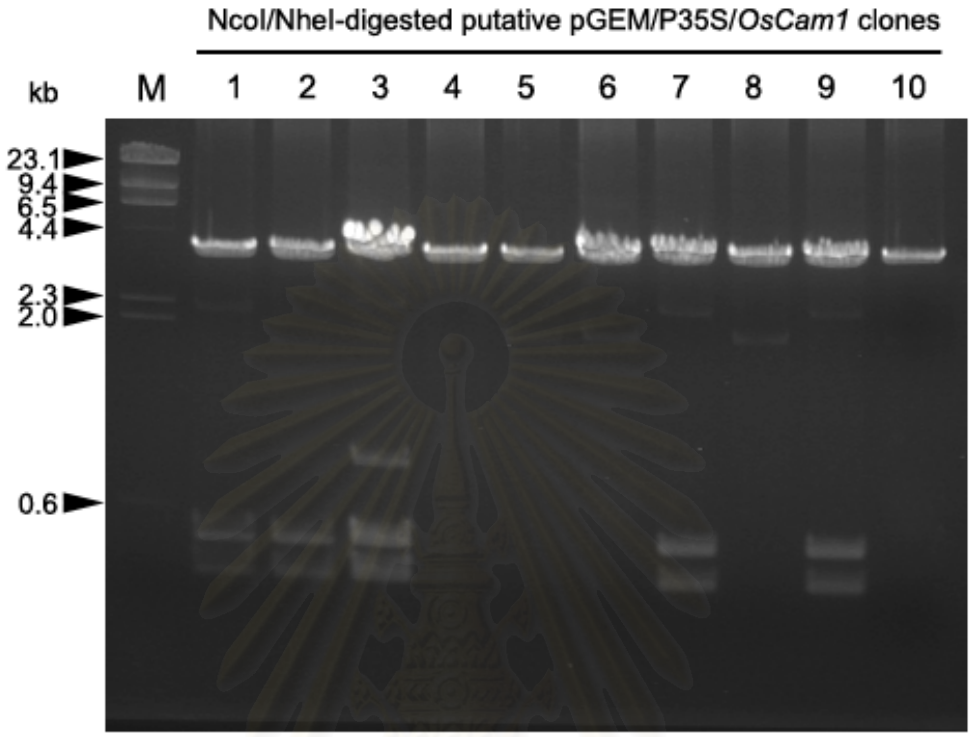


Figure 8 Construction of *35SCaMV/OsCam1-1* gene cassette in pGEM-T. Ten putative clones were doubly digested with *Nco*I and *Nhe*I restriction enzymes. Clones numbers 1, 2, 7, and 9 gave the expected pattern of bands and would be used. Lane M: λ/*Hin*dIII standard marker

gels are shown in Figure 9. The *35SCaMV*/*OsCam1-1* cassette and pCAMBIA1301 were then ligated and transformed into *E. coli* XL1 Blue. A construct of the *35SCaMV*/*OsCam1-1* cassette cloned onto pCAMBIA1301 referred as pCAMBIA1301/*P35S*/*OsCam1-1* was verified by restriction digestion and DNA sequencing in which its results are shown in Figures 10 and 11, respectively.

2. Production of transgenic rice lines containing *35SCaMV-OsCam1-1* gene

After dehusked and surface-sterilized rice seed was planted on callus induction medium supplemented with 2 mg/l of 2,4-D and incubated in the dark at 28 °C for 6 weeks, compact yellowish embryonic calli were obtained. They were separated and subcultured on fresh medium before transformation. The prepared rice calli were transformed with pCAMBIA1301/P35S/*OsCam1-1* or pCAMBIA1301 which would be used as a negative control by *Agrobacterium*-mediated transformation. After the calli were selected in medium containing 50 mg/l hygromycin for 4 weeks, most parts of the calli appeared blackened but there were clusters of yellow cells which were potential transformants. Photographs of hygromycin-resistant calli after 4 and 8 weeks on selection medium are shown in Figure 12. These hygromycin-resistant cells were further selected on the selection medium and used for regeneration.

For regeneration, the hygromycin-resistant cells were transferred to medium containing 4 mg/l BAP. After 4 weeks of culture, green spots and shoots of the resistant calli were observed (Figure 13a). When the shoots were 3.0 to 5.0 cm in height (Figure 13b), they were cut from the calli and subcultured on NB medium without any plant hormone. Healthy plantlets with extensive root system were established on NB medium after 2-3 weeks (Figure 13c). A total of three putative transgenic lines harboring the *35SCaMV-OsCam1-1* and two putative control lines (transformed with pCAMBIA1301 alone) were obtained from regeneration of the hygromycin-resistant calli. No difference was observed in the morphology between the transformed and untransformed rice.

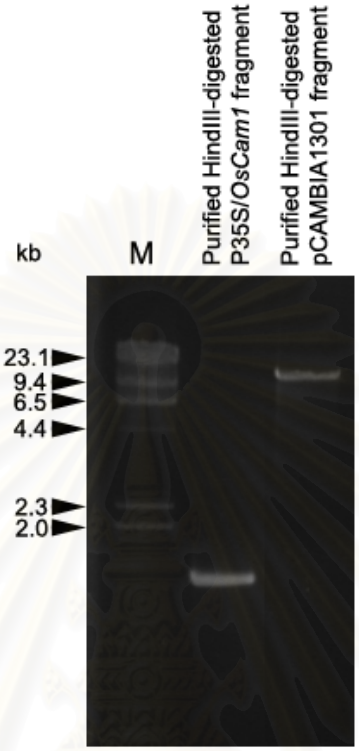


Figure 9 Preparation of DNA fragments for constructing pCAMBIA1301 harboring the *35SCaMV*/*OsCam1-1* cassette. Lane M: λ/*Hin*dIII standard marker.

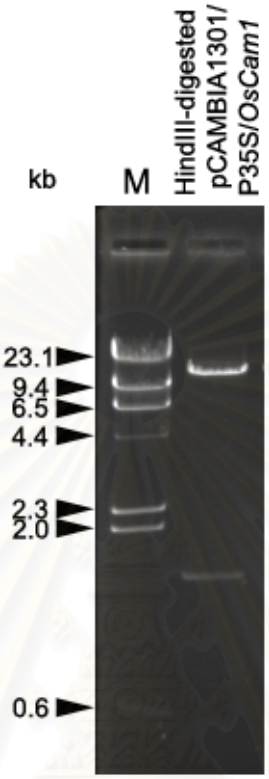


Figure 10 Verification of a putative pCAMBIA1301/*P35S*/*OsCam1-1* by digestion with *Hind*III.

Lane M: λ/*Hin*dIII standard marker

Figure 11 Insertion of the *OsCam1-1* gene into pCAMBIA1301 was verified by DNA sequencing. The *Nco*I and *Nhe*I restriction sequences are highlighted by bold letter. The deduced amino acid sequence of the fusion protein is shown under their corresponding codons.

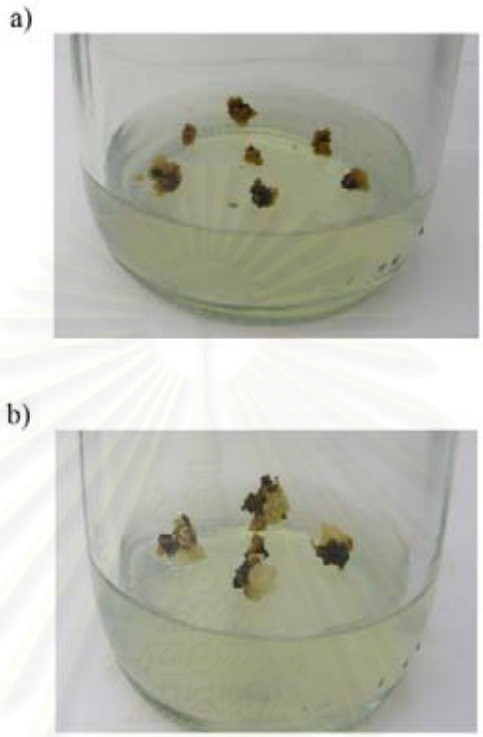


Figure 12 Selection of rice calli on selection medium. a) Brown zones of non-transformed cells were observed after 4 weeks of selection. b) Resistant calli were grown after additional 4 weeks on selection medium.

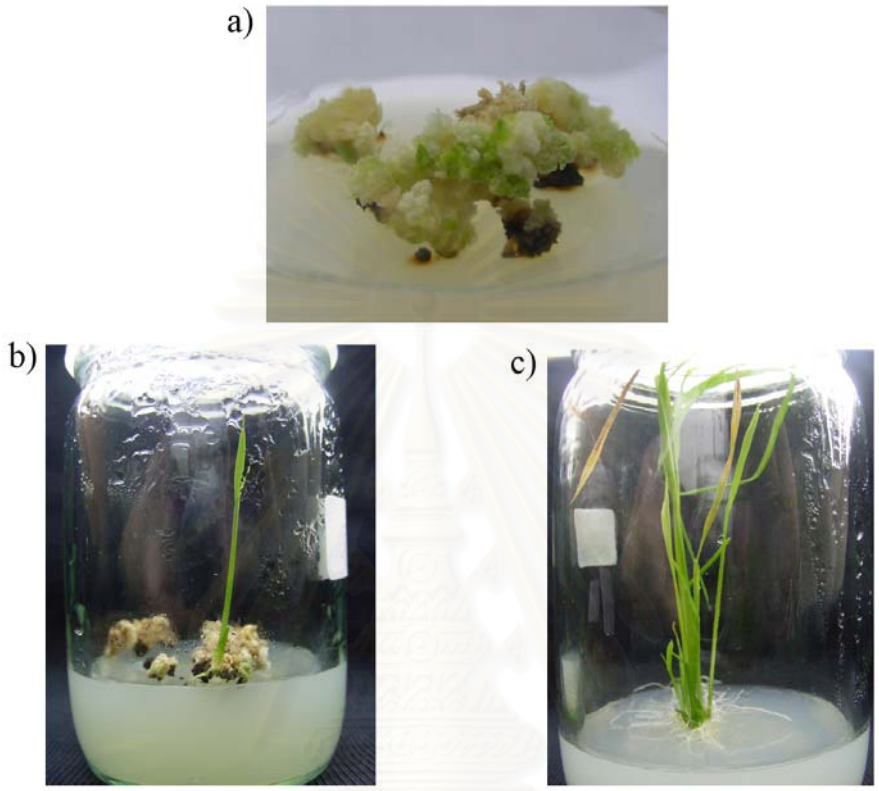


Figure 13 Regeneration of transformed rice calli. a) Green spots appeared within 4 weeks. b) Shoot formation after 6 weeks. c) Plantlet with root system.

3. Histochemical analysis of GUS expression in putative transformants

The binary plasmid pCAMBIA1301 carries the *β-glucuronidase* (*GUS*) reporter gene within the T-DNA. This reporter gene provides an indication that genetic transformation takes place. Putative transgenic rice plants transformed with the binary plasmid pCAMBIA1301 with the inserted *35SCaMV-OsCam1-1* gene or the pCAMBIA1301 alone were subjected to analysis for GUS activity. Different organs of rice tissues were collected and submerged in GUS staining solution. All lines of the putative transgenic rice plants tested positive for GUS. The GUS activity was detected as intense blue staining in calli, leaves and roots from the transgenic plants. The intensity of blue color production was different from plant to plant. No GUS enzyme activity was observed in the tissues from non-transformed plants. Examples of some analyzed tissues are shown in Figure 14.

4. Molecular analysis of the transgenic plants

4.1 PCR analysis of the transgenic plants

To confirm the integration of *OsCam1-1* gene into the rice genomes, the transgenic plants were screened by PCR amplification. First, genomic DNA was isolated from leaves of the transgenic rice plants using a modified CTAB extraction method. The *35SCaMV-OsCam1-1* was expected to give a PCR product of 567 bp using the primers mentioned in the "Procedure" section. In addition, a second pair of primers designed to amplify part of the *35SCaMV-GUS* would give rise to a PCR product of 983 bp. The result shows that both 983 and 567 bp fragments (arrows) were detected in all rice transgenic lines harboring the *35SCaMV-OsCam1-1* (Figure 15). Even though non-specific products were observed from both pairs of primers (dashed arrows), much stronger intensity was obtained from the specific products. In the case of control rice plants transformed with pCAMBIA1301 alone, the specific 983 bp band of the *35SCaMV-GUS* was detected but no 567 bp band from the *35SCaMV-OsCam1-1* was obtained.

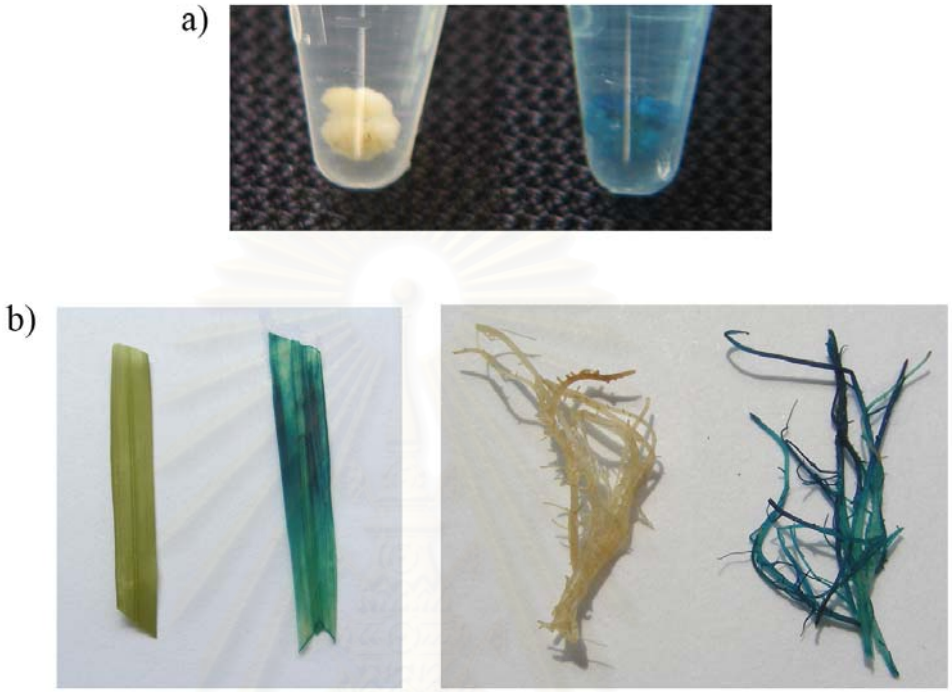


Figure 14 Histochemical analysis of GUS activity in representative transgenic rice. Non-transformed rice was used for comparison (left on each panel). a) Hygromycin-resistant calli after 8 weeks on selection medium. b) Leaf (left panel) and root (right panel) of the non-transformed and transformed rice plants.

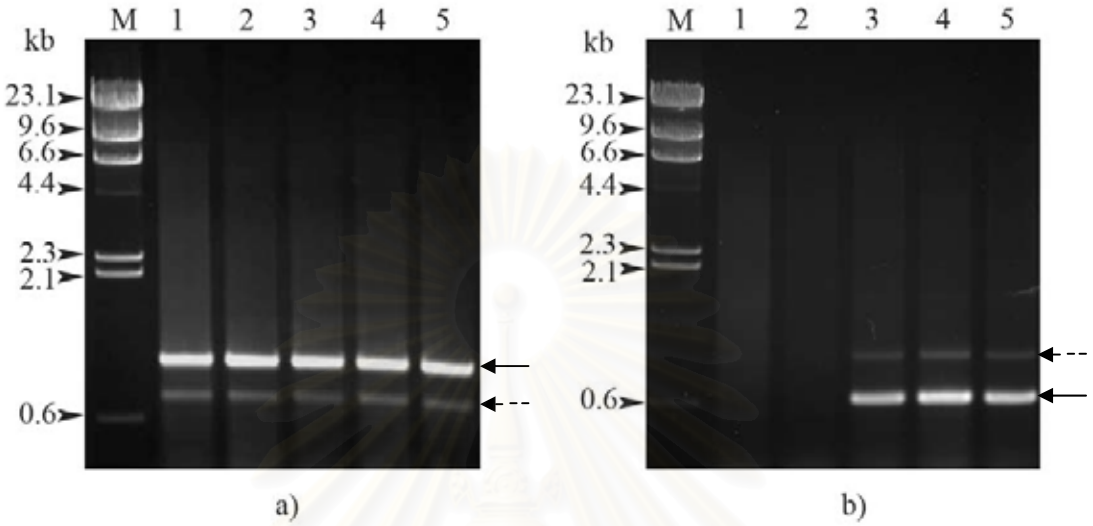


Figure 15 PCR analysis of *GUS* gene and *OsCam1-1* gene insertion into the genomes of the transgenic rice plants. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. Lane M: λ/*Hin*dIII standard marker; lanes 1 and 2: control plants transformed with pCAMBIA1301 alone without *OsCam1-1* gene lines 1 and 2, respectively; and lanes 3, 4 and 5: transgenic rice plants harboring the *35SCaMV-OsCam1-1* lines 1, 2 and 3 respectively. a) Amplification of the *35SCaMV-GUS*. b) Amplification of the *35SCaMV-OsCam1-1*.

4.2 Detection of the *OsCam1-1* mRNA in the transgenic plants by RT-PCR

To determine whether the *OsCam1-1* gene introduced into the transgenic rice was expressed, total RNA was isolated from leaves of all transgenic rice plants and used to perform RT-PCR using primers specific for the inserted *OsCam1-1-nos* region. Amplification of actin transcripts was performed in parallel to ensure the integrity of total RNA. Figure 16 shows that a band of 574 bp as expected from the *OsCam1-1* gene including a partial *Nos* terminator sequence was detected in all transgenic lines except the control transgenic plants transformed with pCAMBIA1301 alone. A control of RT-PCR reaction without adding reverse transcriptase was done in parallel with each experimental reaction using total RNA to ensure that the product obtained could be attributed to the product of the reverse transcriptase reaction. No band was detected in the control RT-PCR reactions. It can be concluded that the inserted *OsCam1-1* gene of the transgenic rice is expressed at the mRNA level.

4.3 Expression level of *OsCam1-1* in transgenic rice plants

To compare the *OsCam1-1* expression level among the transgenic rice plants harboring the *OsCam1-1* gene under the control of the *35SCaMV* promoter, control transgenic plants harboring T-DNA without the *OsCam1-1* gene and wild type plants, northern blot analysis was performed. Total RNA was isolated from leaves, fractionated on formaldehyde agarose gel, and transferred to positively charge nylon membrane. To make a probe, DNA fragments derived from the *OsCam1-1* gene were labeled using [α-^{32}P]dCTP. The blot was incubated with the probe at 40 ^{o}C for 16 hours, washed in 2X SSPE, 0.1% SDS twice and then once in 1X SSPE, 0.1% SDS at room temperature. As a result, positive hybridizing bands were detected by autoradiography. Figure 17 shows high transcript levels of *OsCam1-1* gene in the transgenic rice plants harboring the *35SCaMV-OsCam1-1* compared to those of the wild type plants and the control transgenic plants grown under normal conditions. These results indicate that the *OsCam1-1* gene under the control of the *35SCaMV* promoter is overexpressed in all of the transgenic rice plants harboring the gene construct.

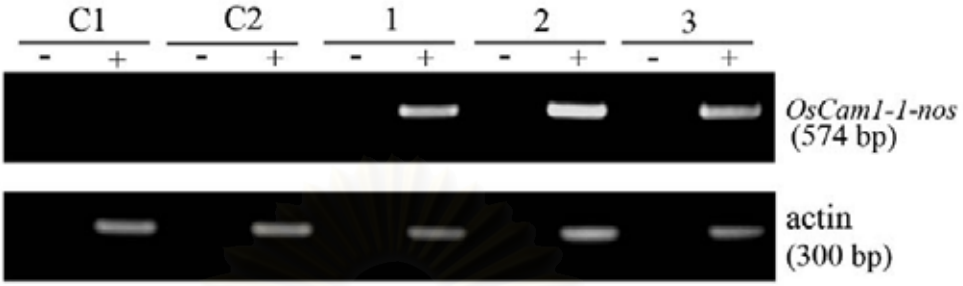


Figure 16 Expression of the *OsCam1-1* gene in transgenic plants by RT-PCR. The total RNA was used in RT-PCR assays either without (-) or with (+) the addition of M-MLV reverse transcriptase. The cDNAs were amplified by PCR using gene specific primers for part of the inserted *OsCam1-1-Nos* region (upper panel) or part of the actin transcript (lower panel). The products were separated in agarose gels and visualized by ethidium bromide staining. Lane C1 and C2: RT-PCR products of control transgenic rice plants lines 1 and 2, respectively; lane 1, 2 and 3: RT-PCR products of transgenic rice plants harboring the *35SCaMV-OsCam1-1* lines 1, 2 and 3, respectively.

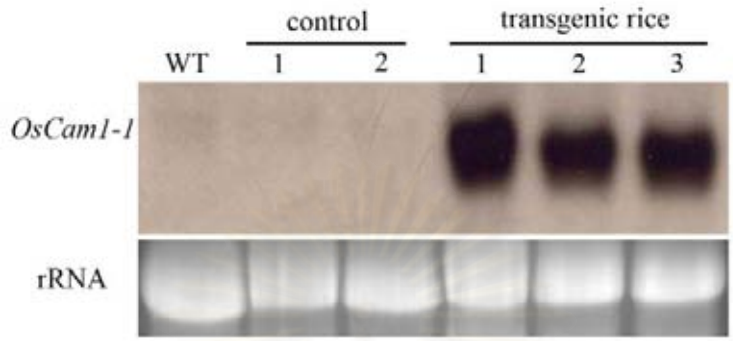


Figure 17 RNA blot analysis of the *OsCam1-1*-overexpressing plants. *OsCam1-1* gene expression of wild type plants (lane WT), control transgenic (lane control) and transgenic rice plants harboring the *35SCaMV-OsCam1-1* (lane transgenic rice) grown under normal conditions was determined. Transgenic line numbers are indicated accordingly. Each lane was loaded with 40 μg of total RNA isolated from leaves. RNA was analyzed by gel blot hybridization with a denatured ^{32}P-oligolabeled *OsCam1-1* probe. An ethidium bromide-stained formaldehyde agarose gel of each RNA sample is shown under its corresponding lane in the autoradiograph.

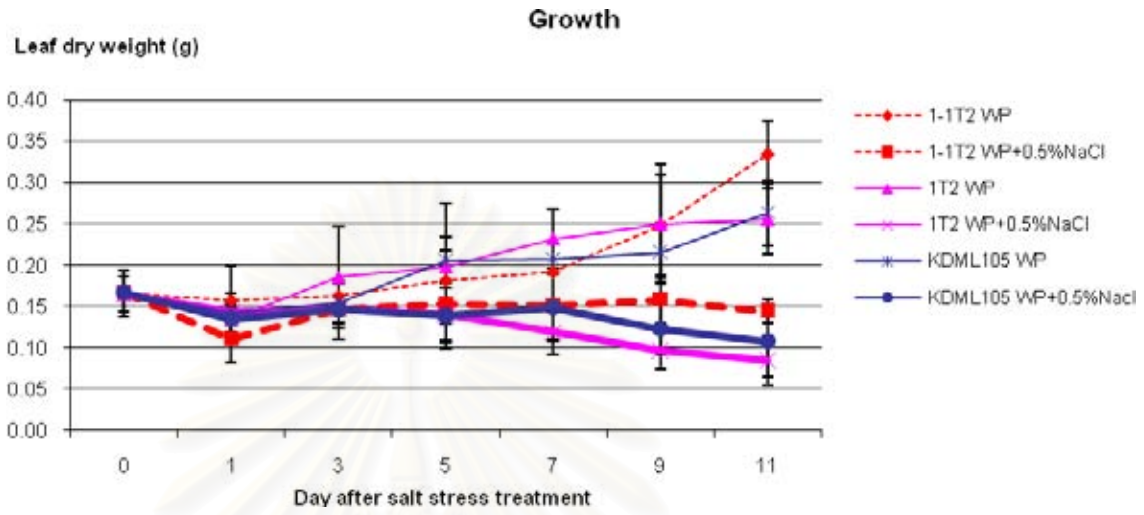
5. Growth of *OsCam1-1*-overexpressing plants

Growth of transgenic rice plants overexpressing the *OsCam1-1* gene under the control of *35SCaMV* promoter was determined. Shoot and root dry weights were measured from plants grown either in normal growing conditions (WP) or under salt stress (WP + 0.5% NaCl). Growth of the control transgenic rice plants that harbor the T-DNA alone without the inserted gene as well as that of wild-type KDML105 plants were determined for comparison. Figure 18 shows growth of all plants determined on day 0, 1, 3, 5, 7, 9, and 11 after treatment in gram dry weight of leaf (Figure 18a) or root (Figure 18b). When growing in normal conditions, the *OsCam1-1*-overexpressing plants have shown higher growth rates than those of the control transgenic and wild-type plants. Specifically, the *OsCam1-1*-overexpressing plants exhibited the trend of better growth during the latter part of growth determination (at day 11 for leaf dry weight and day 9 and 11 for root dry weight). Under salt stress, while all control transgenic and wild-type plants exhibited the decrease in leaf and root dry weights, the *OsCam1-1*-overexpressing plants maintained their leaf weights and had increased root dry weights during the periods of growth determination.

6. ABA contents of *OsCam1-1*-overexpressing plants

ABA was extracted and purified from transgenic rice plants overexpressing the *OsCam1-1* gene as well as from the control transgenic rice plants that harbor the T-DNA alone without the inserted gene and wild-type KDML105 plants for comparison. ABA contents were determined from plants grown either in normal growing conditions (WP) or under salt stress (WP + 0.5% NaCl) and expressed on the basis of dry weight. For the whole period of the experiments, ABA content was found to be significantly higher in the *OsCam1-1*-overexpressing rice plants when grown in normal conditions or under salt stress as shown in Figure 19. Under salt stress, ABA content (Figure 19b) of the *OsCam1-1*-overexpressing rice plants as well as the control transgenic and wild-type plants from day 5 after treatment onwards had a tendency to get higher than that of plants grown in normal conditions (Figure 19a).

(a)



(b)

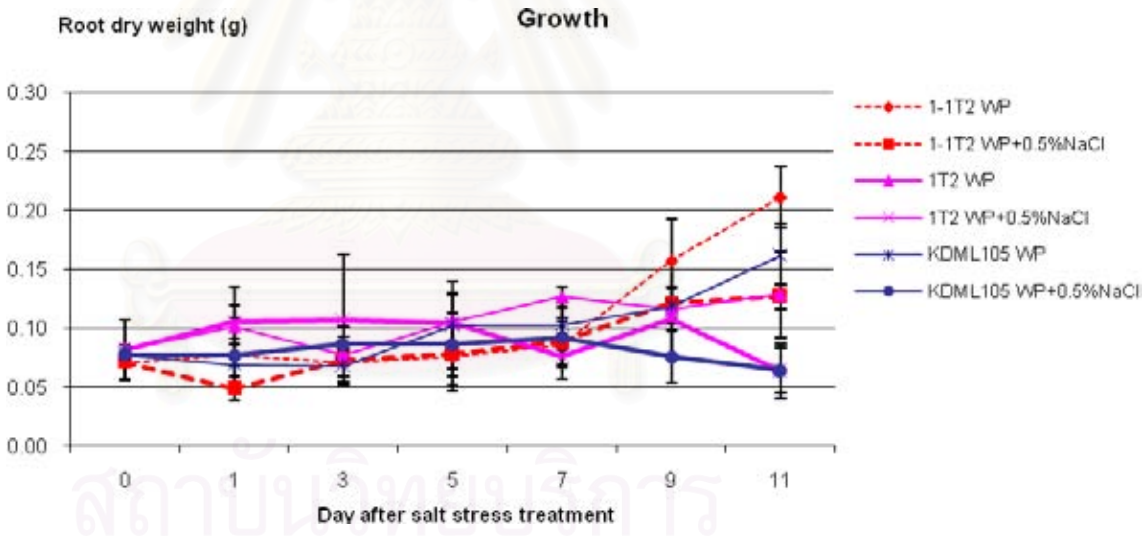
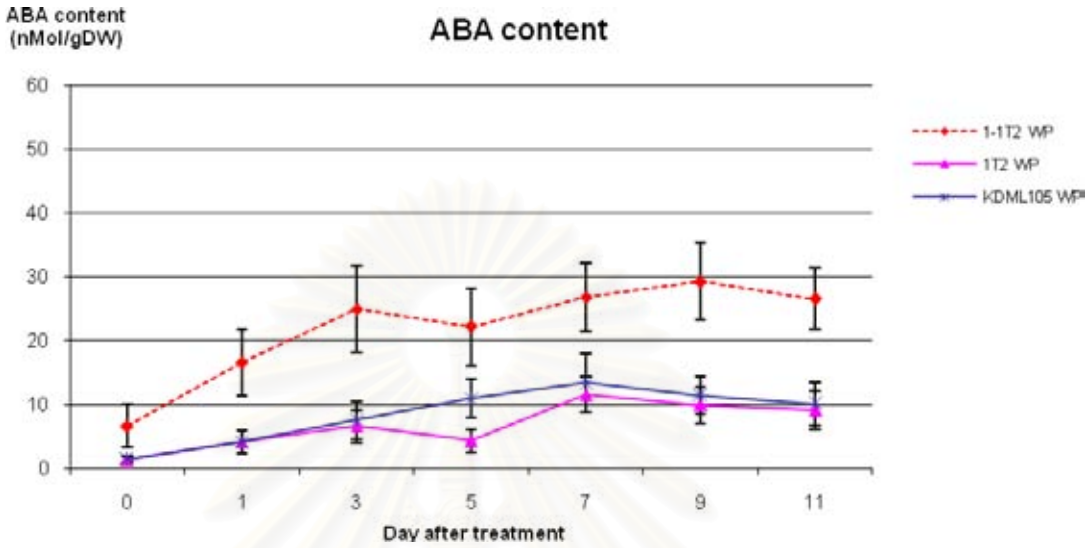


Figure 18 Growth of the *OsCam1-1*-overexpressing plants. Growth of the transgenic plants overexpressing *OsCam1-1* (1-1T2) was determined by dry weights of leaf (a) and root (b) compared with those of the control transgenic (1T2) and wild-type (KDML105) plants. Leaf and root dry weights were determined from plants grown either in normal growing conditions (WP) or under salt stress (WP + 0.5% NaCl) in experiments using the completely randomized design with three replicates.

(a)



(b)

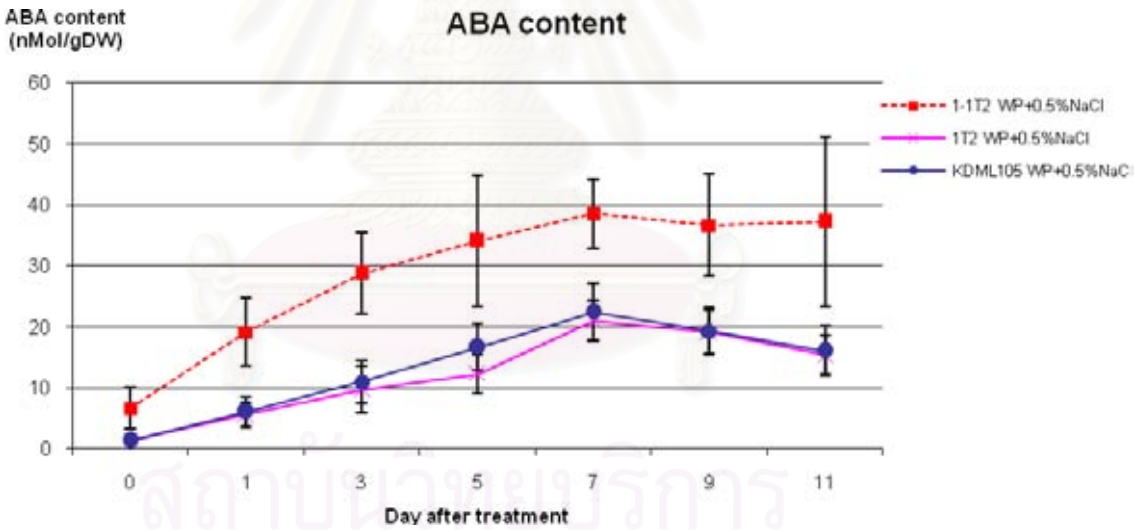


Figure 19 ABA content of the *OsCam1-1*-overexpressing plants. ABA content of the transgenic plants overexpressing *OsCam1-1* (1-1T2) is expressed on the basis of dry weight (DW) compared with those of the control transgenic (1T2) and wild-type (KDML105) plants. ABA content was determined in plants grown in normal conditions (WP) (a) or under salt stress (WP+0.5% NaCl) (b) in experiments using the completely randomized design with three replicates.

บทที่ 5 การอภิปราย (Discussion)

Three putative transgenic rice lines harboring the *35SCaMV-OsCam1-1* and two putative transgenic lines harboring the T-DNA from pCAMBIA1301 alone as negative controls have been produced. Because all constructs contained a *GUS* reporter gene, success of transformation in all transgenic lines was clearly confirmed by GUS activity staining (Figure 14). In addition, integration of the *OsCam1-1* gene into the rice genomes of all *OsCam1-1*-overexpressing transgenic lines was verified by PCR amplification (Figure 15). The results of PCR analysis clearly confirm that all transgenic plants harboring the *35SCaMV-OsCam1-1* with positive histochemical assay of the *GUS* reporter gene contain the *OsCam1-1* transgene in their genomes. It appears that introduction of the gene construct does not alter morphological features of all transgenic lines obtained.

In order to verify expression of the inserted gene, RT-PCR using specific primers encompassing a region within the *OsCam1-1-Nos* terminator sequence which cannot amplify the endogenous *OsCam1-1* gene. As a result, only the *OsCam1-1*-overexpressing lines were shown to produce mRNA resulting from the introduced *OsCam1-1* gene construct (Figure 16). To compare the *OsCam1-1* expression level among the transgenic lines, northern blot analysis was conducted using an *OsCam1-1*-specific probe. Figure 17 shows that bands of the expected size were obtained from all transgenic plant samples, however the *OsCam1-1*-overexpressing plants exhibited bands with much higher intensity than those of the control transgenic and the wild-type KDML105 plants indicating that expression of the *OsCam1-1* gene was highly increased in the transgenic lines harboring the *35SCaMV-OsCam1-1* construct.

To examine the effect of the *OsCam1-1* overexpression in the transgenic rice, growth of the *OsCam1-1*-overexpressing plants was determined. Figure 18a shows that the *OsCam1-1*-overexpressing plants exhibited the growth rate higher than that of the control plants in normal growing conditions. In addition, growth rate of the *OsCam1-1*-overexpressing plants was found to be maintained better than that of the control plants when grown under salt stress (Figure 18b). These results indicate that overexpression of the *OsCam1-1* gene helps improve growth and salt stress tolerance. Previous report has shown that expression of *OsCam1-1* is highly induced in response to salt stress (Phean-o-pas et al, 2005). Taken together, these results suggest that *OsCam1-1* functions to

transduce calcium signals from salt stress into adaptive responses that help increase salt stress resistance.

Finally, *OsCam1-1*-overexpressing plants were shown to contain higher levels of ABA than the control plants when grown in normal conditions or under salt stress (Figure 19). ABA is an important stress hormone that functions to mediate plant responses to drought and salt stress. Increased level of ABA may provide the transgenic rice with faster responses to fluctuations in its surroundings both under normal growing conditions and under salt stress. These results indicate that OsCaM1-1 acts to increase the level of ABA in rice, probably via the increase in ABA biosynthesis activity. ABA biosynthetic genes have been shown to be activated by abiotic stresses such as drought and salt stress via calcium signals (Xiong & Zhu, 2003), therefore OsCaM1-1 may function as an intracellular sensor to mediate responses via the Ca^{2+} signals triggered by salt stress. Since expression of *OsCam1-1* is rapidly and highly induced in response to salt stress, ABA of which content is increased by OsCaM1-1 activity, may act as signals in downstream pathways that lead to further adaptive responses to salt stress.

ข้อสรุป (Conclusion)

Calcium signaling has been implicated in transducing signals from environmental changes into adaptive responses in plants but mechanisms of how calcium signals are used to mediate stress responses have not been fully understood. Here, possible roles of the *OsCam1-1* gene which its expression is rapidly and highly induced were investigated. Transgenic rice plants overexpressing this gene exhibited higher growth rate and better resistance to salt stress than wild-type plants. In addition, *OsCam1-1*-overexpressing plants were shown to contain higher levels of ABA than the control plants both grown in normal conditions and under salt stress. Taken together, the results obtained here suggest that the *OsCam1-1* gene product functions as a sensor for salt stress-induced calcium signals that lead to ABA biosynthesis, which in turn helps the plant to cope with salt stress. This finding will help facilitate further studies of the physiological functions and downstream elements of the product of this gene.

ข้อเสนอแนะ (Suggestion for Further Work)

Further characterization of the *OsCam1-1*-over-epxressing transgenic plants will provide more information on the mechanisms of *OsCam1-1* in mediating stress responses via Ca^{2+} signals. Differential gene and protein expression analyses between the *OsCam1-1*-over-epxressing and the control transgenic plants may lead to identification of downstream elements of transduction pathways carried out by OsCaM1-1.

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