การแสดงออกเกินปกติของยีน *OsCam1-1* ในข้าว *Oryza sativa* L. 'KDML105' และยาสูบ *Nicotiana tabacum* L. 'Virginia Coker' แปลงพันธุ์

นางสาววรินทรา ทักภิรมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

OVEREXPRESSION OF *OsCam1-1* GENE IN TRANSGENIC RICE *Oryza sativa* L. 'KDML105' AND TOBACCO *Nicotiana tabacum* L. 'Virginia Coker'

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2007

Thesis Title	Overexpression of *OsCam1-1* gene in transgenic rice *Oryza sativa* L. 'KDML105' and tobacco *Nicotiana tabacum* L. 'Virginia Coker'
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Field of Study	Biotechnology
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วรินทรา ทักภิรมย์: การแสดงออกเกินปกติของยีน *OsCam1-1* ในข้าว *Oryza sativa* L. 'KDML105' และยาสูบ *Nicotiana tabacum* L. 'Virginia Coker' แปลงพันธุ์ (OVEREXPRESSION OF *OsCam1-1* GENE IN TRANSGENIC RICE *Oryza sativa* L. 'KDML105' AND TOBACCO *Nicotiana tabacum* L. 'Virginia Coker') อ. ที่ปรึกษา : ผศ. ดร.ธีรพงษ์ บัวบูชา, 144 หน้า.

แคลมอดุลิน (CaM) เป็นโปรตีนรับสัญญาณ Ca^{2+} ที่มีส่วนของ EF hand motif ซึ่งเป็นบริเวณที่ใช้ในการจับกับ Ca^{2+} เป็นองค์ประกอบ โดยแคลมอดุลินมีหน้าที่เป็นตัวกลางที่สำคัญในการส่งถ่ายสัญญาณ Ca^{2+} ในการตอบสนองต่อสภาวะเครียดจากสิ่งแวดล้อม จากการศึกษาที่ผ่านมาพบว่า แคลมอดุลินในข้าว *Oryza sativa* L. (*OsCam1-1*) มีการแสดงออกในการตอบสนองต่อสภาวะเครียดออสโมติก และการเกิดบาดแผล เพื่อสร้างข้าวแปลงพันธุ์ และยาสูบแปลงพันธุ์ที่มีการแสดงออกเกินปกติของยีน *OsCam1-1* ได้ทำการส่งถ่ายยีน *OsCam1-1* ภายใต้การควบคุมของโปรโมเตอร์ 35S CaMV เข้าสู่ข้าว (*Oryza sativa* L. cv. KDML105) และ ยาสูบ (*Nicotiana tabacum* L. cv. Virginia Coker) โดยอาศัย *Agrobacterium* เป็นตัวกลางในการส่งถ่ายชิ้นยีน จากการตรวจสอบ GUS activity ร่วมกับการทำ PCR เพื่อตรวจสอบการแทรกของชิ้นยีน *OsCam1-1* ที่เชื่อมต่อกับโปรโมเตอร์ 35S CaMV พบว่า ข้าวแปลงพันธุ์และยาสูบแปลงพันธุ์ที่ได้รับจากการคัดเลือกด้วยการต้านทานต่อไฮโกรมัยซินแสดง GUS activity และมีชิ้นยีน *OsCam1-1* จากการส่งถ่ายแทรกอยู่ การตรวจสอบการแทรกตัวของชิ้นยีนในจีโนมข้าวด้วยวิธี Southern blot analysis พบว่า ข้าวแปลงพันธุ์ที่ได้รับมีอย่างน้อยหนึ่งสายพันธุ์ และข้าวแปลงพันธุ์ชุดควบคุมซึ่งได้รับจากการสอดแทรกด้วยยีน *gus* ของ pCAMBIA1301 ซึ่งใช้เป็นเวคเตอร์ในการส่งถ่ายยีน มีสองสายพันธุ์ จากการทำ RT-PCR พบว่า ข้าวแปลงพันธุ์และยาสูบแปลงพันธุ์มีการแสดงออกของยีน *OsCam1-1* จากการส่งถ่าย และมีการแสดงออกของยีน *OsCam1-1* เพิ่มมากขึ้นเมื่อเปรียบเทียบกับข้าวชุดควบคุมจากการตรวจสอบด้วยวิธี northern blot analysis แสดงให้เห็นว่าพืชแปลงพันธุ์ทั้งสองมีการแสดงออกเกินปกติของยีน *OsCam1-1* ในระดับ RNA เมื่อทำการตรวจสอบการแสดงออกของ *OsCam1-1* ในระดับโปรตีนด้วยวิธี western blot analysis โดยใช้ anti-His-antibody เนื่องจากชิ้นยีน *OsCam1-1* ภายใต้การควบคุมของโปรโมเตอร์ 35S CaMV ถูกเชื่อมต่อกับ His tag ผลปรากฏว่าไม่พบการแสดงออกในระดับโปรตีนของยีน *OsCam1-1* ที่ถูกส่งถ่ายเข้าไปเช่นเดียวกับในพืชแปลงพันธุ์ชุดควบคุม

สาขาวิชา....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต วรินทรา ทักภิรมย์

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

4772458423 : MAJOR OF BIOTECHNOLOGY

KEY WORD: *OsCam1-1* / Overexpression / *Oryza sativa* L. / *Nicotiana tabacum* L.

WARINTRA TAKPIROM: OVEREXPRESSION OF *OsCam1-1* GENE IN TRANSGENIC RICE *Oryza sativa* L. 'KDML105' AND TOBACCO *Nicotiana tabacum* L. 'Virginia Coker'. THESIS ADVISOR: ASSIST. TEERAPONG BUABOOCHA, Ph.D., 144 pp.

Calmodulin (CaM) proteins, members of the EF-hand family of Ca^{2+}-binding proteins are important relays in calcium signals that mediate stress response in plants. In previous study, expression of a *Cam* gene from *Oryza sativa* L. (*OsCam1-1*) was highly induced by osmotic stress and wounding. To generate transgenic rice and tobacco plants overexpressing *OsCam1-1* gene, a gene sequence consisting of the *OsCam1-1* gene driven by the 35SCaMV promoter was introduced into rice (*Oryza sativa* L.) and tobacco (*Nicotiana tabacum* L. cv. Virginia Coker) by *Agrobacterium*-mediated transformation. The transformation of all potential transgenic lines was confirmed by the assay of GUS activity using X-Gluc as a substrate and by PCR amplification of the 35S CaMV promoter-*OsCam1-1* gene cassette using their genomic DNA as template. At least one transgenic rice line and two independent control rice lines, harboring pCAMBIA1301 alone were obtained from the regeneration of hygromycin resistant rice calli and confirmed by Southern blot analysis. The *OsCam1-1* mRNA was detected in all transgenic rice and transgenic tobacco lines by RT-PCR using oligonucleotide primers designed based on its transcript but not found in the control plants harboring pCAMBIA1301 vector used for the introduction of the *OsCam1-1* gene alone. By northern blot analysis transgenic rice plants showed the overexpression of the *OsCam1-1* transcript as compared with the wild type plants. Since, the 35S CaMV promoter-*OsCam1-1* gene cassette was constructed to encode *OsCam1-1* fused with a His tag at its carboxyl terminal, blot analyses of crude proteins from the transgenic rice and transgenic tobacco plants using His antibodies were conducted. All transgenic rice and transgenic tobacco plants did not produce the bands of the recombinant OsCaM1-1 protein and of GUS protein fused with a His tag.

Field of study....Biotechnology... Student's signature Warintra Takpirom

Academic year.....2007..... Advisor's signature

ACKNOWLEDGMENTS

I would sincerely like to acknowledge the efforts of many people who contribute to this work. This thesis could not be achieved without their following assistance, my thesis advisor Assistant Professor Dr. Teerapong Buaboocha for his generous advice, skillful assistance, technical helps, guidance, encouragement, supporting, fruitful and stimulating discussion through the period of my study.

Sincere thanks and appreciation are due to Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Siriporn Sitthipraneed, Assistant Professor Dr. Supachitra Chadchawan and Dr. Sirawut Klinbunga who serv as the members of the master thesis committees, for their helpful suggestion and comments.

The financially support of research scholarship, Chulalongkorn university, is also gratefully acknowledged.

The special thanks are also extended to Srivilai Phean-O-pas, Bongkoj Boonburapong, Veerakorn Hotimavorakul, Mayura Thongchuang, Duangporn Ungsupravate, Montalee Theeraapisakkul and another friends of Biochemistry and Biotechnology for lending a helping hand whenever needed, sharing the great time in laboratory, their warm assistant and friendship.

Finally, I would like to thank the warmest gratitude is extend to the patience, understanding, helping, encouragement, constant support and warmhearted love of my family whilst I have spent many hours with this thesis rather than with them. My thanks are also extended to my patience and sedulous too.

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LIST OF ABBREVIATIONS

A	absorbance, 2′-deoxyadenosine (in a DNA sequence)
A°	angstrom
bp	base pairs
C	2′-deoxycytidine (in a DNA sequence)
^{O}C	degree Celsius
C-terminus	carboxyl terminus
Ca^{2+}	Calcium ion
cDNA	complementary deoxyribonucleic acid
cyt	cytosol
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
G	2′-deoxyguanosine (in a DNA sequence)
g	gram
GUS	β-glucuronidase
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
KDML105	Khao Dawk Mali 105
KOH	potassium hydroxide
l	liter
LB	Luria-Bertani
Mg^{2+}	magnesium ion
µg	microgram
µl	microliter
µM	micromolar

M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
ng	nanogram
NH_4Cl	ammonium chloride
NH_4OH	ammonium hydroxide
nm	nanometer
NMR	nuclear magnetic resonance
N-terminus	amino terminus
OD	optical density
PCR	polymerase chain reaction
pmol	picomole
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolution per minute
SDS	sodium dodecyl sulfate
T	2'-deoxythymidine (in a DNA sequence)
Tris	tris (hydroxyl methyl) aminomethane
UV	ultraviolet
V	voltage
vir	virulence
v/v	volume by volume
w/w	weight by weight
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

CHAPTER I

INTRODUCTION

Calcium signaling

Calcium signaling plays an important role in plants for coordinating a wide range of developmental processes and responses to hormonal and environmental signals such as salinity, cold, light, drought, symbiotic and pathogenic elicators. It appears that different stimuli elicit specific calcium signatures, generated by altering the kinetics, magnitude, and cellular source of the influx (Malhó *et al.*, 1998; Allen *et al.*, 2000, 2001; Evans *et al.*, 2001; Rudd and Franklin-Tong, 2001). Calcium (Ca^{2+}) is important in maintaining the stability of the cell wall, membrane and membrane bound protein, due to its ability to bridge chemical residues among these structures. (Nayyar, 2003). Research during the last two decades has clearly established that Ca^{2+} acts as an intracellular messenger in couping a wild range of extracellular signals to specific responses (Reddy, 2001). The concentration of Ca^{2+} in the cytoplasm of plants cells is maintained low as the nanomolar range (100-200 nM) by being actively pumped into intracellular compartments and extracellular spaces where [Ca^{2+}] is in the millimolar range (1-10 mM) as shown in Figure 1.1 (Reddy, 2001). The export of Ca^{2+} ions from the cytosol to the extracellular space or into intracellular organelles is achieved by ATP-driven Ca^{2+}-pumps and antiporters (Vetter and Leclerc, 2003).

Different stimuli elicit Ca^{2+} transients which are distinct in their subcellular localization, amplitude, duration, frequency of oscillation and mode of spatial propagation (Snedden and Fromm, 2001). These properties are highly coordinated and regulated by the spatial distribution of Ca^{2+}-release channels and Ca^{2+} pump throughout the cell. The influx of Ca^{2+} ions are generated by voltage- and ligand-

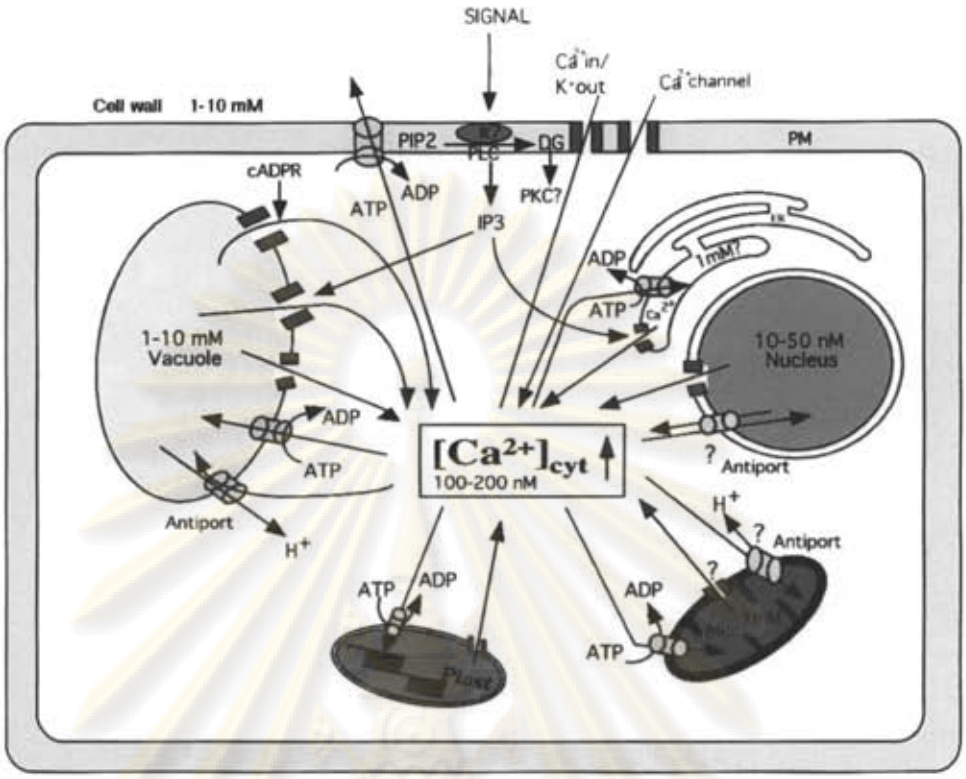

Figure 1.1 Schematic diagram illustrating the mechanisms by which plant cells elevate $[Ca^{2+}]_{cyt}$ in response to various signals and restore Ca^{2+} concentration to resting level. Ca^{2+} channels are shown in red, whereas Ca^{2+} ATPases and antiporters are indicated in yellow. Arrows indicate the direction of Ca^{2+} flow across the plasma membrane, and into and out of cellular organelles (vacuole, plastids, mitochondria, endoplasmic reticulum and nucleus). The estimated concentration of resting levels of Ca^{2+} in different organelles is indicated. Question marks indicate the lack of evidence. $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+}; PLC, phospholipase C; R, receptor, cADPR, cyclic ADP ribose, PIP_2, phosphotidyl inositol-4,5-bisphosphate, DG, diacylglycerol, PKC, protein kinase C, IP_3, inositol-1,4,5-trisphosphate; ER, endoplasmic reticulum; Mt, mitochondria; Plast, plastids; PM, plasma membrane (Reddy, 2001).

gated Ca^{2+}-permeable channels on the plasma membrane. In addition, several intracellular organelles function as Ca^{2+} stores, which can release Ca^{2+} upon stimulation by, for instance, inositol-1, 4, 5-trisphosphate (IP_3) or cyclic ADP-ribose (cADPR). The endoplasmic reticulum (ER) is a major Ca^{2+} stores, but mitochondria and the nucleus also participate actively in the release of Ca^{2+} through the IP_3-receptor. An important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first-round second messengers and by signaling molecules that may themselves be produced as a result of cascades of early Ca^{2+} signals as shown in Figure 1.2. These rounds of signals may have quite different signaling consequences and, therefore, physiological meaning (Xiong *et al.*, 2002).

Ca^{2+}-binding proteins

Transient Ca^{2+} increase in the cytoplasm in response to signals is sensed by several Ca^{2+}-binding proteins and decoded via Ca^{2+}-dependent conformational changes in these sensor polypeptides and interacting with target proteins. Once Ca^{2+} sensor decode the calcium elevation in the cytoplasm, Ca^{2+} efflux into the cell exterior and sequestration into cellular organelles such as vacuoles, ER and mitochondria restores its levels to resting state. A large number of Ca^{2+} sensors can be grouped into four major classes as shown in Figure 1.3. These include (A) Ca^{2+}-dependent protein kinase (CPK) that contains CaM-like Ca^{2+} binding domains and a kinase domain in a single protein. Each individual CPK protein is expected to detect changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity (Roberts and Harmon, 1992), (B) Calmodulin (CaM) which contains four EF-hand domains but have no enzymatic activity themselves and function by interacting

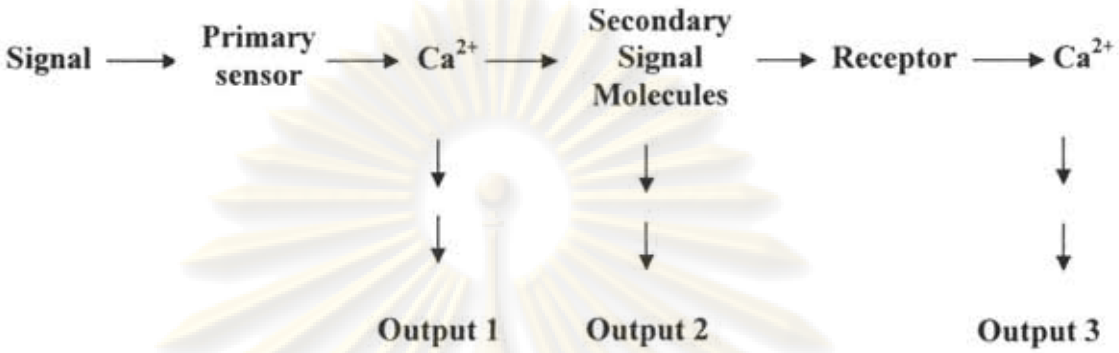

Figure 1.2 Repetitive Ca^{2+} Transients upon the Perception of a Primary Signal. The primary increase in cytosolic Ca^{2+} facilitates the generation of secondary signaling molecules, which stimulate a second round of transient Ca^{2+} increases, both locally and globally. These second Ca^{2+} transients may feedback regulate each of the previous steps (not shown). Ca^{2+} transients from different sources may have different biological significance and result in different outputs, as shown. Secondary signaling molecules such as ROS can also directly regulate signal transduction without Ca^{2+} (Output 2) (Xiong *et al.*, 2002).

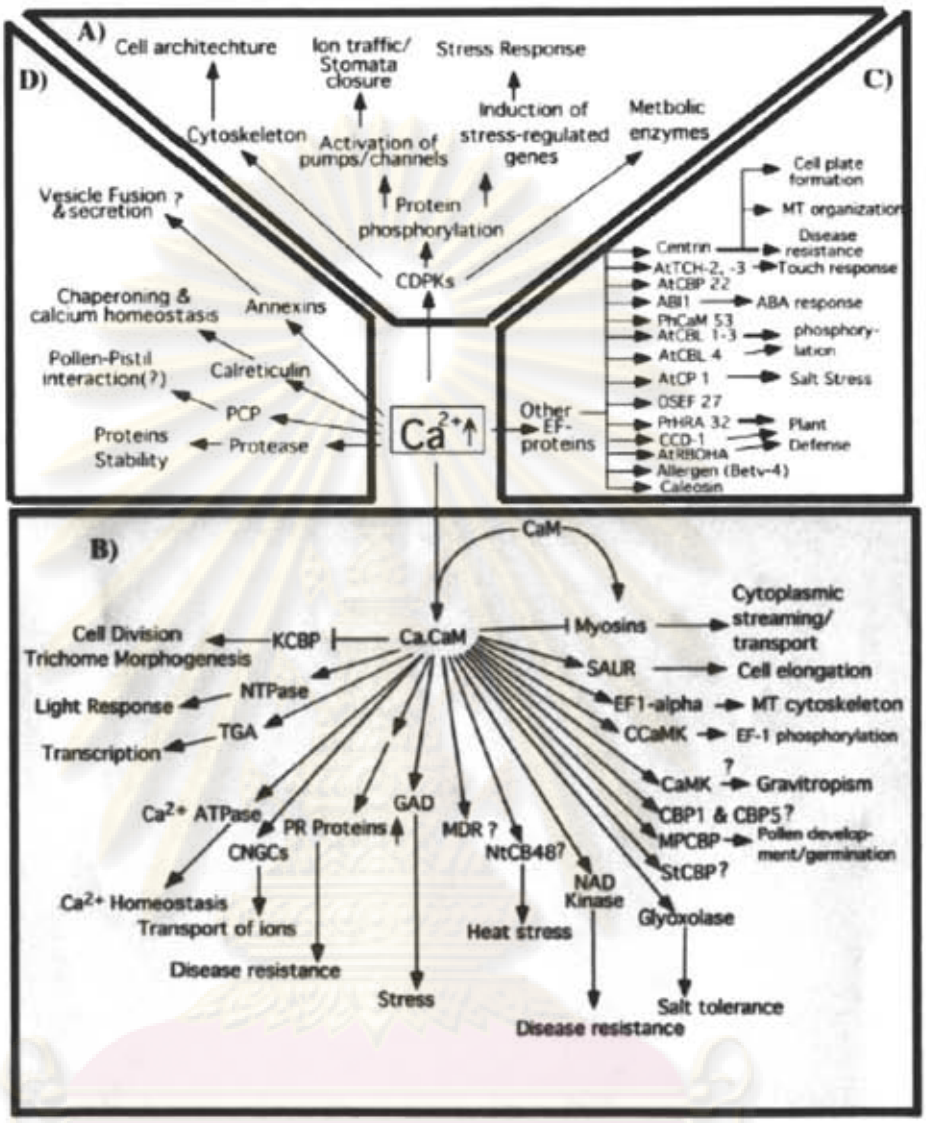

Figure 1.3 Ca^{2+} sensing proteins and their functions in plants. Four major groups of Ca^{2+} sensors (indicated in four boxes) have been described in plants: (A) Ca^{2+}-dependent protein kinase (CDPK), (B) Calmodulin (CaM), (C) other EF-hand motif-containing Ca^{2+}-binding proteins and Calcineurin B-like (CBL) protein, (D) Ca^{2+}-binding proteins without EF-hand motifs (Reddy, 2001).

change and enables Ca^{2+}/CaM to bind to specific CaM-binding domains. The binding of Ca^{2+}/CaM to its target proteins alters their activity in a calcium dependent manner.

Ca^{2+}-bound-calmodulin-mediated signal transduction in plants is shown in Figure 1.5. Biotic and abiotic signals are perceived by receptors, resulting, in some cases, in transient changes in Ca^{2+} concentrations in the cytosol and/or organelles (e.g. nucleus). Increases in free Ca^{2+} concentrations originating from either extracellular pools or intracellular stores are capable of binding to Ca^{2+}-modulated proteins including calmodulin and calmodulin-related proteins. Structural modulations of these proteins enable them to interact with numerous cellular targets that control a multitude of cellular functions, such as metabolism, ion balance, the cytoskeleton and protein modifications. In addition, Ca^{2+} and calmodulin might also regulate the expression of genes by complex signaling cascades or by direct binding to transcription factors. Rapid changes in cellular functions result from direct interactions of calmodulin and calmodulin-related proteins with their targets (within seconds to minutes) while slower responses require gene transcription, RNA processing and protein synthesis (variable times from minutes to days).

The EF hands in CaM are organized into two distinct globular domains, each of which contains one pair of EF hands. Each pair of EF hands is considered the basic functional unit. Pairing of EF hands is thought to stabilize the protein and increase its affinity toward Ca^{2+}. Although each globular domain binds Ca^{2+} and undergoes conformational changes independently, the two domains act in concert to bind target proteins. Upon increase of Ca^{2+} concentration to submicromolar or low micromolar levels, all CaM molecules are activated. Cooperative binding is required for this "on/off" mechanism to function efficiently. The cooperatively of Ca^{2+} binding ensures

with their target proteins (Zielinski, 1998), (C) other EF-hand motif-containing Ca^{2+}-binding proteins and Calcineurin B-like (CBL) protein that are similar to both the regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Klee *et al.*, 1998) and (D) Ca^{2+}-binding proteins without EF-hand motifs. Members of the first three classes of Ca^{2+} sensors contain helix-loop-helix motifs that bind to Ca^{2+} with high affinity (Roberts and Harmon, 1992). However, different Ca^{2+}-binding proteins differ in the number of EF hand motifs and their affinity to Ca^{2+} with dissociating constants (K_ds) ranging from 10^{-5} to 10^{-9} M. Binding of Ca^{2+} to a Ca^{2+} sensor causes a conformational change in the sensor resulting in modulation of its activity or its ability to interact with and modulate function/activity of other proteins (Reddy, 2001).

Calmodulin

Calmodulin is probably the most well characterized Ca^{2+} sensors among these groups of protein. It is a small molecular weight acidic protein of 148 amino acid, highly conserved, soluble, intracellular Ca^{2+}-binding protein ubiquitously found in animals, plants, fungi and protozoa, and is regarded as a major transducer of Ca^{2+} signals in mammalian cells. It has four EF-hands that bind to four Ca^{2+} ion. Many proteins involved in Ca^{2+} signal transduction alter their activity in response to changes in free Ca^{2+} levels, but are themselves not able to bind Ca^{2+} ion. Some of these proteins utilize CaM as a sensor and mediator of the initial Ca^{2+} signal. Calmodulin is a multifunctional protein because of its ability to interact and regulate the activity of a number of proteins as shown in Figure 1.4. CaM relays the Ca^{2+} signal by binding free Ca^{2+} ions to its C- and N-terminal EF-hand pairs, which causes a conformational

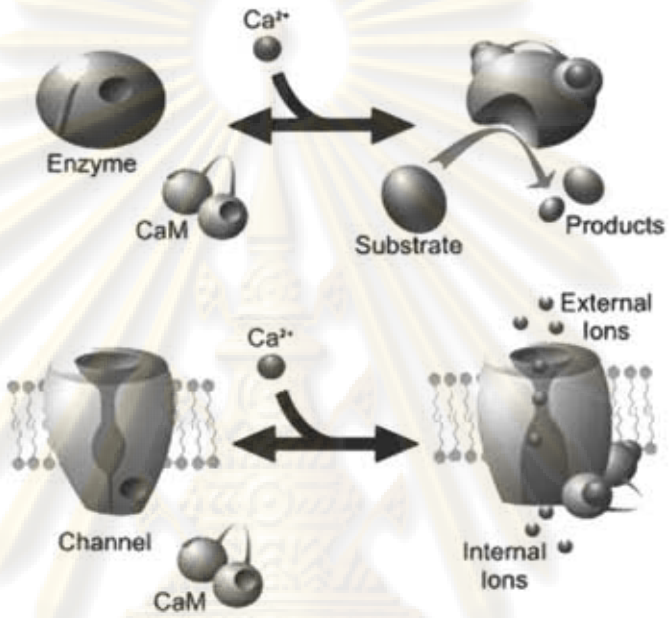

Figure 1.4 Calmodulin acts as a multifunctional protein.

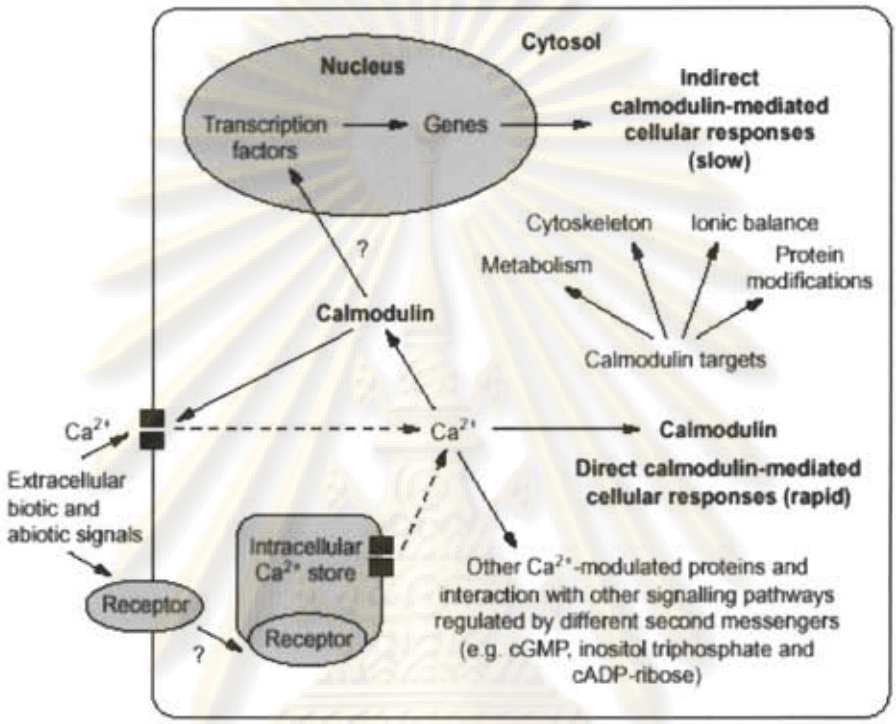

Figure 1.5 The Ca^{2+}-bound-calmodulin-mediated signal transduction in plants. Broken arrows denote Ca^{2+} fluxes from extracellular or intracellular stores, and question marks signify unknown signal transduction intermediates.

interhelical angles between the globular domains. This open conformation exposes a hydrophobic surface within each globular domain and permits the binding of protein targets (Babu *et al.*, 1988; Kuboniwa *et al.*, 1995, Zhang *et al.*, 1995).

Ca^{2+}-CaM binds and regulates the activity of a wide range of proteins that are not necessarily related in structure. How can Ca^{2+}-CaMs bind to so many different proteins? More specifically, the plasticity of the Ca^{2+}-CaM structure must accommodate the variation in both the molecular size and the composition of the target proteins. This issue has been addressed by structural analyses of Ca^{2+}-CaM and target-bound Ca^{2+}-CaM. Figure 1.6C shows that the two globular domains of Ca^{2+}-CaM are connected by a flexible tether that can accommodate peptides of varying sizes. The binding of CaM-binding peptides is largely driven by hydrophobic interaction between hydrophobic anchor residues of the peptide with the hydrophobic surface cavities of CaM. Methionine residues, unusually abundant in CaM, play a particularly important role in the binding of target peptides. The methionine side chains are very flexible and the sulfur atom has a larger polarizability than carbon, resulting in stronger van der Waals interaction. The hydrophobic patche of each lobe is surrounded by several charged residues, creating charged binding channel outlets. The C-terminal end of the peptide-binding channel has a negatively charged rim, whereas the N-terminal hydrophobic patch has clusters of negatively and positively charged residues. This charge distribution on the molecular surface contributes to peptide binding via electrostatic interactions and determines the relative binding orientation of CaM-binding domains in the peptides. Basic residues at the N-terminus of the peptide form salt bridges with acidic residues surrounding the peptide-binding channel of the C-terminal lobe of CaM. Together, the structures of CaM illustrate how

that full activation of the CaM occurs in a narrow region of calcium concentration during a signaling event. The selectivity of CaM toward Ca^{2+} also is an important factor in effective transduction of the Ca^{2+} signal. CaMs bind Ca^{2+} selectively in the presence of high concentrations of Mg^{2+} and monovalent cations in the cell. The cation selectivity is achieved by optimization in the structure folds of the binding loop. For example, discrimination between Ca^{2+} and Mg^{2+} is accomplished through reduction in the size of the binding loop. Binding of Mg^{2+} ions would collapse the EF-hand loop, thereby reducing the distance between negatively charged side chains and destabilizing the CaM- Mg^{2+} complex (Falke *et al.*, 1994). Even small changes in the chemical properties of the Ca^{2+} binding loop (e.g., Glu-12→Gln) can drastically reduce the binding affinity to Ca^{2+} (Beckingham, 1991; Haiech *et al.*, 1991). The Glu-12→Gln mutation changes the carboxylate side chain into carboxylamide, which removes the oxygen ligand for Ca^{2+}. Together, structural analyses in combination with site-directed mutagenesis established that CaMs (and other EF hand–containing proteins, including CBLs) have evolved as highly specific Ca^{2+} sensors (Luan *et al.*, 2002).

The overall structure of Ca^{2+}/CaM is dominated by two EF-hand pairs forming the C- and N- terminal lobes and a long α-helix connecting the two lobes. In vertebrate CaM, the two EF-hand pairs share 48% sequence identity and 75% sequence similarity and the peptide backbone of the two lobes can be superimposed with a mean square derivation of ~0.7 A° (Vetter and Leclerc, 2003). Structural analysis of the Ca^{2+}-free and Ca^{2+}-bound states of CaM proteins reveals the conformational changes induced by Ca^{2+} binding as shown in Figure 1.6. In the Ca^{2+}-free state, CaM adopts a closed conformation. Ca^{2+} binding triggers a conformational change, and the protein adopts an open conformation with nearly perpendicular

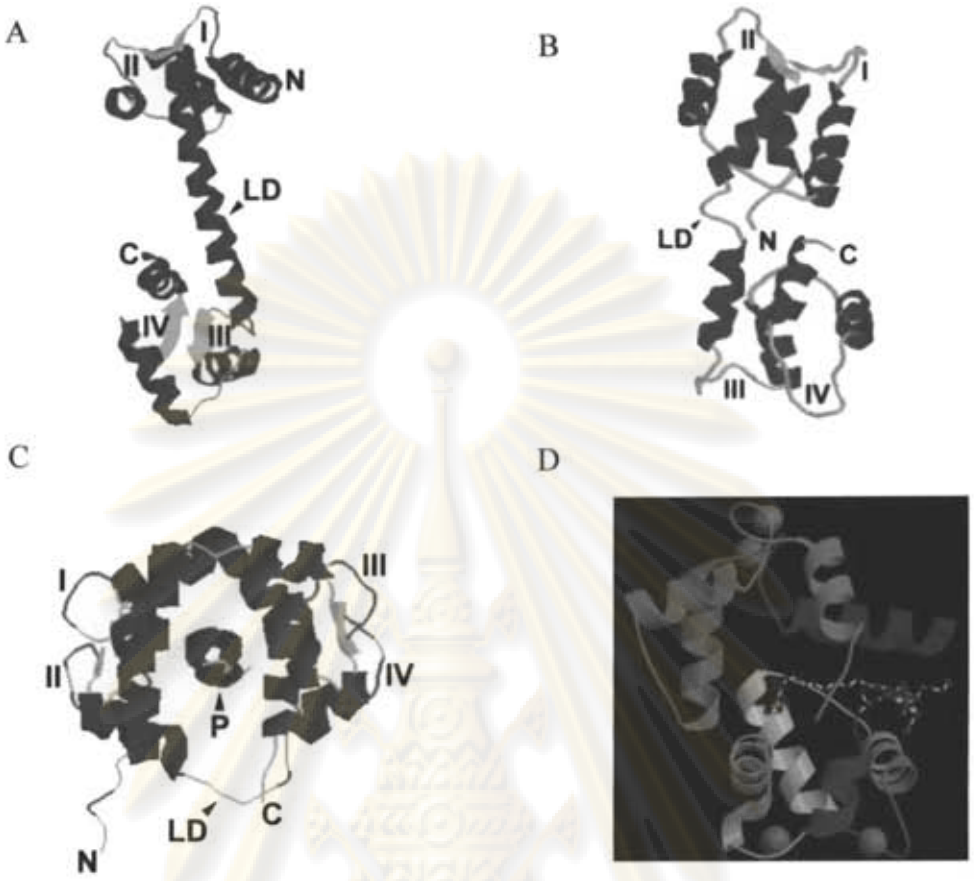

Figure 1.6 Ribbon presentations of Calmodulin. (A) Ca^{2+}/Calmodulin (CaM) determined by X-ray crystallography, (B) globular domain of CaM (apo-CaM) determined by NMR spectroscopy, (C) and (D) The direction of Ca^{2+}/CaM-target peptide interaction; Peptide binding causes disruption of the flexible tether, bringing the globular domains closer to form a channel around the peptide. The majority of contacts between Ca^{2+}-CaM and target peptide are nonspecific van der Waals bonds made by residues in the hydrophobic surfaces. For (A) to (C), I-IV, Ca^{2+}-binding loops in the EF-hands; N, amino- termini of the CaM; C, carboxy-termini of the CaM; LD, central linker domain and P, target peptide. The helices, loop and b-sheet are colored in red, blue and yellow respectively.

this class of proteins can function as extremely efficient Ca^{2+} sensors and on/off switches, allowing them to transduce Ca^{2+} signals with high efficiency and accuracy.

In plants, there are multiple *CaM* genes that code for either identical proteins or proteins containing a few conservative changes. These small changes in amino acid composition of CaM isoforms may contribute to differential interaction of each CaM isoform with target proteins. The striking example for differential regulation of CaMs comes from the studies with soybean CaM isoforms. In soybean there are five CaM isoforms (SCaM1 to -5). SCaM1, -2 and -3 are highly conserved compared to other plant CaM isoforms including Arabidopsis CaM isoforms whereas SCaM4 and -5 are divergent and showed differences in 32 amino acids with the conserved group (Lee *et al.*, 1995). Surprisingly, these divergent CaM isoforms are specifically induced by fungal elicitors or pathogen (Heo *et al.*, 1999). These results provided evidence for the differential regulation of CaM isoforms in plants. Soybean isoforms show differences in their relative abundance *in vivo*. The conserved isoforms are relatively abundant in their expression compared to the divergent forms. All CaM isoforms activate phosphodiesterase (PDE) but differ in their activation of NAD kinase, calcineurin and nitricoxide synthase indicating Ca^{2+}/CaM specificity between CaM isoforms and target proteins (Lee *et al.*, 1997). Although SCaM isoforms show similar patterns in protein blot overlay assays, they differ in their relative affinity in interacting with CaM binding proteins (Lee *et al.*, 1999). In another exsample, two divergent CaM isoforms that are found in Arabidopsis do not interact with proteins that bind to conserved CaM isoforms (Kohler *et al.*, 2000). These studies suggest that conserved and divergent CaM isoforms may interact with different target proteins. Different affinities for Ca^{2+}-CaM interactions with specific target proteins may be sufficient for the differential transduction of the Ca^{2+} signal (Luan *et al.*, 2002).

Recent studies on CaM genes expression in response to different stimuli indicate that different CaM isoforms are involved in mediating a specific signal (Zielinski, 1998). There is considerable evidence to indicate that CaM genes are differentially expressed in response to different stimuli such as drought, salinity, cold and parthogenic microorganism. Three of the six Arabidopsis *Cam* genes (*Cam*1, -2 and -3) are inducible by touch stimulation (Zielinski, 1998) indicating the presence of different *cis*-regulatory elements in their promoters. In potato, only one of the eight CaM isoforms (PCaM1) is induced by touch (Takezawa *et al.*, 1995). In rice, The study of three CaM isoform : *OsCam1*, *OsCam2* and *OsCam3* showed that *OsCam1* and *OsCam3* gene are inducible by salinity and wounding. In contrast, *OsCam2* are not induced by these stimuli indicating that although the expression of genes encoding different calmodulin isoforms is ubiquitous, they are differentially regulated by various stress signals (Phean-o-pas *et al.*, 2005). The presence of multiple CaM isoforms adds further complexity to the Ca^{2+} mediated network in plants which suggests their important and diverse roles in calcium signaling.

Calmodulin in rice

Rice (*Oryza sativa* L.) is one of the most important crops in the world and is the staple food for about three billion people (Toenniessen, 1996). Production and consumtion are concentrated in Asia where more than 90% of all rice is produced and consumed (David, 1991). For Thailand, rice is the stable food and a significant commodity for its economy. By-products of rice is also importance for human and animal consumption (Tassongchant, 1987).

There are three subspecies of *Oryza sativa*, indica (long grain), japonica (round grain) and javanica (medium grain). The indica rice concentrates in the warm

climate belt, from Indochina, Thailand, India, Pakistan, Brazil and Southern U.S.A. The japonica is mostly grown in cold climate including Japan, Korea, northern China and California. The javanica is only grown in Indonesia (Oka, 1991).

Oryza sativa is an annual grass growing best when submerged in water as shown in Figure 1.7. It grows in upland areas, irrigated areas, rainfed lowland areas, and flood-prone areas. Rice is highly adaptable and can be grown in diverse environments. Rice is constantly bombarded with environmental signals, both biotic and abiotic such as soil salinity, drought, cold, disease, and pathogenic microorganism, some of which cause stress and limit the growth and development and affect the yield and quality.

O. sativa was a cereal selected to be sequenced as a priority and has the status of a "model organism". Rice with its relatively small genome size (~430 Mb), ease of transformation, well developed genetics, availability of a dense physical map and molecular markers (Chen *et al.*, 2002; Wu *et al.*, 2002a), high degree of chromosomal co-linearity with other major cereal such as maize, wheat, barley and sorghum (Ohyanagi *et al.*, 2006) and together with its complete genome sequence (Sasaki *et al.*, 2005) is considered a model monocot system. It is being used to understand several fundamental problems of plant physiology, growth and developmental processes ranging from elucidation of a single gene function to whole metabolic pathway engineering. In addition, rice shares extensive synteny among other cereals thereby increasing the utility of this system (Devos and Gale, 2000). These, together with availability of ~28,000 full length cDNAs, a large number of expressed sequence tags, yeast artificial chromosomes, bacterial artificial chromosomes, P1-derived artificial chromosomes, libraries and rich forward and reverse genetics resources (Hirochika *et al.*,2004) have made rice a worthy forerunner among the plants

Figure 1.7 *Oryza sativa* L. (http://en.wikipedia.org/wiki/Image:Koeh-232.jpg)

especially among the cereals.

Khao Dawk Mali 105 (KDML 105), is a famous aromatic rice variety of Thailand and worldwide because of its aromatic, solf and tender cooked rice. KDML 105 is popularly grown under rainfed lowland in the North and Northeast of Thailand which frequently experience the problems about drought and soil salinity. However, KDML105 itself is a tall variety and can not produce high enough grain yield. KDML 105 is also photoperiod sensitive which restricts its multiple cropping per year. Moreover, KDML 105 is susceptible to many insect pets and disease, although it can resist several adversed planting conditions such as modurate degree of drought, salted soil or acid soil (Tassongchant, 1987).

Boonburapong, B. and Buaboocha, T., (2007) studied the Ca^{2+}-binding proteins of rice by phylogenetic analysis based on amino acid sequences similarity and classified proteins with a high degree of identity as "true" CaMs that probably function as typical CaMs. They were named *OsCam1-1*, *OsCam1-2*, *OsCam1-3*, *OsCam*2 and *OsCam3*. *OsCam1-1*, *OsCam1-2* and *OsCam1-3* encode identical protein, 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. It is fascinating that the *OsCam1-1*, *OsCam1-2* and *OsCam1-3* genes encode identical proteins. How these protein sequences have been maintained with the natural selection pressure throughout evolution has no clear answer yet but it is likely that each of these genes has physiological significance. In addition, *OsCam1* amino acid sequences are identical to those of the typical CaMs from barley (*H. vulgare*) and wheat (*T. aestivum*) reflecting the close relationships among monocot cereal plants. On average, OsCaM amino acid sequences share about 99%, 90% and 60% identity with those from plants vertebrate and yeast, respectively.

Use of transgenic plants for characterizing gene functions

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. Gene can be introduced into the plant cell using the bacteria *Agrobacterium tumefaciens*. *Agrobacterium* introduces a plasmid, T-DNA, into the plant cell which integrates randomly in the plant genome. By replacing part of the T-DNA for the gene of a desired protein, the gene can be introduced stably in the plant cell. *Agrobacterium* is exploited by many plant biologists in molecular and genetic studies to introduce DNA into plants. Although best known for this practical application, the transfer of DNA from bacterium to plant comprises fundamental biological processes, many of which are largely uncharacterized.

Agrobacterium carries three genetic components required for plant cell transformation. The T-DNA and the virulence (*vir*) region are located on the large Ti plasmid (Figure 1.8) (Binns and Thmashow, 1988). The T-DNA is the DNA fragment that can be transferred from *Agrobacterium* to the plant cell. The vir region is organized into six complementation groups that are either absolutely essential for (*virA, vir B, vir D and virG*) or that enhance the efficiency of (*virC and virE*) plant transformation. The third bacterial component of the T-DNA transfer process resides in the *Agrobacterium* chromosome. Three chromosomal virulence loci, chvA and chvB and pscA, encode products involved in the binding of *Agrobacterium* to plants cells during the infection process (Zambryski, 1988).

During infection by *Agrobacterium*, a piece of DNA is transferred from the bacterium to the plant cell (Figure 1.8). The piece of DNA is a copy of a segment called the T-DNA (Transferred DNA). It is carried on a specific plasmid, the Ti-

plasmid (Tumer-inducing). The T-DNA is delimited by 25 bp direct repeats that flank the T-DNA. Any DNA between these borders will be transferred to a plant cell (Walden, 1993). Wild-type T-DNA encode enzymes for the synthesis of the plant growth regurators; auxin and cytokinin, and the production of these compounds in transformed plant cells results in the tumorous phenotype. In addition, wild-type T-DNA also encodes enzymes for the synthesis of novel amino acid derivatives called opines. The Ti-plasmid encodes enzymes for their catabolism; hence, *Agrobacterium* has evolved to genetically commandeer plant cells and use them to produce compounds that they can uniquely utilize as a carbon/nitrogen source (Kahl and Weising, 1993).

The processing and transfer of T-DNA are mediated by products encoded by the *vir* (virulence) region, which is also resident on the Ti-plasmid (Stachel and Nesyer, 1986). The *vir* genes, whose products are directly involved in T-DNA processing and transfer, are tightly regulated so that their expression occurs only in the presence of wounded plant cells, the target of infection. Control of gene expression is mediated by the VirA and VirG proteins, a two component regulatory system. Vir A detects the small phenolic componnds released by wounded plants resulting in autophosphorylation VirA phosphorylation of VirG then leads to activation of *vir* gene transcription (Winans, 1992) (Figure 1.8, step 1).

Following *vir* gene induction, the production of a transfer intermediate begins with the generation of the T-strand, a single strand copy of the T-DNA (Stachel *et al.*, 1986). VirD1 and VirD2 are essential for this process (Filichkin and Gelvin, 1993). Together, VirD1/VirD2 recognizes the 25-bp border sequence and produces a single strand endonucleolytic cleavage in the bottom strand of each border (Figure 1.8, step 2). These nicks are used as the initiation and termination sites for T-strand production.

T-strand production is thought to result from the displacement of the bottom strand of the T-DNA between the nicks (Zupan and Zambryski, 1995). After nicking, VirD2 remains tightly associated with the 5′ end of the T-strand. The lone VirD2 at the 5′ end gives the T-complex a polar character that may ensure that, in subsequent steps, the 5′ end is the leading end.

The T-strand must travel through numerous membranes and cellular spaces before arrival in the plant nucleus. Thus, to preserve its integrity, it was hypothesized that the T-DNA likely travels as a single strand DNA-protein complex. VirE2 is an inducible single strand nucleic acid-binding protein encoded by the virE locus which binds without sequence specificity. VirE2 binds tightly and cooperatively, which means that a T-strand would be completely coated (Figure 1.8, step 3). Consequently, degradation by nucleases would be prevented and, indeed, *in vitro* binding of VirE2 renders single strand DNA resistant to nucleolytic degradation. Finally, binding of VirE2 unfolds and extends single strand DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T-strand along with VirD2 and VirE2 are termed the T-complex (Zupan and Zambryski, 1995).

Subsequencely, the T-complex must exit the bacterium cell (Figure 1.8, step 4) passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane (Figure 1.8, step 5). Once inside the plant cell, the T-complex targets to the plant cell nucleus and crosses the nuclear membrane (Figure 1.8 step 7). In the context of experimental chronology and relevant results, step 1 through 3 (Figure 1.8) have been studied. Current and recent research has related to step 4 and 6. Entering the plants cell (step 5) and the mechanics of integration (step 7) are almost completely uncharacterized (Zupan and Zambryski, 1995).

Agrobacterium tumefacians naturally infects only dicotyledoneous plants and many economically important plants, including the cereals, remained accessible for genetic manipulation during long time. For these cases, alternative direct transformation methods have been developed (Shillito *et al*., 1985; Pritrykus, 1991) such as polyethyleneglycol-mediated transfer (Uchimiya *et al.,* 1986), microinjection (de la Pena *et al*., 1987) protoplast and intact cell electroporation (Fromm *et al*., 1986) and gene gun technology (Sanford, 1988). However, *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of transgene, potentially leading to fewer problems with transgenic cosuppression and stability (Hansen *et al*., 1997). In addition, it is a single cell transformation system not forming mosaic plants, which are more frequent when direct transformation is used (Ensiquez-Obregon *et a*l., 1998).

Many Solanaceous plant species, such as tobacco (*Nicotiana tabaccum* L.) are valuable species for biology study because they permit the integration of tools and concepts of genetics, plant physiology, developmental biology, host pathogen interaction, molecular biology and genetic engineering for studying and manipulating all these processes. They are susceptible to *Agrobacterium* infection, and are therefore amenable to current plant transformation technique. Culturing explants of Solanaceous plants species easily gives rise to shoots (McCormick *et al*., 1986).

Several years ago, many scientists have selected Solanaceous plant species for studying foreign genes or expression of tissue specific promoters by *Agrobacterium*-mediated transformation method. The first record on transgenic tobacco plants expressing foreign genes appeared at the beginning of the last decade (Herrera-Estrella *et al.*, 1983). Kosaki *et al*. (1992) transformed the promoter of *RGS-38* gene, which encodes plastidic glutamine synthetase of *Oryza sativa* L., fused to a

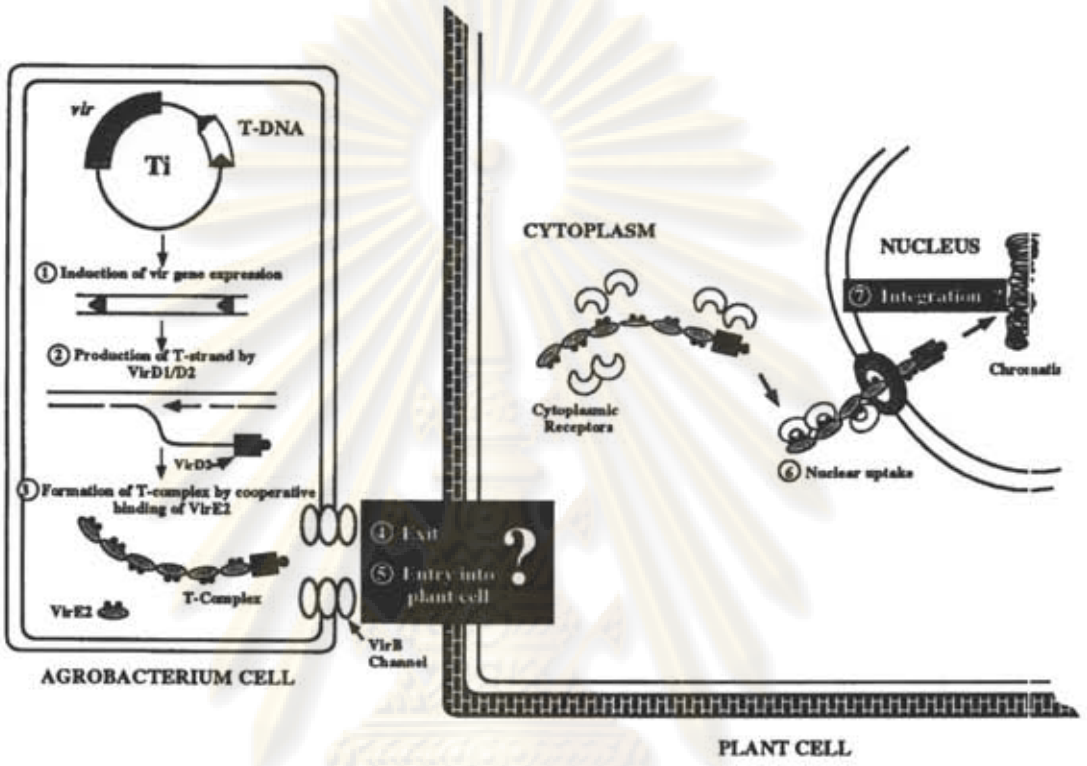

Figure 1.8 Basic step in the transformation of plants cells by *A. tumefaciens* (Zupan and Zambryski, 1995).

β-glucuronidase (GUS) reporter gene, into tobacco plants. The reporter directed GUS expression, both in leaves and roots, and the expression of GUS was regulated by light. Until recently, *Agrobacterium*-mediated gene transfer into monocotyledonous plants was possible when reproducible and efficient methodologies were established on rice (Hiei *et al.*, 1994), corn (Ishida *et al*., 1996) and wheat (Cheng et al., 1997).

pCAMBIA1301 vector system has been developed for efficient transformation of plants. The pCAMBIA1301 which was used in this study contains the GUS (*uidA*) with an intron as a reporter gene, and the hygromycin-resistant gene (*hpt*) as a plant selectable marker within the T-DNA. Each gene was under the control of a CaMV 35S promoter. The double-strand caulifmovirus family has provided the single most important promoter for transgene expression in plants: the cauliflower mosaic virus (CaMV) 35S promoter is a strong as well as a constitutive promoter. It leads to high expression of genes in almost any type of cell of most plants, and in most tissues, although with different efficiency according to plant species. The nptII gene encoding resistance to kanamycin was used as a bacterial selectable marker. This vector harbors the pUC18 polylinker within the lacZα fragment allowed blue/white screening of clones in *E.coli* cloning work. This transformation vectors have a wide-host-range origin of replication from the Pseudomonas plasmid pVS1; the pBR322 origin (pMB9-type) to allow high-yielding DNA preparations in *E. coli*. Intron from castor bean catalase eliminates any possibility of read through or inappropriate GUS and *hpt* production in prokaryotes, such as *Agrobacterium*. This intron is efficiently spiced in dicots and monocots.

A selectable marker is used to select for the specific growth of transformed cells among a background of non-transformed individuals. This is important because transformation frequencies remain normally relatively low. Such markers allow

growth, or at least viability, in the presence of the selective agent. Routinely, resistance to antibiotics or phytotoxins such as herbicides has been used. In the former case, antibiotic resistant genes derived from bacteria have been utilized (e.g. kanamycin and hygromycin), whereas in the latter case genes encoding products which are more tolerant to herbicides, for example glyphosate and phosphinothricin have been used (Walden, 1993).

In pCAMBIA1301, the gene *hpt* isolated from *E. coli* is used. It codes for the enzyme hygromycin phosphotransferase. This gene therefore causes resistance to the antibiotic compound hygromycin. The *hpt*/hygromycin B combination was successfully employed in the genetic transformation of tobacco, *Arabidopsis*, maize and rice (Schortt *et al*., 1995). Hygromycin is a more potent phytotoxin compound than kanamycin; especially in cereal crops (Galun and Breinman, 1997). Hygromycin allows clear discrimination between transformed and non-transformed tissues and problems with albinos or the fertility of regenerants have not been reported (Ayres *et al*., 1994).

To provide a clear indication that genetic transformation did take place, pCAMBIA1301 uses β-glucuronidase (GUS) which is the most widely used system of reporter genes in plants. In general, reporter genes should have the following characteristics: 1) the genetic organization should be well described, 2) the gene products should not be present in the organism or tissue under study, 3) the gene products should be well characterized with regard to biochemical activity, 4) substate dependence and stability, and 5) the product of the reaction catalyzed by the reporter gene product should be stable, easily detectable, and quantifiable (Crazzolara *et al*., 1995).

GUS is encoded by the *Escherichia coli uidA* gene (Jefferson *et al.*, 1986, 1987). The protein has a molecular weight of 68.2 kDa. The best substrate currently available for histochemical localization of β-glucuronidase activity in tissue and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The product of glucuronidase activity on X-Gluc is colorless. Instead, the indoxyl derivative product must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. This dimerization is stimulated by atmospheric oxygen, and can be enhanced by using an oxidation catalyst such as a potassium ferricyanide/ferrocyanide mixture. It is the efficient reporter that can be used to locate its expression in plant tissues and organs without the need to extract the respective tissue. However, the substrate for detection of GUS activity (X-Gluc) is expensive and the X-gluc-stained plant material is killed by the GUS assay; it is therefore not a vital staining (Jefferson *et al.*, 1986, 1987).

Application of biotechnology in plant

Thailand is an agricultural country which produce substantial amount of food for domestic consumption and export. However, agricultural production in Thailand is limited by several factors such as soil salinity, drought, disease and insect pests.

Conventional breeding has been used regularly for crop improvement and production of a new variety. Traditional plant breeding involves making large numbers of crosses between a range of parents which have been selected by the breeder for their desirable attributes. Some of the progeny from these crosses will show a combination of the best traits from the parents. After continued genetic recombination and a number of cycles of selection, a new varieties is obtained which has a combination of desirable characteristics and which distinct from any other variety. This process is simple in outline but complex in practice. Plant breeding

involves a wild range of skills. Given this complexity it can take up to 15 years between the initial cross and the commercial release of a new variety. As a further complication there are various limitations to plant improvement through conventional breeding. Not only are plant-breeding programmes long-term and therefore expensive, they also require the cultivation and analysis of large numbers of plants which takes both time and space. Additionally, in some cases, the breeder has limited genetic variation for incorporation into new varieties. Moreover, varieties derived from breeding program may be somewhat different from their parental lines. Thus techniques which overcome any of these constraints are of interest and potential value to plant breeder (Connett and Barfoot, 1992).

The rapid development of biotechnology particularly plant genetic engineering offers an alternative approach for control of abiotic tolerance and biotic tolerance of plants. Genetic engineering offers an advantage over conventional breeding in a way that only one or two characters will be introduced into crop species. The overall genotypic characters of that species remain unchanged. The improvement of techniques involved in making transgenic plants constitutes one of the major developments which have taken place in plant science. In subsequent years, transgenic plants showing enhanced resistance to herbicides, fungal diseases, insect attacks, and, for better genetic quality of the product, altered levels of hormone or increased level of secondary metabolites have been engineered. These developments have ushered plant molecular biology and biotechnology research into a highly exciting phase with respect not only to the fundamental science but also from the point of view of commercial application.

In spite of these developments, it is still major challenge to genetically engineer crop plants that show improved performance against abiotic stresses. The term abiotic

stress refers to factors such as low and high temperatures stress, salt stress, water stress and oxidative stress. Saijo *et al.*, (2000) studied the physiological function of *OsCDPK7* rice gene encoding a calcium-dependent protein kinase, which is one of the Ca^{2+}-binding protein. They introduced *OsCDPK7* gene which was in the sense orientation downstream of the CaMV 35S promoter into rice calli (*Oryza sativa* L.) by *Agrobacterium*-mediated transformation. A total 14 independent transgenic lines was obtained. The transgenic rice showed constitutive higher levels of *OsCDPK7* protein than non-transgenic rice even in normal condition. Northern blot analysis indicated that the expression of *OsCDPK7* mRNA level in the transgenic rice was higher than in the wild type when induced them with cold and salt stresses. In addition, transgenic rice overexpressing *OsCDPK7* are more tolerant to salt and cold stresses than the wild type. In 2006, Cao *et al.* introduced *OsBIEF3* rice gene encoding the ethylene-responsive element binding protein into tobacco by *Agrobacterium*-mediated leaf disc transformation with a structure containing the *OsBIEF3* ORF under control of CaMV 35S promoter to better understand the function of *OsBIEF3* in disease resistance response and abiotic tolerance. A total 12 independent transgenic lines was obtained from screening by kanamycin resistance. Northern blot analysis of the *OsBIEF3* mRNA level indicated that the level of expression of this gene in the transgenic tobacco was higher than that in the wild type under normal condition. Moreover, the *OsBIEF3*-overexpressing transgenic tobacco plants also showed increased tolerance to salt stress and increased disease resistance response.

Ca^{2+} serves as an intracellular messenger in many cellular processes including plant responses to environmental stresses such as salinity, drought and cold. These stresses have been shown to induce transient elevation of the cytoplasmic Ca^{2+} concentration level. Elevation of the Ca^{2+} concentration is detected by calcium sensor

proteins. The classical calcium sensor is calmodulin (CaM), which regulates activity of its protein targets in a calcium-dependent manner. In plants, CaM may play an important role in transducing Ca^{2+}-mediated signal from salt stress into appropriate adaptive cellular responses. In previous research, Phean-o-pas *et al.*, (2005) studied calmodulin (*CaM*) genes from rice (*Oryza sativa* L.). The result suggests the expression of *OsCam1-1* was highly induced by salt stress and wounding.

Thus, the objective of this study is to generate transgenic rice and transgenic tobacco overexpressing *OsCam1-1* by introducing a construct consisting of the *OsCam1-1* gene driven by a CaMV 35S promoter into rice calli (*Oryza sativa* L. cv. KDML105) and tobacco leaf disc (*Nicotiana tabacum* L. cv. Virginia Coker) by *Agrobacterium*-mediated transformation and to determine the expression level of the *OsCam1-1* gene in transgenic plants compare with in wild type plants. The knowledge obtained from this study will provide a fundamental basis for understanding the physiological function of the *OsCam1-1* gene under stress response and a basic knowledge for improvement of rice plants for abiotic stress tolerance in the future.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plants

Rice (*Oryza sativa* L.) cultivar Khoa Dok Ma Li 105 (KDML105)

Tobacco (*Nicotiana tabacum*) cultivar Virginia Coker

2.1.2 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Automatic micropipette: Pipetman P2, P20, P100, P1000 (Gilson Medical Electronics S.A., France)

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Biophotometer (Eppendorf, Germany)

Film cassette with intensifying screen: 35x43 cm^2 (Kodak, USA)

-20 °C Freezer (Sharp, Japan)

Gel document : Gel Doc™ (Syngene, England)

Gel mate 2000 (Toyobo, Japan)

Gene Pulser : Micropulser™ (Bio-RAD Laboratories, USA)

Hybridization oven : Hybaid shake 'n' Stack (Thermo Scientfic, USA)

Incubator: BM-600 (Memmert Gambh, Germany)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific, UK)

Laminar flow: HS-124 (International Scientific Supply Co., Ltd., USA)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Magnetic stirrer and heater: Cerastir (Clifton, USA)

Mastercycler gradient PCR system (Eppendorf, Germany)

Microcentrifuge: PMC-880 (Tomy Kogyo Co., Ltd., Japan)

Microwave oven (Panasonic, Japan)

pH meter: pH900 (Precisa, Germany)

PCR workstation Model#P-036 (Scientific Co., USA)

Power supply: Power PAC 1000 (Bio-RAD Laboratories, USA)

Refrigerated centrifuge : 5804R (Eppendorf, Germany)

Refrigerated centrifuge : 5417R (Eppendorf, Germany)

Spectrophotometer: DU®640 (Beckman Coulter, USA)

UV chamber : GS Gene Linker™ (Bio-RAD Laboratories, USA)

UV transilluminator: 2001 microvue (San Gabriel California, USA)

Vacuum blotter : model 785 (Bio-RAD Laboratories, USA)

Vacuum pump (Bio-RAD Laboratories, USA)

Vortex mixer: Model K 550-GE (Scientific Inc., USA)

Waterbath: Isotemp210 (Fisher Scientific, USA)

2.1.3 Materials

Filter paper: Whatman No.1 (Whatman International Ltd., England)

KODAK BioMax MS film: 18x24 cm^2 (Kodak, USA)

Microcentrifuge tube 0.6 and 1.5 ml (Axygen Hayward, USA)

0.22µm Millipore membrane filter (Millipore, USA)

Nipro disposable syringe (Nissho, Japan)

Nitrocellulose membrane : Trans-Blot® Blotting Media (Bio-RAD Laboratories, USA)

Paper membrane : Trans-Blot® Blotting Media (Bio-RAD Laboratories, USA)

PCR thin wall microcentrifuge tube 0.2 ml (Eppendorf, Germany)

Pipette tips10, 100, 1000 µl (Axygen Hayward, USA)

Hybond-N^+ membrane (Amersham Biosciences Inc., USA)

2.1.4 Chemicals and reagents

Absolute ethanol (BDH, England)

Abscisic acid (5-[1-hydroxy-2,6,6-trimethyl-4-oxo-cyclohexen-1-yl]-3methyl-{2Z,4E}-pentadienoic acid)

Acetic acid glacial (BDH, England)

Acetosyringone (*Phyto*Technology Laboratories, Inc., USA)

Acrylamide (Merck, Germany)

Agar (Merck, Germany)

Agarose: Seakem LE Agarose (FMC Bioproducts, USA)

Alkaline phosphatase-conjugated AffiniPure Rabbit Anti-Mouse lgG (Jackson ImmunoResearch Laboratories Inc. USA)

Ammoniun sulfate (Sigma Chemical Co., USA)

Ammonium persulfate (Sigma Chemical Co., USA)

Anti-His-Antibody (Amersham Biosciences Inc., USA)

Bacto agar (Difco, USA)

Bacto tryptone (Difco, USA)

Bacto yeast extract (Difco, USA)

6-Benzylaminopurine (BAP) (Sigma Chemical Co., USA)

Beta-mercaptoethanol (Fluka, Switzerland)

Boric acid (Merck, Germany)

Bovine Serum Albumin (Sigma Chemical Co., USA)

5-Bromo-4-chloro-3-indole- β -D-galactopyranoside; X-gal (Sigma Chemical co., USA)

5-Bromo-4-chloro-3-indolyl-β-D-glucoronic acid; X-GLUC (*Phyto*Technology Laboratories, Inc., USA)

Bromophenol blue (Merck, Germany)

Calcium sulfate (Carlo Erba Reagenti, Italy)

Calf thymus DNA (Sigma Chemical Co., USA)

Casein hydrolysate (Merck, Germany)

Cetyltrimethyammonium bromide (CTAB) (Sigma Chemical Co., USA)

Chloroform (Merck, Germany)

dATP, dCTP, dGTP, and dTTP (Promega Co., USA)

2,4-dichlorophenoxy acetic acid (2,4-D) (Sigma Chemical Co., USA)

Dithiothreitol (Sigma Chemical Co., USA)

Diethyl pyrocarbonate: DEPC (Sigma Chemical Co., USA)

Ethidium Bromide (Sigma Chemical Co., USA)

Ethylene diamine tetraacetic acid (EDTA) (Carlo Erba Reagenti, Italy)

Ferrous sulfate (Carlo Erba Reagenti, Italy)

Formamide (Fluka, Switzerland)

Formaldehyde (Sigma Chemical Co., USA)

Ficoll type 400 (Sigma Chemical Co., USA)

GBX developer solution (Kodak, USA)

GBX fixer solution (Kodak, USA)

Glucose (BHD, England)

Glycerol (BDH, England)

Glycine (Sigma Chemical Co., USA)

Glacial acetic acid (Carlo Erba Reagenti, Italy)

Hydrochloric acid (Merck, Germany)

Isoamylalcohol (Merck, Germany)

Isopropanol (Merck, Germany)

Iso-1-thio-β-D-thiogalactopyranoside: IPTG (Serva, Germany)

L-Glutamine (Duchefa Biochemie BV, Netherlands)

L-proline (Duchefa Biochemie BV, Netherlands)

Lambda DNA (Promega Co., USA)

Lithium chloride (Sigma Chemical Co., USA)

Nicotinic acid (Sigma Chemical Co., USA)

N,N'-methylene-bis-acrylamide (Sigma Chemical Co., USA)

Magnesium sulfate (Sigma Chemical Co., USA)

Methanol (Merck, Germany)

Methylene blue (Carlo Erba Reagenti, Italy)

Oligo $(dT)_{15}$ Primer (Promega Co., USA)

Phenol crystal (BDH, England)

Phenol solution (Sigma Chemical Co., USA)

Phenylmethylsulfonyl fluoride: PMSF (USB, USA)

Polyvinyl pyrrolidone (Sigma Chemical Co., USA)

Potassium acetate (Merck, Germany)

Potassium nitrate (BDH, England)

Random hexamers (Promega Co., USA)

Ribonuclease inhibitor (Promega Co., USA)

Sodium acetate (Carlo Erba Reagenti, Italy)

Sodium chloride (Carlo Erba Reagenti, Italy)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium dihydrogen orthophosphate (Carlo Erba Reagenti, Italy)

di-Sodium dihydrogen orthophosphate anhydrous (Carlo Erba Reagenti, Italy)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

Sugar (Mitr Phol Sugar crop., Ltd., Thailand)

Triethanolamine (Sigma Chemical co., USA)

Tri-Reagent® (Molecular research center, Inc., USA)

Tris-(hydroxyl methyl)-aminomethane (Fluka, Switzerland)

Triton X-100 (Merck, Germany)

Xylene Cyanol FF (Sigma Chemical co., USA)

2.1.5 Enzymes

DNA polymerase, Large (Klenow) fragment (New England Biolabs, Inc., USA)

Restriction endonucleases: *Bst*EII, *Eco*RI, *Eco*RV, *Hin*dIII, *Nco*I, *Nhe*I

(Fermentus, Inc., USA)

MMLV reverse transcriptase (Promega Co., USA)

RNaseA (Promega Co., USA)

RQ1 RNase-free DNase (Promega Co., USA)

T4 DNA ligase (Promega Co., USA)

Taq DNA Polymerase (Fermentus, Inc., USA)

2.1.6 Kits and Plasmids

InsT/Aclone ™ PCR Product Cloning Kit (Fermentus, Inc.,USA)

(Appendix A)

QIAquick™ Gel Extraction kit (Qiagen, Germany)

QIAprep Miniprep kit (Qiagen, Germany)

pGEM®-T vector system I (Promega Co., USA) (Appendix A)

pCAMBIA1301 (Appendix A)

2.1.7 Radioactive

[α-^{32}P] dCTP (1000-3000 Ci/mmole) (Amersham Biosciences Biotech)

2.1.8 Antibiotics

Ampicilin (Sigma Chemical Co., USA)

Cefotaxime (UTOPIAN Co., Ltd., Thailand)

Hygromycin B (*Phyto*Technology Laboratories, Inc., USA)

Kanamycin (Sigma Chemical Co., USA)

Rifampicin (Sigma Chemical Co., USA)

2.1.9 Oligonucleotide primers

The oligonucletide primers were synthesized by Bioservice Unit (BSU), Thailand., Operon, Germany and Qiagen, Germany.

2.1.10 Microorganisms

Escherichia coli

strain DH5α *(F' / end*A1 *hsd*R17 (r_k^- m_k^+) *supE44 thi*-1 recA1 *gyr*A96 *(Nal^r) relA1Δ(lacZ*YA-*arg*F)*U169 deo*R *(Φ*80d*lacZ*Δ(lacZ)M15)

strain *XL1-Blue* (F':: Tn*10 proA$^+$B$^+$lacI^q Δ(lacZ)M15/recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_k^- m_k^+) supE44 relA1 lac*)

Agrobacterium tumefaciens strain EHA105 (pEHA105); a hypervirulent, L,L-succinamopine helper strain. (pEHA105 is a T-DNA deletion derivative of pTibo542, the hypervirulent Ti plasmid of *A. tumefaciens* strain A281).

2.2 Bacterial growth medium

Luria-Bertani broth (LB medium) (Maniatis et al., 1982)

LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.2 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121° C. If needed, a selected antibiotic drug was then supplemented.

2.3 Methods

2.3.1 *OsCam1-1* and 35SCaMV-gus-nos PCR product cloning

2.3.1.1 Primer design

Two pairs of oligonucleotide primers were designed band on the *OsCam1-1* cDNA sequence and the coding sequence of *gus* gene under the control of 35SCaMV promoter and *nos* gene of pCAMBIA1301 (*35SCaMV-gus-nos*). The cDNA sequence endcoding the *OsCam1-1* gene (Os.2226) and its deduced amino acid sequence are shown in Figure 2.1 Base on the *OsCam1-1* cDNA sequence, a pair of primers for amplifying the coding region of the *OsCam1-1* gene (Os.2226) was designed with *Nco*I and *Nhe*I restriction sites engeneered at the 5′ and 3′ ends, respectively while that for amplifying the *35SCaMV-gus-nos* cassette was designed with *Hin*dIII restriction sites engeneered at both 5′ and 3′ ends. The sequence and the length of the oligonucleotide primers are shown in Table 2.1.

2.3.1.2 PCR amplification

The coding region of *OsCam1-1* gene and the *35SCaMV-gus-nos* cassette were amplified from the *OsCam1-1* cDNA clone which was obtained from The National Institute of Agrobiological Sciences, Japan, and the plasmid pCAMBIA1301, respectively. The amplification reaction were performed in 50 µl reaction volume containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 50-100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase (Fermentus, Inc., USA). PCR amplification was performed as follows : pre-denaturation at 94 °C for 5 minutes, 30 cycles of denaturation at 94°C for 3 minutes, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The final extension step was performed at 72 °C for 20 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3.1.3 Agarose gel electrophoresis

For Agarose gel electrophoresis, the standard method was used for separation DNA fragments on the basis of their molecular weights and the rough estimation of DNA on the basis of its direct relationship between the amount of DNA and the level of the fluorescence after ethidium bromide staining. The DNA was run on 1.2% agarose gel in Tris-acetate-EDTA (TAE) buffer. The gel was prepared by adding 0.6 g of agarose to 50 ml of 1X TAE. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50-60°C before pouring into a plastic gel former with a present well-forming comb. The concentration of agarose gel used varies with the size of the DNA fragments to be separated. Generally, 1.0-1.8% gel was used.

```
5′
       CATTCTCTCCGCGACGGTCTCGTCTTCCCCACCCCTCGCCTCCTCGCGCTCGG
TGAGAGAAGCGAAGAAGAAGAAGAGGAGGAGGAAGAAGCCAGGCTAAGCCCAGCGGC
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
TCTGTTAAGGCTTGCTCTGATCTCTCCGAAAAAAAAAAAAAAAAAAAAA
                                                          3′
```

Figure 2.1 The cDNA and the deduced amino acid of *OsCam1-1* gene. The deduced amino acid sequence is shown with the Ca^{2+}-binding motifs underline.

Table 2.1 Sequences of the oligonucleotides primers used for amplification of the *OsCam1-1* gene and the *35SCaMV-gus-nos* cassette. The restriction sites designed for cloning were underlined.

Primer	Length (bp)	Sequence (5′-3′)
Os2226 F (NcoI)	20	CCATGGCGGACCAGCTCACC
Os2226 R (NheI)	22	GTGCTAGCCTTGGCCATCATGA
Pcb F	28	AAGCTTTCTCGAGCTGGCGTAATAGCGA
Pcb R	28	CTGAAGCTTTAATTCCCGATCTAGTAAC

The ladder obtained from restriction enzyme digestion was analyzed according to their sizes. The size was estimated from the standard curve of molecular weight markers, i.e., fragments of Lambda DNA digested with *Hin*dIII and fragments of pBR322 digested with *Msp*I. The calibration curve was plotted between logarithmic molecular massed and their relative mobilities.

The DNA were mixed with 10% (v/v) of DNA gel loading Buffer (0.1M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue). The mixture was loaded into slots of the gel which was submerged in the electrophoretic chamber filled with TAE. Electrophoresis was carried out at constant 100 volts. The duration of the running times depended on the size of DNA. Generally, the gel was run until the bromophenol blue moved to 1 cm from the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 3-5 minutes and was destained with an appropriate amount of water with gentle shaking for 10 minutes to remove unbound ethidium bromide from the agarose gel. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed.

2.3.1.4 Extraction of DNA fragment from agarose gel

The amplification products generated by PCR were recovered from agarose gel by using QIAquick gel extraction protocol (QIAGEN, Germany). After electrophoresis, the desired DNA fragment was excised as gel slice from an agarose gel using a scalpel and transferred to a microcentrifuge tube. Three volumes of buffer QG (supplied by manufacturer) were added and incubated for 10 minutes at 50°C or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3

minutes during the incubation period. The gel mixture should be in yellow after the gel is completely dissolved. The mixture was transferred into a QIAquick column and centrifuged at 12,000 rpm for 1 minute. The flow through solution was discarded. Another 500 µl of buffer QG was added and centrifuged at 12,000 rpm for 1 minute. After this step, a 750 µl of buffer PE (supplied by manufacturer) was added to the QIAquick column and centrifuged at 12,000 rpm for 1 minute. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace element of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted by an addition of 30 µl of steriled water to the center of the QIAquick column and was let the column standing for 1 minute, and then centrifuged at 12,000 rpm for 1 minute. The DNA concentration was determined by agarose gel electrophoresis.

2.3.1.5 Ligation of *35SCaMV-gus-nos* PCR product to pGEM®-T vector

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in a ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The *35SCaMV-gus-nos* PCR product purified by the QIAquick Gel Extraction Kit (see in 2.3.1.4) was ligated to the pGEM®-T vector (see in Appendix 1). The ligation reaction was performed in the total volume of 10 µl containing 330 ng of the PCR product, 50 ng pGEM®-T vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-

HCl pH 7.8, 2 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG 8000), 3 units of T4 DNA ligase. All components were mixed and incubated at 4 °C overnight. The ligation product was electrotransformed into *E. coli* XL1-Blue.

2.3.1.6 Transformation of ligated products to *E. coli* host cells by electroporation

a) Preparation of *E. coli* competent cells

Competent *E. coli* strain XL1-Blue was prepared according to the method of Sambrook *et al.* (1989). The glycerol stock of *E.coli* was streaked onto LB agar plate (2.2) and incubated at 37 °C overnight. Ten milliliters of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) was inoculated with a single colony of *E. coli* strain XL1-Blue and incubated at 37 °C with shaking at 250 rpm overnight. One liter of LB broth was inoculated with the starting culture and the culture was incubated at 37 °C with shaking at 250 rpm for 3-4 hours until the optical density at 600 nm (OD_{600}) of cells reached 0.4-0.6. The cells were chilled on ice for 15-30 minutes and harvested by centrifugation at 8,000 rpm, 4 °C for 15 minutes. The supernatant was removed as much as possible. The cell pellet was washed with 100 ml of cold sterile deionized water, resuspended by gently mixing and centrifuged at 8,000 rpm, 4 °C for 15 minutes. The supernatant was discarded. The cells was resuspended and centrifuged further with 50 ml cold sterile deionized water, followed by 20 ml of cold steriled 10% glycerol twice. Finally, the cells were resuspended in 300 µl of cold 10% glycerol. The cell suspension was devided into 45 µl aliquots and stored at -80 °C for later use.

b) Electoporation of recombinant DNA

The competent cells were gently thawed on ice. Forty five microliters of competent cells were mixed well with 1 to 3 µl of the ligation mixture (2.3.1.5), and then placed on ice for 1 minute. The cells were transferred to a cold cuvette, chilled on ice previously and transformed by setting the GENE pulser apparatus (Bio-RAD) as follows: 25 F, 200 Ω of the pulse controller unit, and 2.50 kV. After that, 1 ml of LB broth was added immediately to the cuvette and quickly resuspended with a pasture pipette. The cell suspension was transferred to a new tube and incubated at 37 °C with shaking at 250 rpm for 60 minutes. Finally, the cell suspension was spread on LB agar plates which contained 100 µg/ml ampicillin, 10µl of 25mg/ml Iso-1-thio-β-D-thiogalactopyranoside (IPTG), and 70µl of 25mg/ml 5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside (X-gal) and incubated at 37 °C overnight. The recombinant clones containing inserted DNA were white while those without inserted DNA were blue. The white colonies containing potential recombinant plasmids were selected.

2.3.1.7 PCR product cloning by using InsT/Aclone™ PCR Product Cloning Kit

a) ligation of *OsCam1-1* PCR product to pTZ57R/T vector

The purified *OsCam1-1* PCR product (see in 2.3.1.4) was ligated to the pTZ57R/T. The 30 µl ligation reaction was composed of 165 ng of plasmid vector pTZ57R/T, 459 ng of the PCR product, 3 µl of 10x ligation buffer (400 mM Tris-HCL, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8), 3 µl of 10x PEG 4000 solution, and 5 weiss units of T4 DNA ligase. The reaction components were gently mixed and incubated at 22 °C overnight.

b) Preparation of *E. coli* competent cells

The glycerol stock of *E.coli* strain XL1-Blue was streaked onto LB agar plate (2.2) and incubated at 37 ºC overnight. Two milliters of TransformAid™ C-medium was inoculated with a single colony and incubated at 37 ºC with shaking at 250 rpm for overnight. 150 µl of overnight culture was added to 1.5 ml of the pre-warmed C-medium and incubated in a shaker at 37 ºC for 20 minutes. The cells were harvested by centrifugation at 14,000 rpm for 1 minute at 4 ºC, resuspended in 300 µl of cold TransformAid™ T-Solution (mixed equal volumes of T-Solution (A) and T-Solution (B)) and incubated on ice for 5 minutes. The pelleted cells were recentrifuged as above, resuspended in 120 µl of TransformAid™ T-Solution and incubated on ice for 5 minutes. The cell suspension was divided to 50 µl aliquots.

c) Transformation system

The 50 µl of competent cells were mixed well with 2.5 µl of the ligation mixture and incubated on ice for 5 minutes. Finally, The total mixture was spread on a selective LB agar plate containing 100 µg/ml ampicillin, 25µl of 25mg/ml Iso-1-thio-β-D-thiogalactopyranoside (IPTG), and 25µl of 25mg/ml 5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside (X-gal) and incubated at 37 ºC overnight. The white colonies containing potential recombinant plasmids were selected.

2.3.1.8 Analysis of recombinant plasmids

a) Plasmid DNA isolation by alkaline lysis method

A single colony of *E. coli* harboring potential recombinant plasmids was selected and grown in 1 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract,

and 1% (w/v) NaCl) containing 100 µg/ml of ampicillin overnight at 37 ºC with shaking at 250 rpm. The cells were spun in a microcentrifuge at 14,000×g for 1 minute at 4 ºC. The cells were resuspended in Lysis buffer (50mM of Tris base, 10mM of $Na_2EDTA.H_2O$ and 100 µg/ml of RNaseA) and mixed by pipetting up and down. The suspension was allowed to stand at room temperature for 5 minutes, then 300 µl of Alkaline-SDS solution (200mM NaOH and 1% SDS) was added. The suspension was inverted several times to mix and allowed to stand on ice of 5 minutes. 300 µl of High salt solution (3M of potassium acetate) was added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 14,000 rpm, 4 ºC for 15 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was precipitated by adding 480 µl (0.6 volumes) of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 30 minutes to collect the precipitated DNA. The pellet was resuspended in 90 µl of sterile water and the suspension was vortexed gently. 10 µl of 3M sodium acetate, pH7 and 300 µl of cold absolute ethanol were added to the mixture. The mixture was mixed and chilled on ice overnight. The DNA was collected by centrifuging at 14,000×g for 20 minutes at 4 ºC. The pellet was rinsed with 300 µl of 70% ethanol and allowed to dry for 10-15 minutes. The plasmid DNA was resuspended with 50 µl of sterile water and stored at -20 ºC.

b) Restriction enzyme analysis

Restriction endonuclease was used to cut DNA based on its specific binding property and cleaving double-stranded DNA at a specific sequence. The recombinant plasmids isolated by alkaline lysis method (2.3.1.8 a) were analyzed for the presence

of the interested cloned fragments by digestion with appropriate restriction endonucleases. The *OsCam1-1* recombinant plasmids were digested with *Nco*I and *Nhe*I while the 35SCaMV-gus-nos recombinant plasmids were digested with *Nhe*I. Each of the reactions was carried out in 20 µl mixture at 37 ºC. The products were analyzed by 1% agarose gel electrophoresis. The size of DNA insert is compared with λ / *Hin*dIII ladder.

2.3.2 Construction of *OsCam1-1* gene under control of 35SCaMV promoter

a) Digestion of recombinant plasmids

The concentration of the isolated *OsCam1-1* and 35SCaMV-*gus-nos* recombinant plasmids (2.3.1.8 a) were estimated by measuring the absorbance at 260 nm, and calculated in µg/ml unit, using the following equation:

$$[\text{DNA}] = A_{260} \times \text{dilution factor} \times 50^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the DNA of approximately 50 µg/ml (Sambrook *et al.*, 1989).

The recombinant plasmids were digested with *Nco*I and *Nhe*I using the condition recommended by the manufacturer. Each reaction contains about 5 µg of DNA in a final volume of 50 µl containing 1X enzyme reaction buffer and 2-5 units of restriction enzyme. The reaction was incubated at 37 ºC overnight. The restriction products were analyzed on agarose gel and the expected fragment were extracted from the agarose gel using QIAquick gel extraction Kit (2.3.1.4). The DNA fragments were dissolved in 30 µl of sterile water.

b) Replacement of *gus* gene with *OsCam1-1* gene in pGEM® containing the 35SCaMV-*gus-nos* cassette

The *Nco*I and *Nhe*I-digested *OsCam1-1* gene was ligated to pGEM® containing the 35SCaMV-*gus-nos* cassette digested with the same restriction enzymes to replace the fragment of the removed *gus* gene. The ligation reaction was performed in the total volume of 10 µl containing 250 ng of the purified *OsCam1-1* fragment (2.3.1.9), 50 ng of the digested pGEM® recombinant plasmid with *gus* gene removed, 1X T4 DNA ligase buffer (50 mM Tris-HCL pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA), 5.5 weiss units of T4 DNA ligase. The reaction components were mixed and incubated at 16 °C overnight. The ligation product was electrotransformed into *E. coli* XL1-Blue. After transformation, white colonies were randomly selected. The recombinant plasmids were extracted and analyzed by *Nco*I and *Nhe*I digestion. The restricted products were analyzed by agarose gel electrophoresis.

2.3.3 Construction of plant expression vector under control of 35SCaMV promoter

The pCAMBIA1301 was employed for plant transformation. The pCAMBIA1301 contains a GUS (*uid*A) with an intron as a reporter gene, and a hygromycin-resistant gene (*hpt*) as a plant selectable marker within the T-DNA. Each gene was under the control of a 35S promoter from cauliflower masaic virus (CaMV). The *npt* II gene encoding resistance to kanamycin was used as a bacterial selectable marker.

The 35SCaMV-*OsCam1-1-nos* cassette was excised from pGEM®-35SCaMV-*OsCam1-1-nos* by *Hin*dIII digestion and ligated with pCAMBIA1301

digested with the same restriction enzyme to generate pCAMBIA-35SCaMV-*OsCam1-1-nos*. The ligation reaction was performed in the total volume of 20 µl containing the ratio between pCAMBIA1301 vector and 35SCaMV-*OsCam1-1-nos* cassette of 1:6, 1X T4 DNA ligase buffer (50 mM Tris-HCL pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA), 5.5 weiss units of T4 DNA ligase. The reaction mixture was incubated at 16°C overnight. The ligation product was electrotransformed into *E. coli* XL1-Blue. The white colonies were selected. The potential recombinant plasmids were extracted and analyzed by *Hin*dIII digestion. The restricted products were separated by agarose gel electrophoresis. The resulting recombinant plasmid was called pCAMBIA1301-35SCaMV-*OsCam1-1-nos* which contained the expression cassette of the 35SCaMV promoter, the *OsCam1-1* coding sequence and the nos terminator.

2.3.4 Sequence analysis of pCAMBIA1301-35SCaMV-*OsCam1-1*-Nos

The pCAMBIA1301-35SCaMV-*OsCam1-1*-Nos recombinant plasmids were extracted by QAIprep Miniprep kit (Qiagen, Germany) and nucleotide sequences of the inserts were determined. DNA sequencing was carried out at Macrogen, Korea. The sequences of forward and reverse primers used for sequencing were as follows:

Os.2226check-F	5′-AACCTGATGGCACGCAAGAT-3′
Os.2226check-R	5′-TCGTCGGTCAGCTTCTCGCC-3′

2.3.5 Preparation of plants for transformation

2.3.5.1 Rice calli

Seeds of the *indica* rice cultivar (*Oryza sativa* L.) KDML 105 were obtained from Kasetsart University. Mature seeds were dehusked and first sterilized with 70%

ethanol for 2 minutes and then with 15% Clorox for 1 hour with shaking. The seeds were rinsed 4 times with sterile water. These were cultured on 2NB medium [Li *et al*., 1993; NB basal medium supplemented with 2 mg/l of 2,4-dinitrophenoxy acetic acid (2,4-D)] for callus induction. The cultures were incubated in the dark at 28 ºC for 6 weeks. The calli observed as being yellowish and granular were separated with sterile scalpel and subcultured on fresh medium. The growing calli (1-2 mm in diameter) were used for plant regeneration and transformation experiments.

2.3.5.2 Tobacco leaf discs

Tobacco plants (*Nicotiana tabacum* cv. Virginia Coker) culturally grown on MS medium (Murashige and Skoog, 1962) were obtained from Department of Botany, Faculty of Science, Chulalongkorn University, and subcultured to fresh MS basal. The cultures were incubated at 25 ºC under a 16/8 hours light/dark photoperiod. The leaves of tobacco from were detached from *in vitro* plantlets and placed on a Petri dish. The leaves were cut off as square sections approximately 1.0 cm^2 and made 1-2 mm of wounds on the square leaves by scalpel.

2.3.6 Transformation of pCAMBIA1301-35SCaMV-*OsCam1-1-nos* and pCAMBIA1301 to *A. tumefaciens* EHA105 host cells by electroporation

a) Making competent *A. tumefaciens* EHA105 cells

A. tumefaciens EHA105 was streaked on solid LB medium containing 25 µg/ml rifampicin and incubated at 30 ºC for 2 days. Ten milliliters of LB-broth supplemented with 25 µg/ml rifampicin was incubated with a single colony and incubated at 30 ºC for 8 hours by shaking. One liter of LB-broth was inoculated with the starter culture and incubated at 28 ºC with shaking to the OD_{600} of 1.0 to 1.5. The

cells were harvested by centrifugation in a cold rotor at 5,000 rpm for 15 minutes. The pellet was resuspended in 1 liter of cold sterile distilled water and centrifuged as above. The supernatant was carefully discarded. The cell pellet was resuspended in 500 ml of cold sterile distilled water and centrifuged again. Then, the pellet was resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and supernatant was removed. Finally, the cell pellet was resuspended 1 ml of 10% glycerol. The cell suspension was divided to 45 µl aliquots. These cells could be used immediately or kept at -80 ºC for 6 months.

b) Electrotransfomation of binary vectors to *A. tumefaciens* EHA105

A. tumefaciens EHA105 were transformed with the pCAMBIA1301-35SCaMV-*OsCam1-1-nos* recombinant vector and pCAMBIA1301 vector by electrotransfomation as described above (2.3.1.6 b). The electroporated cells were immediately removed from the cuvette and added to a new tube containing 1 ml of LB medium. The cell suspension was incubated at 30 ºC with shaking for 2 hours. Fifty microliters of the cell suspension was spread LB agar plates containing 50 µg/ml of kanamycin, 25 µg/ml rifampicin and incubated at 30 ºC for 2 days.

c) Restriction enzyme analysis of *A. tumefaciens* transformant

The selected clones were grown in 5 ml of LB broth containing 50 µg/ml of kanamycin, 25 µg/ml rifampicin and incubated overnight at 30 ºC with shaking. The plasmid DNA was isolated by alkaline lysis method (2.3.1.8 a). The recombinant plasmids were analyzed by digestion with *Hin*dIII. The reaction was carried out in 20 µl mixtures at 37 ºC overnight. At the end of digestion, the products were analyzed and compared with λ/*Hin*dIII marker by 1% agarose gel electrophoresis.

2.3.7 Transformation of binary vectors by co-cultivation with *Agrobacterium*

2.3.7.1 Co-cultivation of rice calli

A. tumefaciens strain EHA105 harboring pCAMBIA1301-35SCaMV-*OsCam1-1*-Nos and pCAMBIA1301 without the gene, used to generate control plant, were streaked on solid AB medium (Appendix B) containing 25 μg/ml rifampicin and 50 μg/ml kanamycin, and incubated at 30 °C for 2 days. A single colony was collected by scraping from plates with a loop. The bacteria were resuspended in AAM medium (Appendix B) supplemented with 300 μM of acetosyringone with shaking. The optical density at 600 nm of the bacterial suspension was adjusted to 1.0 by diluting with AAM medium. The calli from section 2.3.5.1 were subcultured to fresh medium and incubated in the same condition for 4 days before using in co-cultivation. The calli were immersed in the bacterial suspension for 30 minutes with occasional shaking. Subsequently, the calli were blotted dry on sterile filter papers and then transferred to co-cultivation medium (2NB-AS; 2NB containing 300 μM of acetosyringone). The calli were incubated in the dark at 25 °C for 3 days.

2.3.7.2 Selection and regeneration of transformed rice calli

After three-day co-cultivation, the calli were washed with sterile water containing 250 μg/ml cefotaxime for 15 minutes to discard the excess of *Agrobacterium*, and rewashed with sterile water. Then, The calli were blotted dry on sterile filter papers. The co-cultivated calli were transferred to selection medium (2NB-CH; 2NB containing 500 μg/ml cefotaxime and 50 μg/ml hygromycin) and incubated at 28 °C for 4 weeks. The hygromycin resistant calli obtained after the first round selection were subcultured for more cycles of selection on fresh 2NB-CH medium every 2 weeks. The hygromycin resistant calli were then transferred to

regeneration medium without any antibiotics (NB-RE) and incubated at 28 ºC under 16 hours light photoperiod for 3-4 weeks. Green buds / shoots were observed after 4 weeks. When the transformed shoots became 1.0 to 1.5 cm height, they were cut from calli and transferred to NB medium for stimulation of roots and stem elongation for 4 weeks. The transgenic plants were subcultured onto fresh NB medium every 4 weeks.

2.3.7.3 Co-cultivation of tobacco leaf disc

A. tumefaciens strain EHA105 harboring pCAMBIA1301-35SCaMV-*OsCam1-1*-Nos and pCAMBIA1301 without the gene, used to generate control plant, were separately streaked on solid LB medium containing 50 µg/ml kanamycin and incubated at 30 ºC for 2 days. Ten milliliters of LB broth were inoculated with single colony and incubated at 30 ºC with shaking for 2 days. Then, the cell suspension was diluted with equal volume of sterile liquid MS medium containing 0.5 mg/l MES. Leaves discs (2.3.2.2) were immersed in the cell suspension for 5 minutes. After that, the co-cultivated leaves were transferred onto CM medium (MS basal medium supplemented with 1 mg/l of BA and 1 mg/l of NAA) and incubated at 25 ºC under a 16/8 hours light/dark photoperiod for 2 days.

2.3.7.4 Selection and regeneration of transformed tobacco

After 2 days, the co-cultivated leaves were transferred to selection medium (CM medium supplemented with 50 mg/l of hygromycin and 250 mg/l cefotaxime) and incubated at 25 ºC under a 16/8 hours light/dark photoperiod for 7 days. Then, these leaf explants were transferred to shoot induction medium (MS basal medium supplemented with 1 mg/l of BA and 250 mg/l cefotaxime) and subcultured to fresh medium every 2 weeks until shoots appeared. The healthy shoots were separated from

the rest of leaf explants and transferred to MS basal medium for stimulation of roots. The transgenic tobaccos were subcultured on free medium every 4 weeks.

2.3.8 Charaterization of transgenic plants by histochemical analysis for β-Glucuronidase (GUS) activity

Histochemical GUS assays were made after co-cultivation of calli with EHA105 (pCAMBIA1301) and after leaves and roots were regenerated. The tissues were placed in microcentrifuge tubes and 500 µl of histochemical (X-Gluc) straining solution composed of 1mM of X-Gluc, 100 mM NaH_2PO_4/Na_2HPO_4, 0.5 mM K Ferrocyanide, 0.5 mM K Ferricyanide, 0.1% (v/v) Triton X-100 and 10 mM EDTA was added. The tissues were vacuum infiltrated for ten minutes and incubated at 37 ºC overnight. To facilitate detection of the blue color, 70% of ethanol was added to remove the chlorophyll and to detect the formation of blue color.

2.3.9 Molecular analysis of transformed plants

2.3.9.1 Analysis of *gus* and *OsCam1-1* genes in genomic DNA of transformed plants by PCR

a) Genomic DNA extraction

Tissues of the transformed plants were harvested and ground to a fine powder in liquid nitrogen using chilled mortars and pestles. The frozen powder was immediately transferred to 1.5 ml microcentrifuge tubes containing 700 µl of preheated extraction buffer (60 ºC) (100 mM Tris-HCl (pH8.0), 40 mM EDTA (pH7.5), 1.4 M NaCl, and 2% (w/v) CTAB) and 3 µl of 2-mercaptoethanol. Then, the mixture was inverted several times to mix and incubated at 60 ºC for 30 minutes. During the incubation time, the mixture was inverted gently every 10 minutes to

ensure efficient extraction. After that, the mixture was left at room temperature for 5 minutes. Then, 500 µl of chloroform:isamyalcohol (24:1) was added and mixed for 5 minutes followed by centrifuged at 14,000 rpm for 10 minutes at 4 °C to separate phases. The upper aqueous phase was transferred to a new tube. Genomic DNA was precipitated by the addition of 0.1 volumes of 3 M NaOAc and 0.6 volumes of isopropanol. The suspension was mixed and incubated at -20 °C for 30 minutes. The DNA pellet was collected by centrifugation at 14,000 rpm for 10 minutes at 4 °C and air-dried for 10-15 minutes. The genomic DNA was resuspended with 100 µl of TE buffer (10 mM Tris-HCl, pH7.4 and 1mM EDTA). RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA and the mixture was incubated at 37 °C for 1 hour. After that, an equal volume of phenol:chloroform:isamyalcohol (25:24:1) was added. The sample was mixed gently and centrifuged for 1 minute. The upper aqueous phase was transferred to a fresh tube and extracted once with an equal volume of chloroform-isoamyl alcohol. The mixture was centrifuged at 14,000 rpm for 1 minute at 4 °C and the top aqueous phase was collected. 0.1 volume of 3 M NaOAc and 2.5 volumes of cold absolute ethanol were added and mixed gently. The suspension was allowed to stand for 30 minutes at -20 °C and then centrifuged at 14,000 rpm for 10 minutes to collect the genomic DNA. The pellet was rinsed with 100 µl of 70% ethanol and centrifuged at 14,000 rpm for 5 minutes. The genomic DNA was air-dried and resuspended with 50 µl of sterile water. The DNA was analyzed by agarose gel electrophoresis and the concentration was estimated by measuring the absorbance at 260 nm as described in 2.3.2 a).

b) Verification of *gus* and *OsCam1-1* gene in transformed plants

The putative transformants were screened using 35Scheck-F and OsCam1check-R primers for checking *OsCam1-1* gene while *gus* gene was screened with 35Scheck-F and GUScheck-R primers. Their sequences were as follows:

35Scheck-F	5′-TGAAGATGCCTCTGCCGACACTGGT-3′
OsCam1check-R	5′-GTCAGCTTCTCGCCGAGGTTGGTCA-3′
GUScheck-R	5′-CAGGTGTTCGGCGTGGTGTAGAGCA-3′

The amplification reactions were performed in a 50 µl reaction volume containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 50 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase (Fermentus, Inc., USA). The reaction was pre-denatured at 94 °C for 5 minutes following by 30 cycles of denaturation at 94°C for 3 minutes, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes. After that, 15 µl of the reaction product was analyzed and compared with a λ/*Hin*dIII by agarose gel electrophoresis.

2.3.9.2 Southern Blot Analysis

a) Digestion genomic DNA with restriction enzymes

The extracted genomic DNA (2.3.2.7.1 a) was digested with *Hin*dIII. The reaction contains 50 µg of DNA, 1X enzyme reaction buffer and 30 units of restriction enzyme in final volume 20 µl. The reaction was incubated at 37 °C overnight. After that, 2 µl of the reactions were analyzed by 1% agarose gel electrophoresis, visualized on an UV transilluminator and photographed.

b) Agarose gel electrophoresis

Forty five micrograms of the digested genomic DNA were mixed with 10% (v/v) of DNA gel loading buffer (0.1 M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue. and loaded into a 0.8% (w/v) TAE agarose gel. Electrophoresis was carried out at constant 80 volts. The gel was run until the tracking dye reached 1/4 from the bottom of the gel.

c) Southern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 5 gel volumes of denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 20 minutes with gentle agitation and in 5 gel volumes of neutralization solution (0.5 M Trisma base, 3 M NaCl) for 15 minutes with gentle agitation, twice. A tray was filled with 20xSSPE (2.98 M NaCl and 0.2 M $NaH_2PO_4.H_2O$) and a supporting platform was placed on the tray. A piece of nylon membrane was cut to the dimensions of the gel and pre-wetted with distilled water. Three sheets of filter paper were cut in the same size of the gel and one as long to be used as a wick. The platform was covered with a wick so that the edges were submerged in the transfer buffer. The filter paper, same size of the gel was placed on top of the wick. The gel was laid face down on the filter paper. Any air bubbles between the gel and the wet filter were removed. The gel was immediately overlaid with the nylon membrane and any air bubbles were again removed. Strips of saran wrap were cut and laid on each edge of the membrane and draped over the side of the tray. Another sheet of filter paper was place on the nylon membrane. Paper towels, same size as the membrane were cut and stacked on top of the filter to the height of

about 10 cm. Then a weigh was placed on top of stack. When three-day transfer was completed, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The DNA was immobilized on the blotted membrane by UV cross-linking in Bio-Rad GS Gene Linker™ UV chamber and the membrane was stored at room temperature.

d) Preparation of probe

For preparation of the *gus* probe, a partial *gus* gene was taken by digesting pCAMBIA1301 with *Eco*RV. The digested DNA was separated by agarose gel electroporesis and purified by the QIAquick Gel Extraction Kit (2.3.1.4).

Nine microliters of 200 ng of DNA were mixed with 1 µl of 60 ng/µl of random hexanucleotide in the final volume of 10 µl. The mixture was incubated in boiling water for 5 minutes. Then, the denatured DNA was chilled on ice for 30 seconds, and any condensation was collected by a 2-second spin in a minicentrifuge. White tube was held behind a plexiglass shield, the labeling was started by adding 2 µl of 1X klenow buffer, 5 µl of [α-^{32}P]dCTP, 2 µl of H_2O and 1 µl of 5-6 units of klenow fragment of DNA polymerase. The mixture was incubated for 60 minutes at room temperature and the reaction was stopped with 25 mM Na_2EDTA. The solution was passed through a spin column (Bio-Rad) to remove the unincorporated [α-^{32}P]dCTP. To prepare the Bio-Rad spin column, excess liquid was removed and the column was packed by spinning for 2 minutes at 2500 rpm. Then the sample was loaded to the center of the packed resin and collected by spinning for 4 minutes at 2500 rpm. To prepare for hybridization, the probe was incubated in boiling water for at least 5 minutes, then immediately added to the hybridization mixture.

e) Southern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50%(v/v) deionized formamide, 5x SSPE/NaOH (pH 7.4), 20mM $Na_2EDTA.2H_2O$, 1X Denhardt's solution (100x Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone(PVP) at 2%[w/v] each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 µg/ml denatured DNA stock (calf thymus DNA and TF buffer, pH 8.0) was prepared and warmed to 40 °C. The prehybridization solution was added to the blotted membrane placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at 40 °C overnight. When prehybridization was completed, the prehybridization solution was discarded and replaced with the hybridization solution (same as prehybridized solution, but without denatured DNA stock) previously equilibrated to 40 °C, then the prepared denatured ^{32}P-oligolabeled DNA probe was added. After removing bubbles, the bag was sealed by a heat-sealer and incubated for at least 16 hours at the desired temperature. When hybridization was completed, the filter was removed and immediately washed in 2x SSPE, 0.1% SDS twice and once in 1X SSPE, 0.1% SDS at room temperature. If the general level of cpm was too high, washes would be repeated in 1X SSPE, 0.1% SDS at a higher temperature. After that the damp filter was wrapped in plastic wrap and placed in an x-ray cassette with a KODAK BioMax MS film scientific imaging film. The film was exposed overnight at -80 °C. When the blot contained barely enough cpm, exposures of 4 to 7 days were necessary in some cases. For detection, the X-ray film was developed in a developer solution and a fixer solution for 1 minute each and air-dried. The melting temperature (T_M) of an

oligonucleotide hybridized to a target sequence can be calculated using the following equation:

$$T_m = 16.6(\log[Na^+]) + 0.41(GC\%) + 81.5 - (0.7C)(\%\ \text{formamide})$$
$$- \%\ \text{mismatch} - 500/n;\ \text{if } n < 100$$

When the oligonucleotide is used as a probe, hybridization is usually carried out at 5-12°C below the calculated T_m, and prehybridization is usually performed at the same temperature.

2.3.9.3 Expression analysis of *OsCam1-1* mRNA in transformed plants

The transcription of *OsCam1-1* gene in transformed plant was examined using RT-PCR method and northern blot analysis.

2.3.9.3.1 Total RNA extraction

Total RNA was extracted from 50-100 mg of leaves of transformed plants using Tri-Reagent® (Invitrogen Life Technologies). Plant tissues were ground to a fine powder in liquid nitrogen using chilled mortars and pestles, and transferred to microcentrifuge tubes containing 1 ml of Tri-Reagent® immediately. The mixture was homogenized by vortexing and incubated at room temperature for 5 minutes. Consequently, 200 µl of chloroform was added and vortexed again. The mixture was left at room temperature for 15 minutes and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The upper aqueous phase was transferred to a new tube. The total RNA was recovered by adding 500 µl of isopropanal and mixing thoroughly. The mixture was left at room temperature for 5-10 minutes and then centrifuged at 12,000 rpm for 8 minutes. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 12,000 rpm for 15 minutes. The RNA pellet was air-dried

and dissolved in diethylpyrocarbonate (DEPC)-treated water by incubating at 55-60 °C for 10 minutes. The concentration of total RNA was estimated by measuring the optical density at 260 nm, and calculating in µg/ml unit, using the following equation:

$$[RNA] = A_{260} \times \text{dilution factor} \times 40^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook et al., 2001).

2.3.9.3.2 RT-PCR

a) First strand cDNA synthesis

Five micrograms of the total RNA sample (2.3.9.3.1) were added to the DNase treatment mixture (1X M-MLV Reverse Transcriptase (M-MLVRT) buffer, 40 units of recombinant RNasin® ribonuclease inhibitor, and 1 unit of RQ1 DNase). The reaction was incubated at 37 °C for 60 minutes. Consequently, the reaction was incubated in boiling water for 2 minutes and quick-chilled on wet ice to terminate the reaction. One microgram of the DNase-treated RNA was added to the reverse transcription mixture (1X M-MLVRT buffer, 100 ng of oligo $(dT)_{15}$ primer, 100 ng of the dNTP mix, and 200 units of M-MLVRT). The reaction was incubated at room temperature for 10 minutes, then at 42 °C for 60 minutes and immediately placed in boiling water for 3 minutes and quick-chilled on ice to terminate the reaction. The reactions were stored at -20 °C or used to assemble PCR reactions immediately on ice.

b) RT-PCR amplification

The presence of the OsCam1-1 mRNA in putative transformed plants was detected by RT-PCR. The first-strand cDNAs of various transformants were screened

by PCR amplification using Os.2226-F (NcoI) and Os.2226-R (nos) oligonucleotide primers. Their sequences are as follows:

Os.2226-F (NcoI)	5′-CCATGGCGGACCAGCTCACC-3′
Os.2226-R (nos)	5′-TAATCATCGCAAGACCGGCAACAGG-3′

The amplification reaction was performed in 25 µl total volume containing 1X *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 50-100 ng of DNA template, 100 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase (Fermentus, Inc., USA). The PCR profile was composed of initial denaturation at 94 ºC for 5 minutes followed by 30 cycles of denaturation at 94 ºC, 1 minute, annealing at 58 ºC for 30 seconds and extension at 72 ºC for 1 minute. The final extension was performed at 72 ºC for 10 minutes. After amplification, 10 µl of the reaction product was analyzed by 1% agarose gel electrophoresis and its size was compared with λ/*Hin*dIII ladder.

2.3.9.3.3 Northern Blot analysis

a) Formaldehyde-agarose gel electrophoresis

First, one-hundred milliliters of 1.5% (w/v) formaldehyde agarose gel was prepared as follows. A 1.5 g of agarose was mixed with 2.5 ml of Gel buffer 40X, 90 ml of H_2O and was boiled until complete solubilization, and allowed to cool to 50 ºC. The melted agarose gel was mixed with 8.4 ml formaldehyde in 100 ml agarose gel and poured into a chamber set treated with diethylpyrocarbonate solution and washed again with (DEPC)-treated water.

Forty micrograms of the total RNA (2.3.9.3.1) were mixed with sample buffer and incubated at 65 ºC for 10 minutes. The sample was loaded into the 1.5% (w/v) formaldehyde agarose gel. The RNA Ladder (Fermentas, USA) was used as a standard RNA marker. Electrophoresis was carried out in reservoir buffer at 70 volts, until Bromphenol blue reached approximately ¾ of the gel length. The total RNA was visualized as fluorescent bands using a UV transilluminator and photographed.

b) Northern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 250 ml of 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 twice for 20 minutes each time and then transferred to positively charged nylon membrane by the same method of southern blotting (see in section 2.3.9.2 c).

c) Preparation of probe

For preparation of *OsCam1-1* probe, the partial *OsCam1-1* gene was amplified using OsCam1-F and OsCam1-R oligonucleotide primers. Their sequences were as follows:

OsCam1-F	5′-AGGAGCTGGGAACCGTGA-3′
OsCam1-R	5′-GTTGATCTGGCCGTCACC-3′

The PCR reaction was performed in 50 µl total volume containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 50-100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase (Fermentus, Inc., USA). PCR amplification was performed as follows: predenaturation at 94 ºC for 5 minutes, 30 cycles of denaturation at 94°C for 3

minutes, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. The final extension step was performed at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The resulting PCR products were purified as described in 2.3.1.4. The DNA-labeling was performed as indicated in 2.3.9.2 d.

d) Northern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50%(v/v) deionized formamide, 5x SSPE/NaOH (pH 7.4), and 20mM $Na_2EDTA.2H_2O$, 1X Denhardt's solution (100x Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone(PVP) at 2%[w/v] each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 µg/ml denatured DNA stock (calf thymus DNA and TE buffer, pH 8.0) was prepared and warmed to 40 °C. The prehybridization solution was added to the blotted filter placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at 40 °C overnight. When prehybridization was completed, the prehybridization solution was discarded and replaced with the hybridization solution (same as prehybridized solution, but without denatured DNA stock) previously equilibrated to 40 °C, then the prepared denatured ^{32}P-oligolabeled DNA probes was added. After removing bubbles the bag was sealed by a heat-sealer and incubated for at least 16 hours at the desired temperature. When hybridization was completed, the filter was removed and immediately washed in 2x SSPE, 0.1% SDS twice and once in 1X SSPE, 0.1% SDS at room temperature. If the general level of cpm was too high, washes would be repeated in 1X SSPE, 0.1% SDS at a higher temperature. After that the damp filter

was wrapped in plastic wrap and placed in an x-ray cassette with a KODAK BioMax MS film scientific imaging film. Film exposure and signal detection were performed as indicated in 2.3.9.2 e.

2.3.10 Western blot analysis of OsCam1-1 protein in transformed plants

a) Preparation of protein sample

Leaves of transformed plants were harvested and ground in ice-cold homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM EDTA, 20 mM dithiothreitol (DTT) and 0.5 mM PMSF using chilled mortars and pestles. The homogenate was centrifuged at 50,000 xg for 30 minutes and the supernatant was collected and used for protein concentration determination.

b) Protein concentration determination

The concentration of each protein sample was determined according to Bradford's method (Bradford, 1976). One microliter of each protein samples was mixed with 99 µl of distilled water to make a total volume 100 µl. Subsequently, the diluted protein sample was added with 1 ml of Bradford working buffer (0.1% (w/v) Serva Blue G, 10% (v/v) of 85% phosphoric acid and 5% (v/v) of 95% ethanol) and was vortexed. After 5 minutes, the absorbance of the blue color generated was measured at 595 nm using BSA as the standard.

c) Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE)

The gel sandwich was assembled according to the menufacter's instruction. The separating gel (12.5% acrylamide) was prepared by mixing 16.7 ml of

acrylamide, 30% A: 0.8% B, 5 ml of 8x resolving gel buffer, 100 µl of 20% SDS and 18 ml of distilled water in a flask. Then 200 µl of 10% ammonium persulfate and 20 µl of TEMED were added and mixed rapidly by swirling. The solution was introduced into the preset glass using a Pasteur pipette to a level about 2 mm below the bottom of the teeth of the gel comb. After that, the separating gel was overlaid with distilled water. The gel was allowed to polymerize, distinguished by clear interface between the separating gel and water. The water was poured off before the stacking gel was set.

The stacking gel (5% acrylamide) solution was prepared by mixing 2.6 ml of acrylamide, 30%A:0.8%B, 5 ml of 4x stacking gel buffer 50 µl of 20% SDS and 12.3 ml of distilled water. The solution was swirled and then 150 µl of 10% ammonium persulfate and 10 µl of TEMED were added and mixed rapidly. The stacking gel mixture was poured on the separating gel until solution reached top of the short plate. The well-forming comb was inserted. The gel was allowed to set for at least 1 hour. After stacking gel was polymerized, the comb was removed carefully. Then, the gel was placed into electrophoresis chamber. A 1X reservoir buffer (APPENDIX C) was added into the inner and outer reservoir.

Eighty micrograms of protein samples were mixed with 5X sample buffer and boiled for 5 minutes. After that, the sample was loaded into 12.5% SDS polyacrylamide gel. The protein ladder was used as a standard protein marker. The molecular weight marker proteins were β-galactosidase (116,000 Da), Bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactase dehydrogenase (35,000 Da), restriction endonuclease Bsp98I (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da). Electrophoresis was carried out in reservoir buffer at 13 mA. After the dye completely entered the separating gel, the power was then increased to

18 mA. The gel was run until Bromophenol blue reached approximately ¼ from the bottom of the gel.

d) Protein staining

The protein bands on the gel were visualized by staining with Coomassie staining solution (0.25% (w/v) Coomassie Blue R-250, 7% (v/v) glacial acetic acid and 50% (v/v) methanol) for at least 20 minutes. Destaining was performed by immersing the gel in destaining solution (7% (v/v) glacial acetic acid and 10% (v/v) methanol) followed by several change of destaining solution until a clear gel background was obtained (APPENDIX C). The Coomassie Brilliant Blue stained polyacrylamide gel was photographed.

e) Western blotting and detection

Electrophoresed protein was transferred to a nitrocellulose membrane by mini Trans-Blot Electrophoretic Transfer cell (Bio-RAD Laboratories, USA). After electrophoresis, the stacking gel was removed and soaked in 1X transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 30 minutes, twice. A piece of nitrocellulose membrane (Bio-RAD Laboratories, USA) and two sheets of filter paper was cut to the dimensions of the gel and pre-wetted with transfer buffer. The blotted sandwich was assembled in a large tray containing 1X transfer buffer in the following order: two pieces of fiber pad saturated with 1X transfer buffer, electrophoretic gel, nitrocellulose membrane and covered with two layers of saturated filter papers. Air bubbles between layers of component were removed by rolling a clean glass rod across the surface of each component. Finally, the electroblotting of proteins on the gel to nitrocellulose membrane was carried out at 200 mA, room temperature for 5

hours. When the transfer was completed, the membrane was incubated in blocking buffer (5% milk powder in TBS buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl), 0.1% (v/v) Tween 20) for two and a half hours at room temperature. After blocking, the diluted Anti-His-Antibody (1:3,000) (Amersham Biosciences Inc., USA) was added in the blocking buffer and the membrane was incubated for 1 hour by shaking. Then, the membrane was washed twice in TBS-Tween buffer (TBS buffer, 0.05% (v/v) Tween 20) for 10 minutes each time at room temperature. After that, the membrane was incubated in a new blocking solution containing diluted Alkaline Phosphatase-conjugated AffiniPure Rabbit Anti-Mouse lgG (1:5,000) (Jackson ImmunoResearch Laboratories Inc. USA) for 1 hour and was washed twice in TBS-Tween buffer for 10 minutes each time. In the detection step, the membrane was incubated in 10 ml of alkaline phosphatase (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM $MgCl_2$) containing 33 µl of BCIP-T (50 mg/ml in dimethylformamide) and 44 µl of NBT (75 mg/ml in 70% (v/v) dimethylformamide) with gentle shaking. The reaction was terminated by washing the membrane several times in distilled water.

CHAPTER III

RESULTS AND DISCUSSION

3.1 *OsCam1-1* and 35SCaMV-*gus-nos* PCR product cloning

An *OsCam1-1* cDNA clone, obtained from the National Institute of Agrobiological Sciences, Japan, and pCAMBIA1301 plasmid were used as templates for PCR amplification of the coding region of *OsCam1-1* gene and the cassette of the 35SCaMV promoter, the *gus* gene and the *nos* gene (35SCaMV-*gus-nos*), respectively. Base on the *OsCam1-1* cDNA sequence shown in Figure 2.1, a pair of primers for amplifying the coding region of the *OsCam1-1* gene was designed with restriction endonuclease sites for *Nco*I and *Nhe*I engineered at its 5′- and 3′-ends, respectively. The sequence of the forward primer, designated as Os.2226F (*Nco*I), was 5′- CC**ATG**GCGGACCAGCTCACC -3′. The initiation codon ATG was in bold letters and the *Nco*I site added to the 5′ end of the amplified DNA was underlined. The sequence of the reverse primer, designated as Os.2226R (*Nhe*I), was 5′–GTGCTAGCCTTGGCCATCATGA-3′. The *Nhe*I site added to the 3′ end of the amplified DNA was underlined. To amplify the 35SCaMV-*gus-nos* cassette from pCAMBIA1301, another pair of primers designated as PcbF and PcbR, was designed. The sequence of the PcbF primer was 5′-AAGCTTTCTCGAGCTGGCGTA ATAGCGA-3′ and that of the PcbR primer was 5′-CTGAAGCTTTAATTCCCGA TCTAGTAAC-3′. The *Hin*dIII sites designed to engineer both ends of the 35SCaMV-*gus-nos* cassette were underlined.

The amplified PCR products of approximately 0.53 and 3.3 kb, which were the expected sizes of the *OsCam1-1* gene and the 35SCaMV-*gus-nos* cassette, respectively were obtained as shown by agarose gel electrophoresis in Figure 3.1.

Each DNA fragment was purified using the QIAquick gel extraction kit. The purified *OsCam1-1* PCR product was ligated into pTZ57R/T, while the purified 35SCaMV-*gus-nos* PCR product was ligated into pGEM®- T by T4 DNA ligase. The competent XL1-Blue cells were transformed with the ligation reactions. The transformants were selected by blue/white colony screening on ampicillin agar plates containing X-gal and IPTG. White colonies were randomly selected and cultured in 1 ml LB broth containing 100 µg/ml of ampicillin at 37 °C overnight and the cultures were subjected to plasmid extraction. To verify the insertion of the PCR products into their respective vectors, the potential recombinant plasmids containing the *OsCam1-1* were digested with *Nco*I and *Nhe*I while the potential 35SCaMV-*gus-nos* recombinant plasmids were digested with only *Nhe*I at 37 °C overnight. These reactions were analyzed by 1.2% agarose gel electrophoresis. The results showed that DNA fragments of *OsCam1-1* and 35SCaMV-*gus-nos* of approximately 0.53 and 6.3 kb in length, respectively were obtained as expected as shown in Figure 3.2 and Figure 3.3.

3.2 Construction of *OsCam1-1* gene under control of 35SCaMV promoter

To replace the *gus* gene in the 35SCaMV-*gus-nos* cassette with the *OsCam1-1* gene, both recombinant plasmids (from 3.1) were extracted and digested with *Nco*I and *Nhe*I. The DNA fragments of approximately 0.53 kb of the *OsCam1-1* and 4.3 kb of the pGEM®-35SCaMV-gus-nos with the *gus* removed recombinant plasmid (pGEM®-35SCaMV-nos) were obtained and purified. The purified products were analyzed by 1.2% agarose gel electrophoresis (Figure 3.4). The *Nco*I and *Nhe*I-digested *OsCam1-1* fragment was ligated into the same restriction sites in the pGEM®-35SCaMV-nos recombinant plasmid. The ligation products were transformed to *E. coli* XL1-Blue. The transformants were selected by ampicillin resistance and

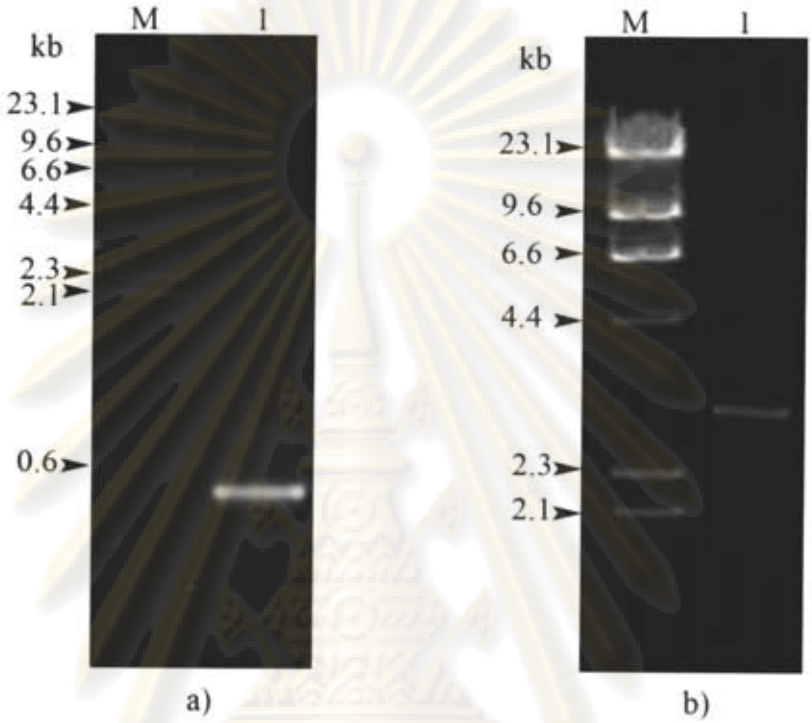

Figure 3.1 Agarose gel electrophoresis of the amplified fragments of the *OsCam1-1* gene and the 35SCaMV-*gus-nos* cassette. The PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 *OsCam1-1* fragment

b) Lane M λ/*Hin*dIII standard marker

Lane 1 35SCaMV-*gus-nos* fragment

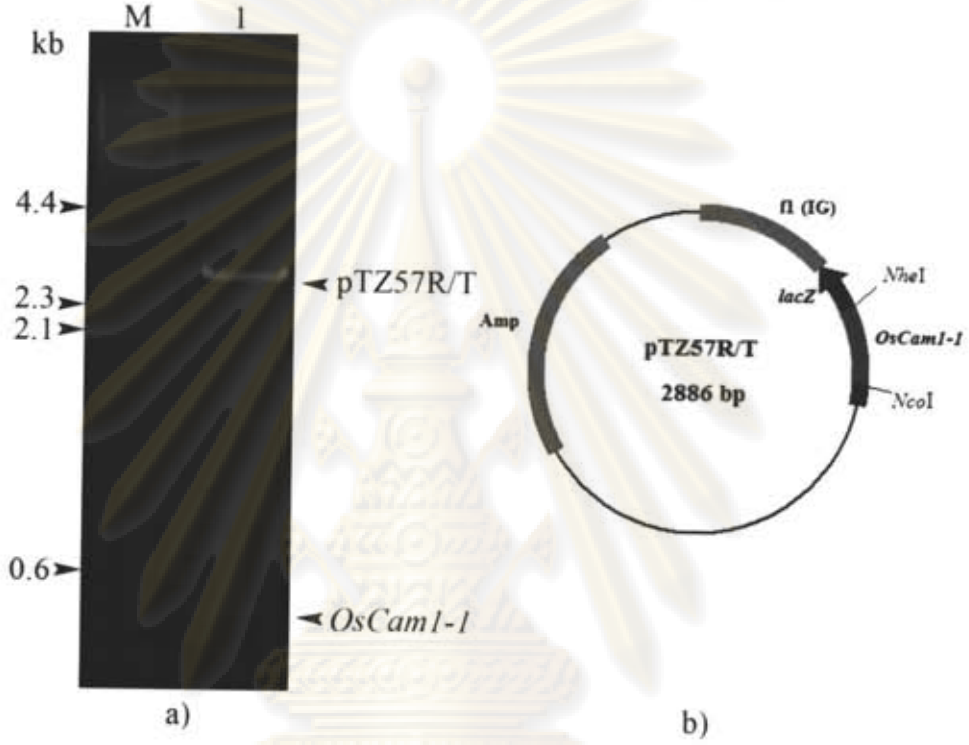

Figure 3.2 Agarose gel electrophoresis of recombinant plasmid pTZ57R/T-*OsCam1-1* on 1.2% agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pTZ57R/T-*OsCam1-1* digested with *Nco*I and *Nhe*I

b) Schematic diagram of the recombinant pTZ57R/T containing *OsCam1-1* gene (pTZ57R/T-*OsCam1-1*)

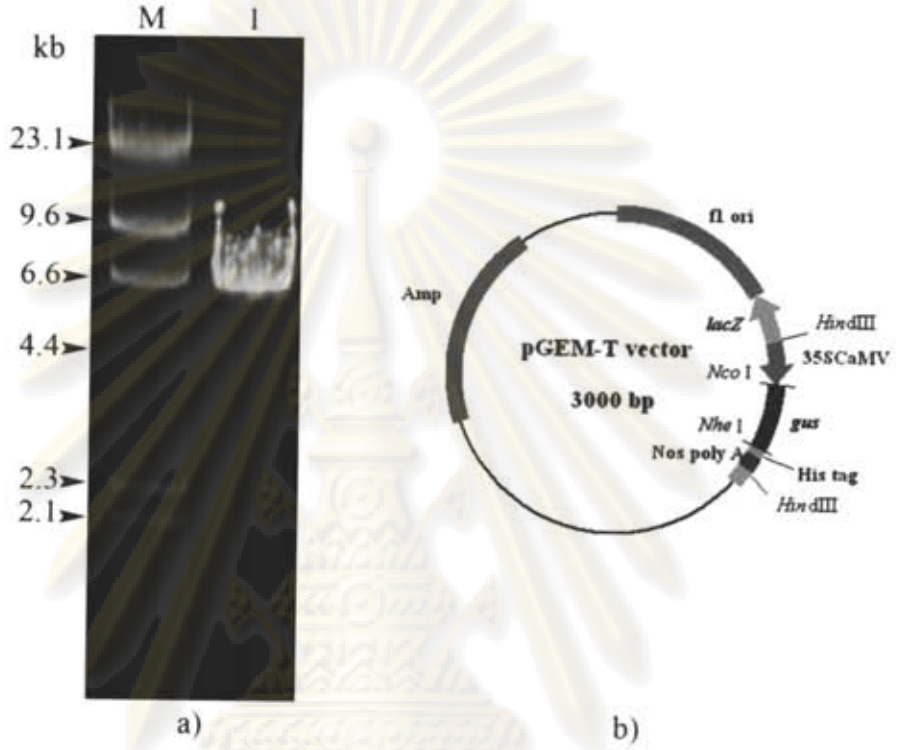

Figure 3.3 Agarose gel electrophoresis of recombinant plasmid pGEM®-35SCaMV-*gus-nos* analyzed on 1.2% agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM®-35SCaMV-*gus-nos* digested with *Nhe*I

b) Schematic diagram of the recombinant pGEM®-T containing 35SCaMV-*gus-nos* (pGEM®-35SCaMV-*gus-nos*)

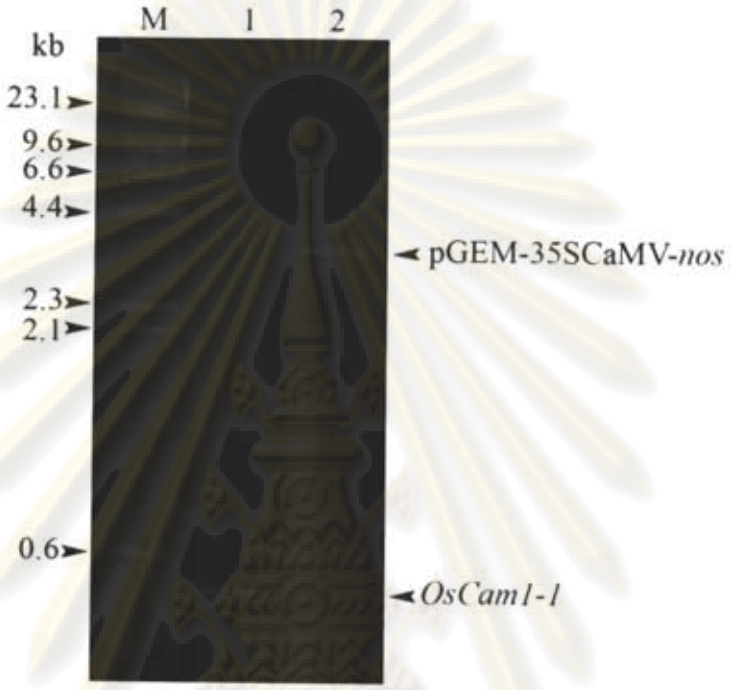

Figure 3.4 Purified products of the *OsCam1-1* gene and the pGEM®-35SCaMV- *nos* analyzed on 1.2% agarose gel.

Lane M λ/*Hin*dIII standard marker

Lane 1 purified *Nco*I/*Nhe*I digested *OsCam1-1* fragment

Lane 2 purified *Nco*I/*Nhe*I digested pGEM®-35SCaMV- *nos* fragment

colonies were randomly picked for plasmid extraction and analyzed by *Nco*I and *Nhe*I digestion. Restriction endonuclease analysis of a recombinant plasmid released three fragments of approximately 4, 0.5 and 0.3 kb (Figure 3.5). The DNA fragment of approximately 0.5 kb was the expected size of the *OsCam1-1* gene. The resulting recombinant plasmid was called pGEM®-35SCaMV-*OsCam1-1-nos* which the coding region of the *OsCam1-1* gene without its stop codon was fused in frame with the 6x His tag coding sequence derived from the removed *gus* gene next to the *Nhe*I site.

3.3 Construction of plant expression vector under control of 35SCaMV promoter

For plant transformation, pCAMBIA1301 was employed. To use pCAMBIA1301 as a binary vector to transfer the 35SCaMV-*OsCam1-1-nos* gene cassette into plants, the pGEM®-35SCaMV-*OsCam1-1-nos* and pCAMBIA1301 were digested with *Hin*dIII. The desired *Hin*dIII-digested 35SCaMV-*OsCam1-1-nos* fragment of approximately 1.8 kb was separated by 1% agarose gel electrophoresis (Figure3.6). This fragment was purified and ligated with the compatible site of pCAMBIA1301. The ligation mixture was transformed into *E. coli* XL1-Blue strain. The pCAMBIA1301 contains a β-glucuronidase (*uid*A) as a reporter gene, a kanamycin-resistant gene (*npt* II) as a bacterial selectable marker and a hygromycin-resistant gene (*hpt* II) as a plant selectable marker within the T-DNA. After transformation, four white colonies were randomly picked and the recombinant plasmids were extracted. The potential recombinant plasmids were digested with *Hind*III and the expected DNA fragment of approximately 1.8 kb was obtained from all of the resistant colonies (Figure 3.7). The resulting plasmid was designated as pCAMBIA1301-35SCaMV-*OsCam1-1-nos* which contains the *OsCam1-1* gene fused

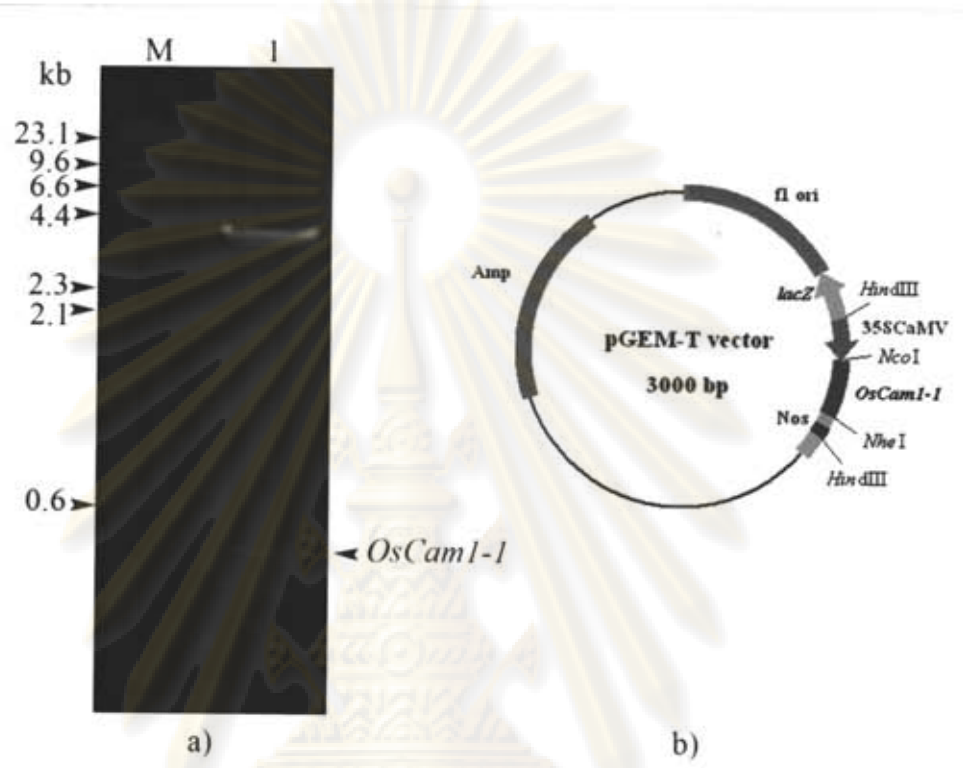

Figure 3.5 Agarose gel electrophoresis of the recombinant plasmid pGEM®-35SCaMV-*OsCam1-nos* analyzed on 1.2% agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM®-35SCaMV-*OsCam1-1-nos* digested with *Nco*I and *Nhe*I

b) Schematic diagram of the recombinant plasmid pGEM®-35SCaMV-*OsCam1-nos*

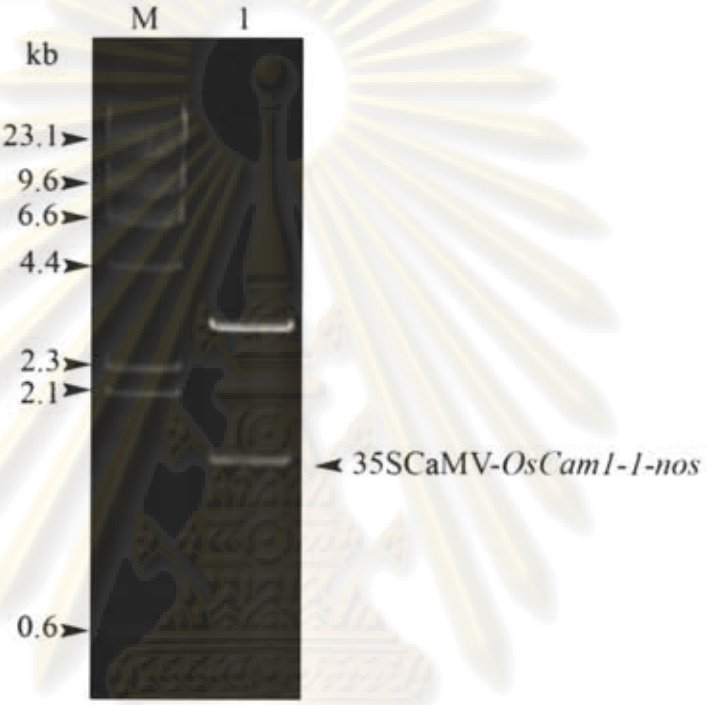

Figure 3.6 Agarose gel electrophoresis of the recombinant plasmid pGEM®-35SCaMV-*OsCam1-nos* analyzed on 1.2% agarose gel.

Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM®-35SCaMV-*OsCam1-1-nos* digested with *Hin*dIII

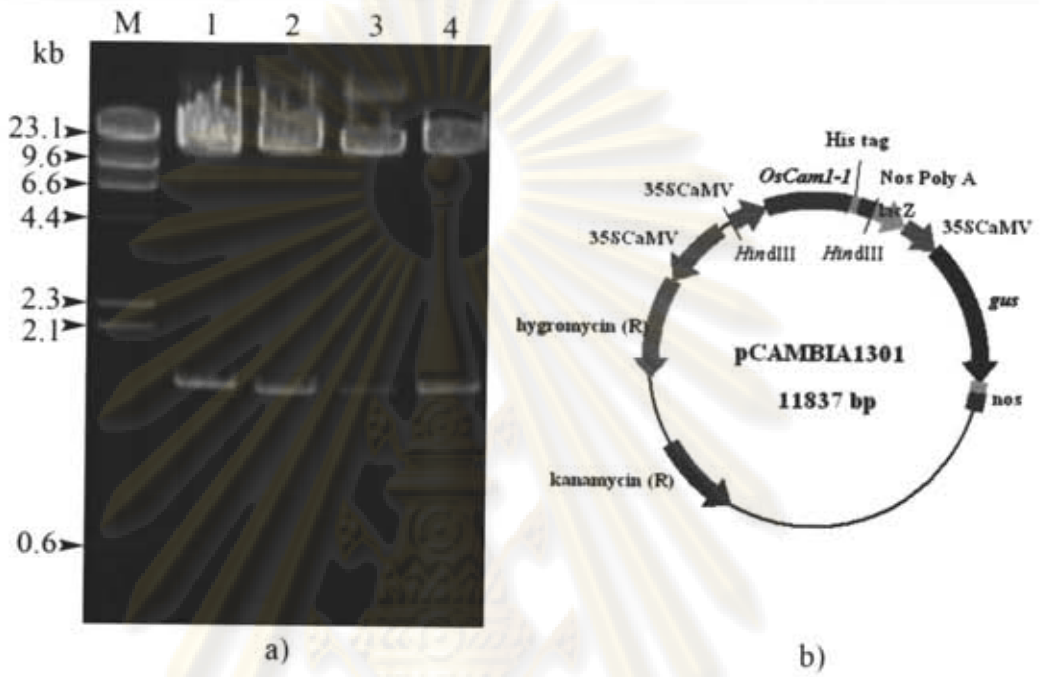

Figure 3.7 Agarose gel electrophoresis of the recombinant pCAMBIA1301-35SCaMV-*OsCam1-nos* analyzed on 1.2% agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane1, 2, 3, 4 pCAMBIA1301-35SCaMV-*OsCam1-1-nos* digested with *Hin*dIII

b) Schematic diagram of the recombinant pCAMBIA1301-35SCaMV-*OsCam1-nos*

in-frame with six histidine codons under the control of the 35SCaMV promoter and *nos* terminator (*nos* Poly A) of the nopaline synthase gene.

To confirm the nucleotide sequence of *OsCam1-1* gene inserted into pCAMBIA1301, a recombinant plasmid was subjected to DNA sequencing using Os.2226checkF and Os.2226checkR as primers (2.3.4). The result showed that the sequence of the *OsCam1-1* gene in the recombinant plasmid shared 100 % identity with the cDNA sequence of *OsCam1-1* as shown in Figure 3.8 and the direction of 35SCaMV-*OsCam1-1-nos* cassette inserted into pCAMBIA1301 was shown in Figure 3.9.

3.4 Plant transformation

To generate transgenic plants that harbor a gene sequence consisting of the *OsCam1-1* gene driven by the 35SCaMV promoter, rice and tobacco were used. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including major economic crops, vegetables, fruits and trees (Birch, 1997). *Agrobacterium*-mediated transformation has remarkable adventages over direct transformation methods because of its lower the copy number of the transgene, potentially leading to fewer problems with transgene cosuppresion and instability (Koncz *et al.*, 1994, Hansen *et al.*, 1997), the high efficiency of transformation, and the transfer of relatively large piece of DNA (Hiei *et al.*, 1994). Systems of *Agrobacterium*-mediated transformation have been well established for many dicotyledonous plants. However, monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens*. However, the studies on rice, maize, wheat and barley transformation mediated by *A. tumefaciens* have

Figure 3.8 Nucleotide and deduced amino acid sequence of the *OsCam1-1* gene in the recombinant plasmid. The underlined letters represent primer binding sites and the bold letters indicate start and stop codons. The green letters show the nucleotide sequence of a part of the 35SCaMV promoter and the blue letters show the nucleotide sequence of the terminator of the *nos* gene.

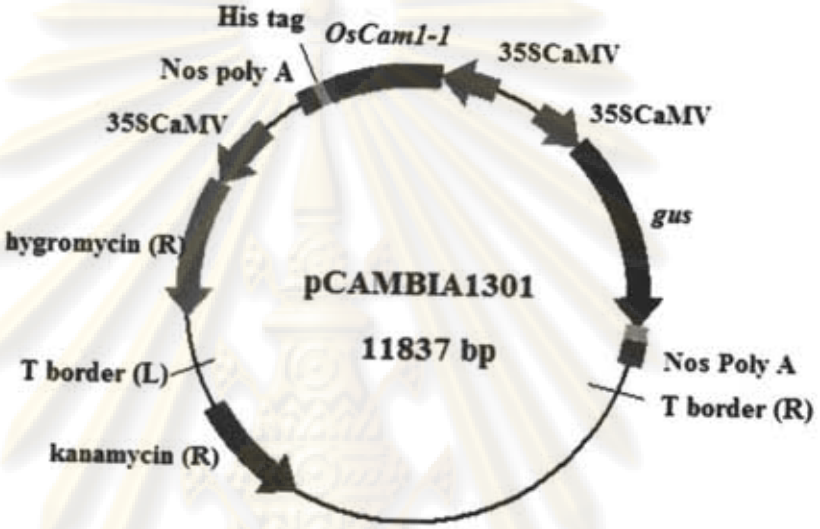

Figure 3.9 The direction of the 35SCaMV-*OsCam1-1-nos* cassette inserted into pCAMBIA1301.

provided strong support that monocotyledons can be transformed as dicotyledons (Hiei *et al.*, 1994; Ishida *et al.*, 1996 and Cheng *et al.*, 1997).

3.4.1 Transformation of rice with *Agrobacterium tumefaciens*

For rice transformation, *A. tumefaciens* strain EHA 105 harboring either pCAMBIA1301-35SCaMV-*OsCam1-1-nos* or pCAMBIA1301 alone to generate control plants were used. The seeds of rice (KDML105) were sterilized and grown in callus induction NB medium. Callus is a mass of undifferentiated plant cells which, depending on the presence of different growth substances, can be induced to form shoots or roots (Walden, 1993). The calli observed as being yellowish, compact, and granular. The 6-week old rice calli were used as target tissues for *Agrobacterium*-mediated transformation (Figure 3.10).

After co-cultivation with *Agrobacterium* carrying the recombinant plasmid, calli were transferred to the selection medium containing 50 mg/l hygromycin to inhibit growth of non-transformed rice cells and supplemented with 500 mg/l cefotaxime to inhibit *A. tumefaciens* growth. Any calli that turned brown within 10 day were found to be untransformed. The non-transformed calli did not show continuous growth and died in the selection medium while the hygromycin resistant calli which survived after 4 weeks of selection were obtained (Figure 3.11). These calli were subcultured onto the second round of selection on fresh selection medium for additional 4 weeks in the dark at 28°C. A total of 7 hygromycin resistant calli was obtained from the transformation of 90 explants for an apparent transformation efficient of 7.78%. All these calli were further grown on callus induction medium and transferred to regeneration medium. Stringent selection was maintained throughout callus growth; however, hygromycin was not included during regeneration. Within 4

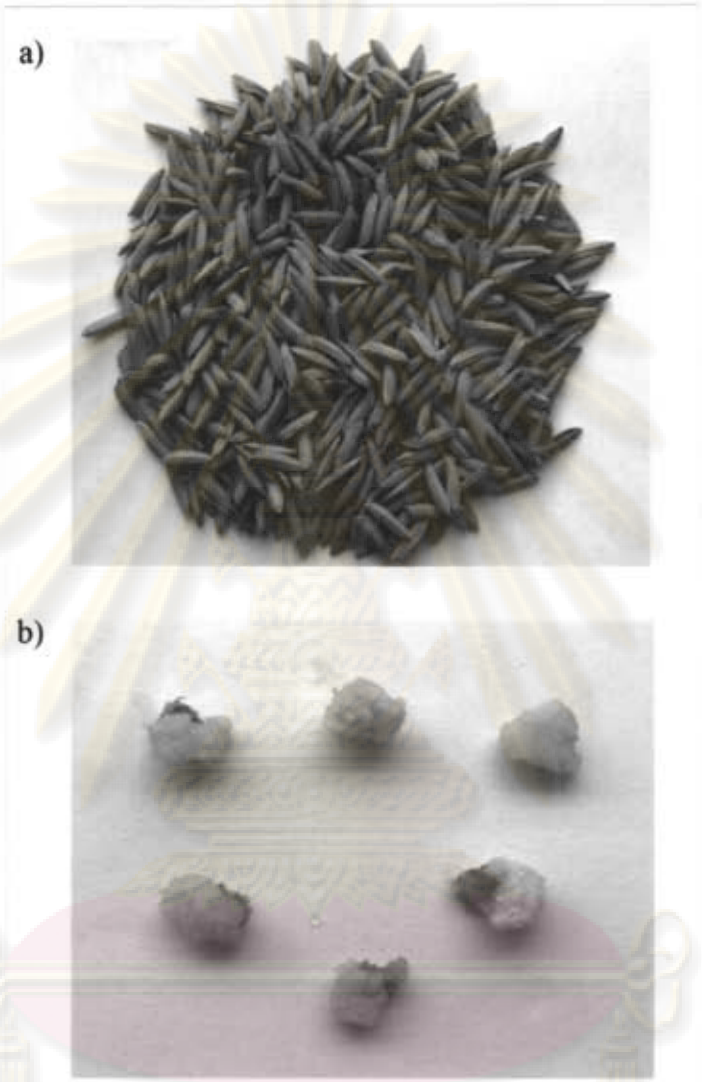

Figure 3.10 Tissue culture of rice *O. sativa* cultivar KDML105

a) Mature seeds of rice

b) Rice calli formed on callus induction medium

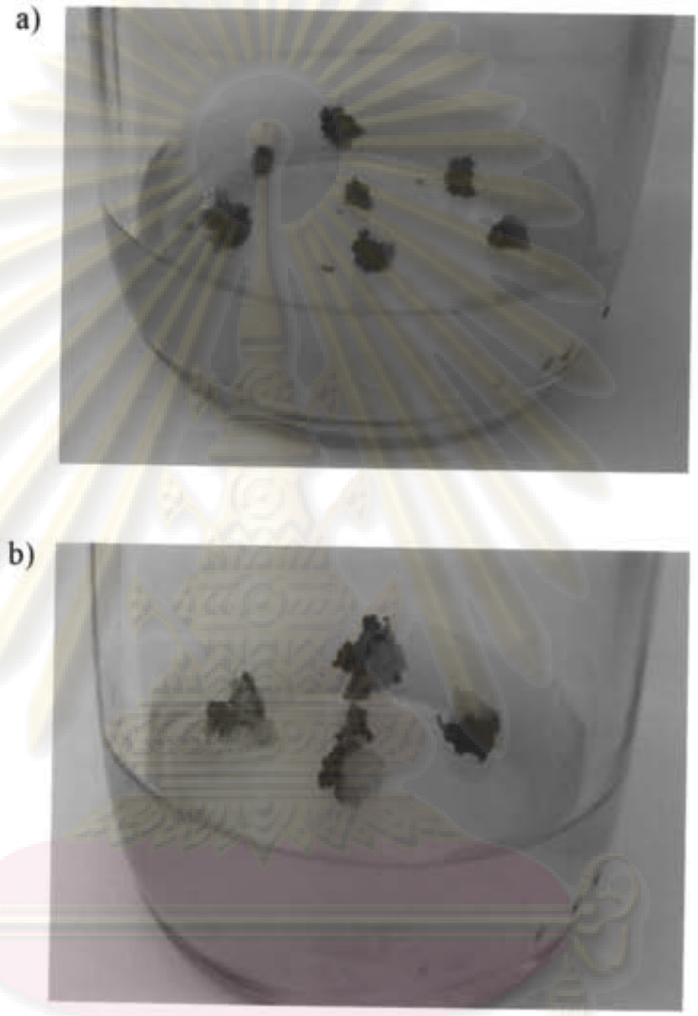

Figure 3.11 Selection of transformed rice calli on selection medium containing 50 mg/l hygromycin and 500 mg/l cefotaxime.

a) Brown zone of non-transformed callus after 4 weeks of selection

b) Resistant callus growing on selective hygromycin medium after 4 weeks

weeks of culture, green spots and shoots of the resistant calli were observed (Figure 3.12 a). When the shoots were 3.0 to 5.0 cm in height (Figure 3.12 b), 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 3.12 c). A total of 3 putative transgenic lines were obtained from regeneration of the hygromycin-resistant calli. No differences were observed in the morphology between the transformed and untransformed plants.

The transformation efficiency varies with both plant genotype and with the transformation system used. In general, *japonica* cultivars of rice are more tissue culture-responsive and give higher frequency of transformation than *indica* cultivars. Like many *indica* varieties KDML105 was viewed as recalcitrant to both tissue culture and transformation. Transformation efficiency obtained in this work was 7.78% which has been as high as those reported for rice (Hiei *et al.,* 1994, Khanna and Raina, 1999, Zhang *et al.,* 1988 and Rashid *et al.,* 1996). The regeneration efficiency of the transformed calli was very low. The loss of the regeneration potential in transformation could have resulted from the long term cultures and the length of culture time in the presence of hygromycin (Raineri *et al.*, 1990 and Peng *et al.*, 1992).

3.4.2 Transformation of tobacco with *Agrobacterium tumefaciens*

A. tumefaciens harboring either pCAMBIA1301-35SCaMV-*OsCam1-1-nos*, which carries the *OsCam1-1* gene, the β-glucuronidase (*gus*) reporter gene and the hygromycin-resistance selectable marker (*hpt*) gene, or pCAMBIA1301 alone to generate the control plants were used for transformation of tobacco. Leaves of *in vitro* tobacco, no more than one-month-old, (Figure 3.13) were used as the starting material

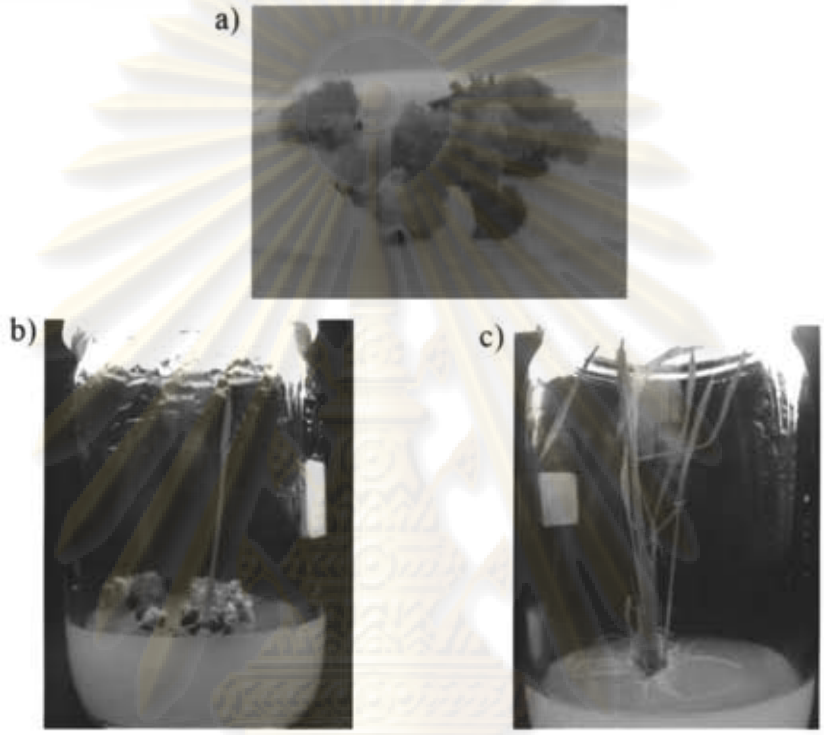

Figure 3.12 Regeneration of transformed rice plants

a) Green spots appeared

b) Shoot formation after 6 weeks

c) Plantlet with root system

for leaf disc transformation by co-cultivation with *A. tumefaciens* carrying the recombinant plasmid (Figure 3.13). After the co-cultivation, co-cultivated leaves were transferred to the selection medium supplemented with 50 mg/l hygromycin and 250 mg/l cefotaxime for 7 days to select transformed cells and eliminate Agrobacterial overgrowth, respectively (Figure 3.13). Then, these leaves were transferred to the shoot induction medium. As a result, a lot of shoots, regenerated from hygromycin resistant cells, on the cut edges of the explants were observed within 6 weeks. They were left to elongate on the shoot induction medium until they were 3.0 to 5.0 cm long (Figure 3.14), then they were excised from the leaf explants and subcultured onto MS medium to generate the root system for 2 weeks (Figure 3.14). Many independent transgenic lines were obtained from regeneration of the hygromycin-resistant cells. No differences were observed in the morphology between the transformed and nontransformed plants.

3.5 Histochemical analysis of GUS expression in putative transformants

The binary plasmid pCAMBIA1301 carried the β-glucuronidase (*gus*) reporter gene within the T-DNA. This reporter gene provides an indication that genetic transformation takes place. Both rice and tobacco transformed with the binary plasmid pCAMBIA1301-35SCaMV-*OsCam1-1-nos* and pCAMBIA1301 alone as the control plants were subjected to analysis for GUS activity. The blue staining by the activity of β-glucuronidase enzyme was observed on the transformed tissues.

The histochemical analysis for GUS activity was analyzed in the rice plants transformed with pCAMBIA1301 alone and the transgenic rice plants transformed with pCAMBIA1301-35SCaMV-*OsCam1-1-nos*. Different organs of rice tissues were collected and submerged in staining solution. All lines of the transgenic rice

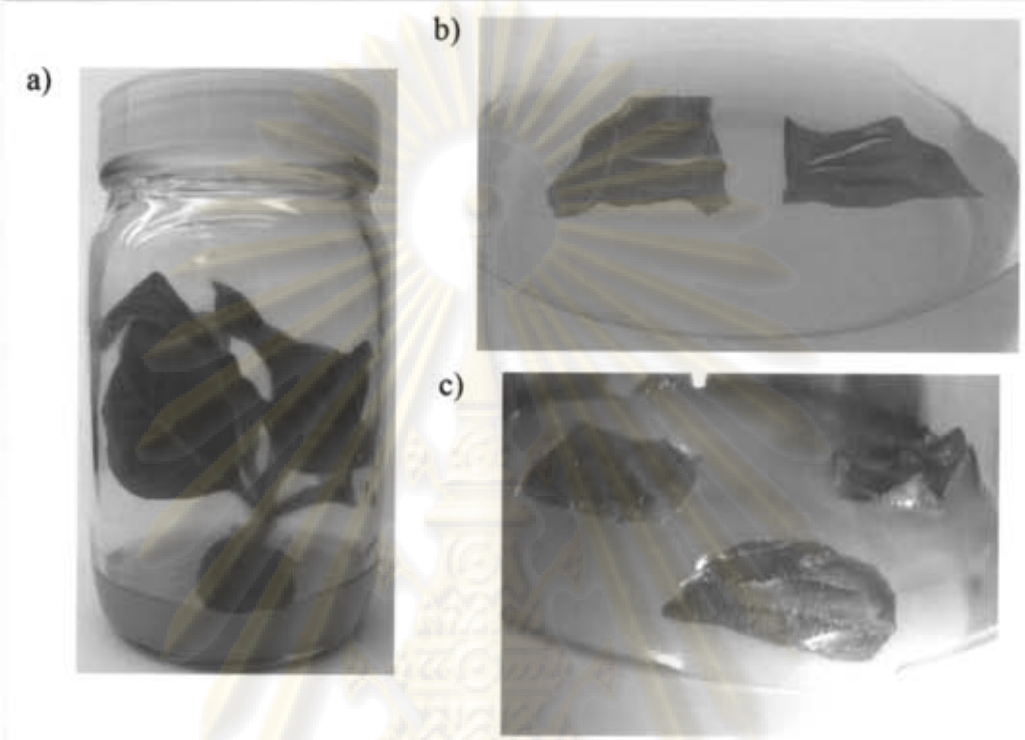

Figure 3.13 Tissue culture of tobacco (*Nicotiana tabacum*) cultivar verginiakuger and selection of transformed leaf discs

a) Tobacco plantlet *in vitro*

b) Tobacco leaf discs after co-cultivation with *Agrobacterium*

c) Resistant tobacco leaf discs on selective hygromycin medium after 7 days

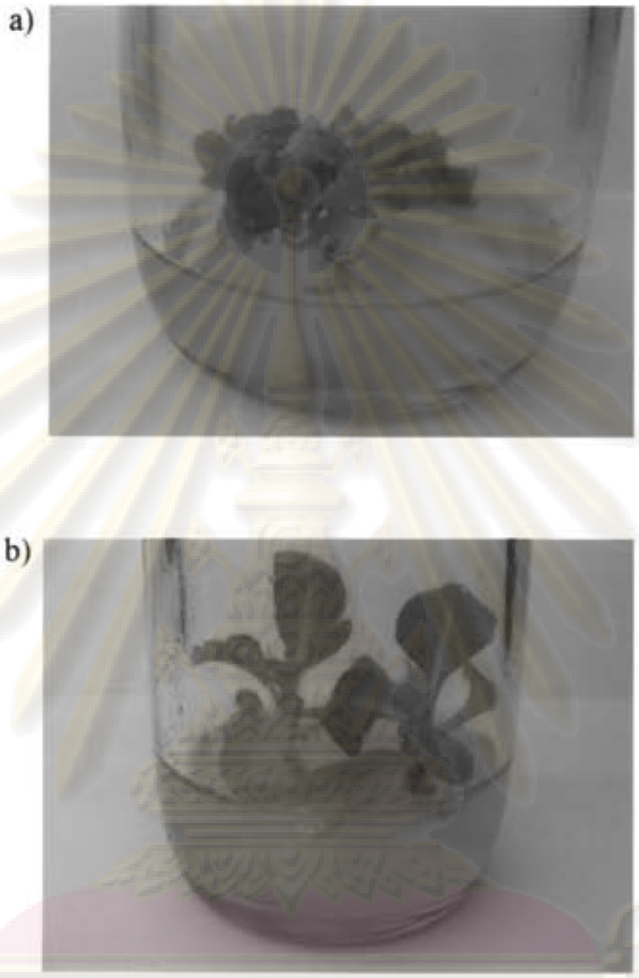

Figure 3.14 Regeneration of transformed tobacco

a) Shoot induction on shoot induction medium for 6 weeks

b) Transformed shoots on selective root induction medium

transformed with pCAMBIA1301 alone and the three lines of the transgenic rice transformed with pCAMBIA1301-35SCaMV-*OsCam1-1-nos*, tested positive for GUS. The GUS activity revealed intense blue staining in callus, surface of leaf pieces and roots (Figure 3.15). 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.

The GUS activity was analyzed in leaves of putative tobacco transformants. The control plant transformed with pCAMBIA1301 alone and six chosen independent transgenic lines showed GUS activity (Figure 3.16). The intensity of the blue color production was different from plant to plant. Tissues from non-transformed plants did not show any GUS expression.

The result from the histochemical analysis confirms that both transgenic rice and transgenic tobacco plants obtained from screening of hygromycin-resistant plants were inserted with the constructed vector which has the *gus* gene as a reporter gene. The absence of GUS expression in some transgenic plants, despite hygromycin selection, could reflect development of antibiotic resistance or genetic instability after integration.

3.6 Molecular analysis of transgenic plants

3.6.1 PCR analyses of putative transformants

To confirm the integration of *OsCam1-1* gene into the plant genomes, the transformants were screened by PCR amplification to amplify parts of the 35SCaMV-*gus* gene and the 35SCaMV-*OsCam1-1* gene. The genomic DNA was isolated from leaves grown *in vitro* from the control plants transformed with pCAMBIA1301 alone

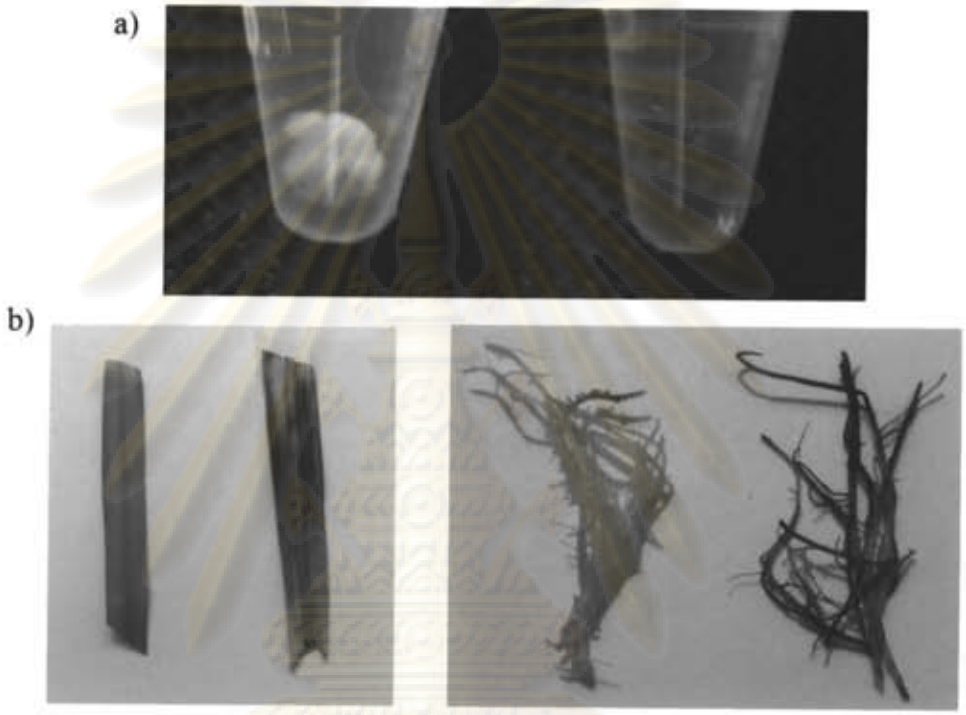

Figure 3.15 Histochemical analysis for GUS activity in rice transformants

a) Hygromycin resistant calli of transformed rice after 8 weeks on selection medium

b) Leaf and root of transformed rice plants. Non-transformed rice was used for comparison.

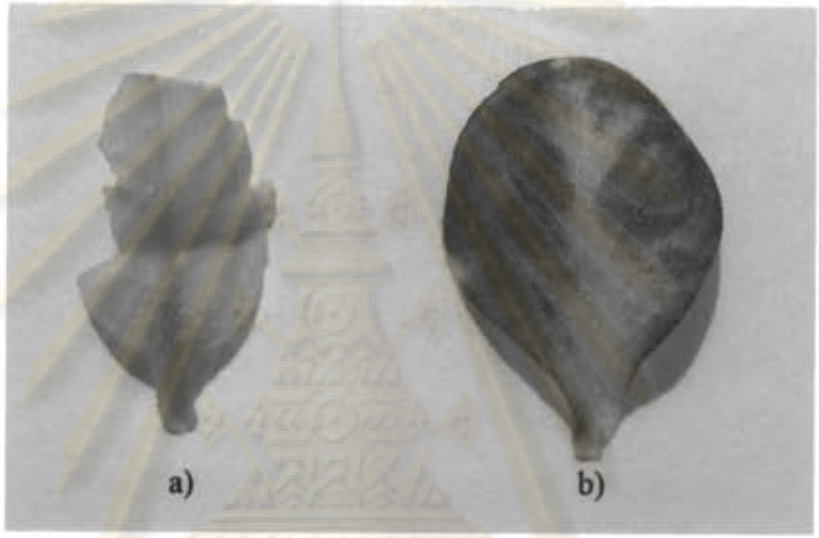

Figure 3.16 Histochemical analysis for GUS expression in leaf of tobacco transformant. Non-transformed tobacco was used as a control.

a) Leaf of non-transformed tobacco

b) Leaf of transformed tobacco plants

and the transgenic rice or tobacco plants harboring 35S CaMV-*OsCam1-1-nos* using a modified CTAB extraction method. DNA concentrations were spectrophotometrically determined by measuring the absorbance at 260 nm (A_{260}). An A_{260} of 1.0 corresponds to a concentration of 50 µg double strand DNA/ml. Approximately 1-5 µg of nucleic acid were obtained from 100 mg of plant tissue. The ratio of A_{260}/A_{280} was 1.8-2.0 which indicated that relatively clean DNA was obtained.

Existence of the 35SCaMV-*gus* gene and the 35SCaMV-*OsCam1-1* gene in the chromosomal DNA of three transformed rice plants and six transformed tobacco plants were verified by PCR. The DNA of plants transformed with pCAMBIA1301 alone was used as a control. The presence of the 35SCaMV-*gus* cassette was analyzed by PCR amplification using 35Scheck-F and GUScheck-R as primers while the 35SCaMV-*OsCam1-1* cassette was amplified with 35Scheck-F and OsCam1check-R primers. The putative rice and tobacco transformants containing the transgene were identified by the presence of amplified PCR products of 983 and 567 bp as expected from the 35SCaMV-*gus* and the 35SCaMV-*OsCam1-1* gene cassette, respectively. The result showed that the specific 983 and 567 bp bands were detected in all putative rice transgenic lines (Figure 3.17) and putative tobacco transgenic lines (Figure 3.18). In the case of control rice and tobacco plants transformed with pCAMBIA1301 alone, the specific 983 bp band of the 35SCaMV-*gus* cassette was detected whereas no 567 bp band from the 35SCaMV-*OsCam1-1* cassette was obtained.

The result of PCR analysis certainly confirms that all transgenic plants with positive histochemical assay of GUS reporter gene previously detected contain the *OsCam1-1* transgene in their genome.

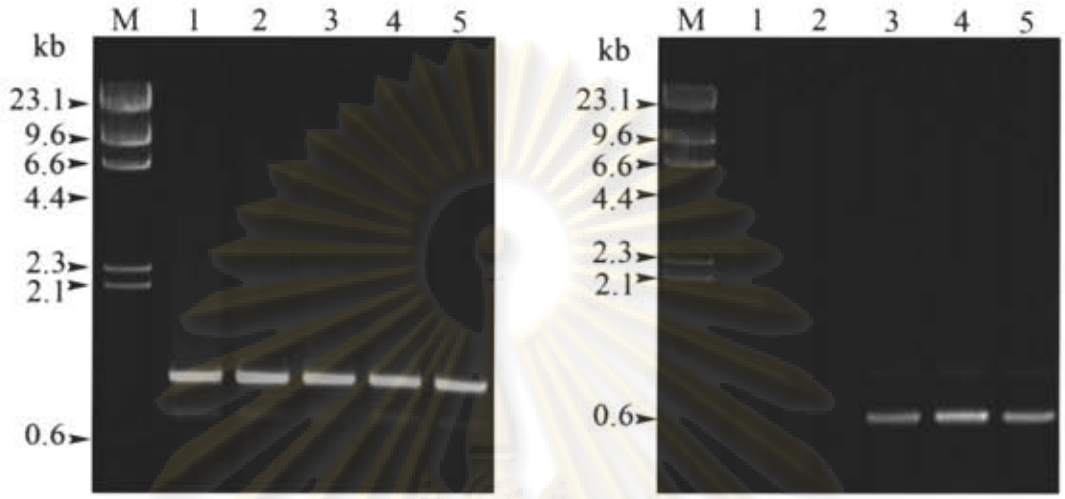

Figure 3.17 PCR analysis of *gus* gene and *OsCam1-1* gene insertion in the genome of transformed rice plants transformed with pCAMBIA1301-*OsCam1-1*-Nos. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

a) The amplification of 35SCaMV-*gus* gene cassette

Lane M λ/*Hin*dIII standard marker

Lane 1, 2 control plants transformed with pCAMBIA1301 without the *OsCam1-1* gene

Lane 3, 4, 5 transgenic rice plants line 1, 2 and 3, respectively

b) The amplification of 35SCaMV-*OsCam1-1* gene cassette

Lane M λ/*Hin*dIII standard marker

Lane 1, 2 control plants transformed with pCAMBIA1301 without the *OsCam1-1* gene

Lane 3, 4, 5 transgenic rice plants line 1, 2 and 3, respectively

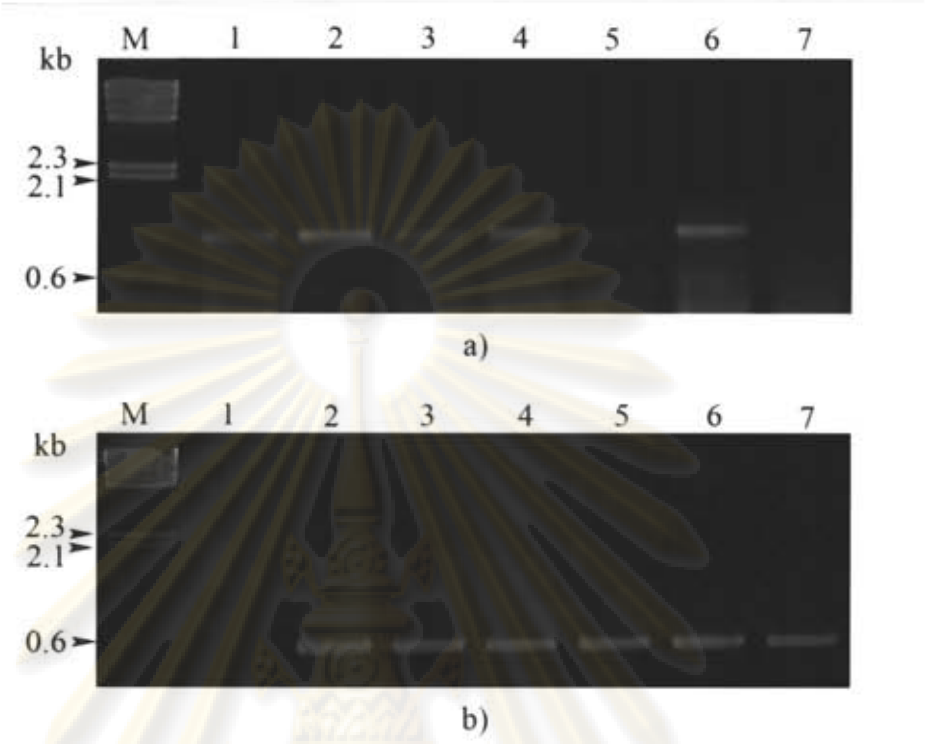

Figure 3.18 PCR analysis of *gus* gene and *OsCam1-1* gene insertion in the genome of transformed tobacco plant transformed with pCAMBIA1301-*OsCam1-1*-Nos. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

a) The amplification of 35SCaMV-*gus* gene cassette

Lane M	λ/*Hin*dIII standard marker
Lane 1	control plants transformed with pCAMBIA1301 without the *OsCam1-1* gene
Lane 2-7	transgenic tobacco plants line 1, 2, 3, 4, 5 and 6, respectively

b) The amplification of 35SCaMV-*OsCam1-1* gene cassette

Lane M	λ/*Hin*dIII standard marker
Lane 1	control plants transformed with pCAMBIA1301 without the *OsCam1-1* gene
Lane 2-7	transgenic tobacco plants line 1, 2, 3, 4, 5 and 6, respectively

3.6.2 Genomic DNA blot analysis of putative *OsCam1-1* transgenic rice plants

To determine whether the transgenic rice lines, isolated previously from screening of the hygromycin-resistant calli, were independent lines, genomic DNA was isolated from leaves grown *in vitro* from two control plants transformed with pCAMBIA1301 alone and three transgenic rice lines harboring 35S CaMV-*OsCam1-1-nos*. Sixty micrograms of genomic DNA isolated from each plant were cleaved completely with *Hin*dIII, separated by agarose gel electrophoresis, and transferred to a charged nylon membrane. A specific probe was made from a partial *gus* gene sequence of pCAMBIA1301. To prepare the probe, pCAMBIA1301 was completely digested with *Eco*RV (Figure 3.19). The purified DNA fragment of approximately 0.2 kb in size was labeled with [α-^{32}P]dCTP and used for hybridization which was carried out at 40°C for 16 hours. The blot was washed in 2X SSPE, 0.1% SDS twice and then once in 1X SSPE, 0.1% SDS at room temperature. Positive hybridization bands were detected by autoradiography. Figure 3.20 shows that the two control plant lines gave hybridizing restriction fragments corresponding to fragments of different size of approximately 14.75 and 10.94 kb (Lanes 1, 2) whereas the three transgenic lines harboring 35S CaMV-*OsCam1-1-nos* gave single hybridizing restriction fragments of approximately 16.14 kb in size (Lanes 3-5).

This analysis supports the conclusion that the three transgenic lines, obtained previously from the regeneration of seven hygromycin resistant calli, were the same line. It might be that the hygromycin resistant calli which were isolated previously in the selection medium were not actually independent calli another possibility that they might be independent line but producing hybridizing fragment of similar size. While two control plants lines transformed with pCAMBIA1301 alone were independent lines. Generally, observation of different integration patterns can be explained in

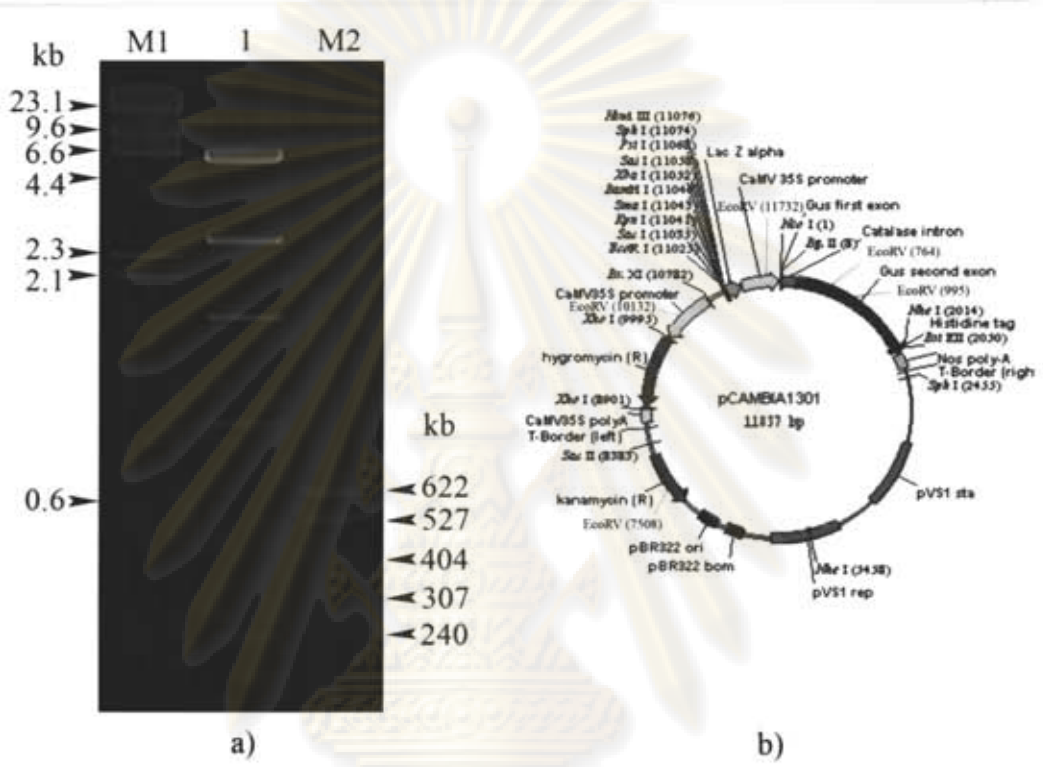

Figure 3.19 Agarose gel electrophoresis of the pCAMBIA1301 digested with *Eco*RV. The DNA was separated on a 1% agarose gel and visualized by ethidium bromide staining.

a) Lane M1 λ/*Hin*dIII standard marker

Lane 1 pCAMBIA1301 digested with *Eco*RV

Lane M2 pBR322/*Msp*I

b) The restriction site of *Eco*RV on pCAMBIA1301

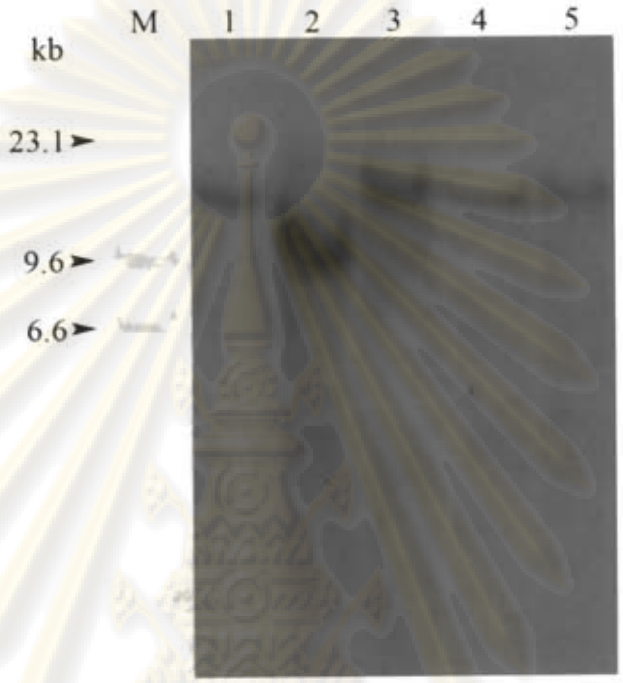

Figure 3.20 Genomic DNA blot analysis of the transgenic rice plants. Rice genomic DNA was digested with *Hin*dIII and hybridized with part of the *gus* gene inserted into genomic DNA of transgenic rice at using the same hybridization conditions earlier. Positive hybridizaing bands were detected by autoradiography.

Lane M1	λ/*Hin*dIII standard marker
Lane 1, 2	Control plants transformed with pCAMBIA1301 alone
Lane 3, 4, 5	Transgenic rice plants harboring 35SCaMV-*OsCam1-1-nos*

terms of stable genetic integration either in different chromosomes or the number of copies that have been delivered into a plant genome (Valdez-Ortiz *et al.*, 2005). Nevertheless, the genomic DNA of all transgenic lines were digested with only one restriction enzyme, *Hin*dIII giving single hybridizing fragment, therefore the information on the copy number of integration is not conclusive .

3.6.3 Detection of the *OsCam1-1* mRNA in transformed plants by RT-PCR

To determine whether the *OsCam1-1* gene inserted into genomic DNA of the transgenic rice and tobacco was expressed at the mRNA level, total RNA was isolated from leaves grown *in vitro* from control plants, transgenic rice and tobacco plants harboring 35S CaMV-*OsCam1-1-nos* using TRI REAGENT® and used to perform reverse transcription PCR (RT-PCR) using primers specific for the inserted *OsCam1-1-nos* cassette. The concentration of total RNA was approximately 5-6 μg per 100 mg plant tissue. An A_{260}/A_{280} ratio in the range of 1.8 to 2.0 indicated relatively cleans RNA (Figure 3.21). Total RNA extracted from leaves was reverse transcribed. The first strand cDNA was used as a template for amplification of the *OsCam1-1-nos* transcript. Amplification of actin transcripts was performed in parallel to ensure the integrity of RNA. The presence of 574 bp RT-PCR product corresponding to size of *OsCam1-1* including a partial *nos* gene was expected from amplifying the *OsCam1-1-nos* transcript. 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. Figure 3.22 shows that a band of the expected size of 574 bp was detected in all transformants except the control plants transformed pCAMBIA1301 alone. No band was detected in the control RT-PCR reactions. It should be note that the relative

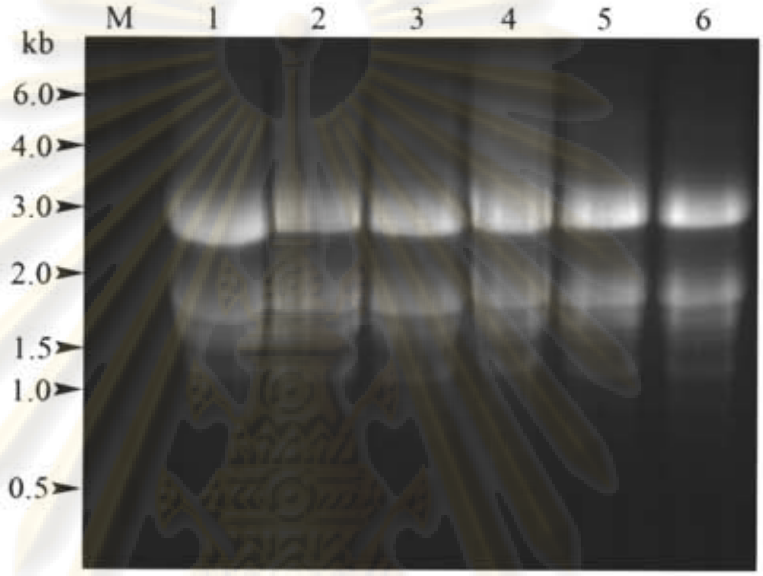

Figure 3.21 Total RNA extracted from leaves of rice electrophoresed on a 1.5% formaldehyde agarose gel and visualized by ethidium bromide staining.

Lane M RNA marker

Lane 1 Wild type plants

Lane 2, 3 Control plants line 1 and 2, respectively

Lane 4, 5, 6 Transgenic rice plants harboring 35SCaMV-*OsCam1-1-nos*

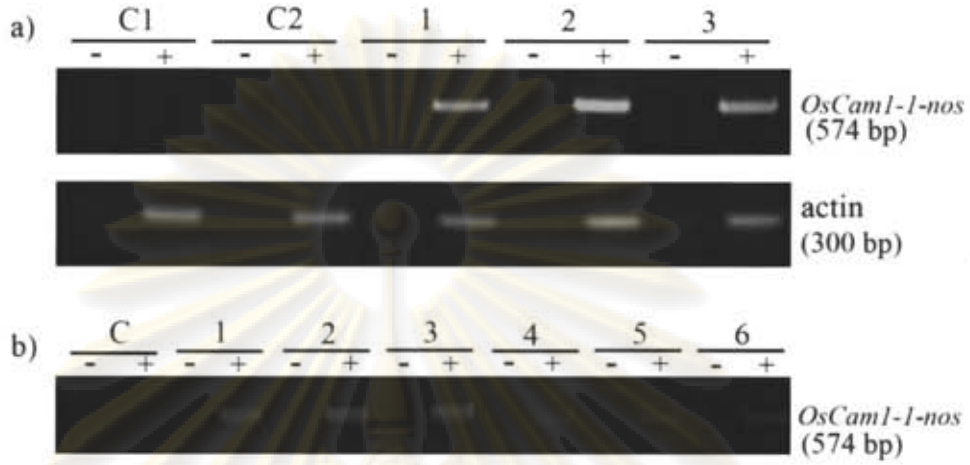

Figure 3.22 Expression of the *OsCam1-1* gene. The total RNA was used in RT-PCR assays either without (-) or with (+) the adding of M-MLV reverse transcriptase. The cDNA were amplified by PCR using gene specific primers for the inserted *OsCam1-1-nos* gene cassette. Amplification of actin transcript was performed in parallel. The products were separated in agarose gels and visualized by ethidium bromide staining.

a) C1, C2 RT-PCR product of control rice plant line 1 and 2, respectively

Lane 1-3 RT-PCR product of transgenic rice plants harboring 35SCaMV-*OsCam1-1-nos*

b) C1 RT-PCR product of control tobacco plant

Lane 1-6 RT-PCR product of transgenic tobacco plants harboring 35SCaMV-*OsCam1-1-nos* line 1, 2, 3, 4, 5 and 6, respectively

abundance of *OsCam1-1* mRNA was not determined, therefore comparison of the expression levels between transformants can not be made. Nevertheless, it can be concluded that the inserted *OsCam1-1* gene of the transgenic rice and tobacco is expressed at the mRNA level.

3.6.4 Detection of the altered expression level of *OsCam1-1* in transgenic rice plants

To compare the *OsCam1-1* expression level among the transgenic rice harboring the *OsCam1-1* gene regulated by the 35SCaMV promoter, control plants containing the 35SCaMV-*gus* gene and wild type plants, northern blot analysis of total RNA was performed. Total RNA was isolated from leaves grown *in vitro* using TRI REAGENT®. The concentration of total RNA was approximately 5-6 μg per 100 mg plant tissue. An A_{260}/A_{280} ratio in the range of 1.8 to 2.0 indicated relatively clean RNA. Forty micrograms of total RNA were fractionated on 1.5% formaldehyde agarose gel and transferred to positively charge nylon membrane. DNA fragments made from a partial *OsCam1-1* gene by PCR amplification were used to prepare a specific probe using a pair of primers given in the "Materials and Methods" section. The PCR product of the partial *OsCam1-1* gene corresponding to an expected size of 324 bp (Figure 3.23) was incorporated with [α-^{32}P]dCTP and used for hybridization which was carried out at 40°C for 16 hours. The blot were washed in 2X SSPE, 0.1% SDS twice and then once in 1X SSPE, 0.1% SDS at room temperature. Positive hybridizing bands were detected by autoradiography.

Figure 3.24 shows a high transcript level of *OsCam1-1* gene in the transgenic rice plants (Lanes 3-5) as compared to that in the wild type plants (Lane 1) and control plants containing only the 35SCaMV-gus gene (Lanes 2, 3) under normal

Figure 3.23 Agarose gel electrophoresis of amplified fragment of the partial *OsCam1-1* gene. The DNA was separated on a 1% agarose gel and visualized by ethidium bromide staining

Lane M1 λ/*Hin*dIII standard marker

Lane 1 PCR product of the partial *OsCam1-1* gene

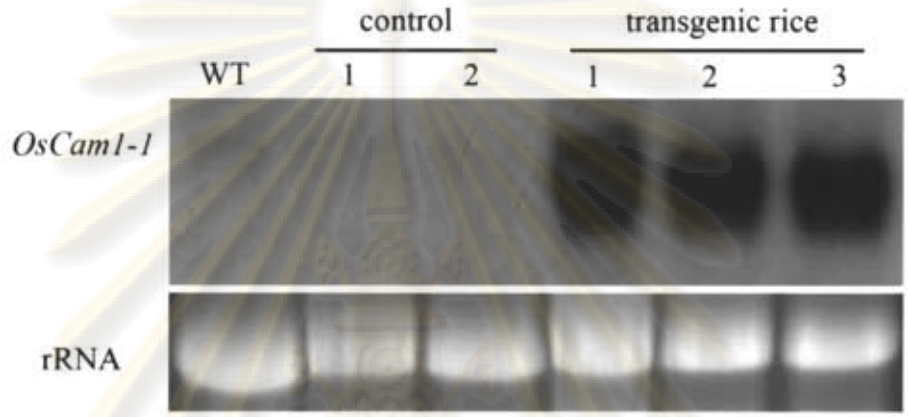

Figure 3.24 RNA blot analysis and quantitative comparison of *OsCam1-1* gene under *in vitro* normal growth condition of wild type plants, two independent control rice plants lines and three transgenic rice plants. Each lane was loaded with total RNA isolated from leaves. RNA was analyzed by gel blot hybridization with a denatured ^{32}P-oligolabeled *OsCam1-1* probe. An ethidium bromide-staining gel of each analysis is shown under its corresponding autoradiography.

growth condition. These results support the conclusion that the *OsCam1-1* gene regulated by 35SCaMV is over-expressed in the transgenic rice plants.

The double-strand caulimovirus family has provided the single most important promoter for transgenic expression in plants: the cauliflower mosaic virus (CaMV) 35S promoter. The *35SCaMV* is expressed in most cells of most plants, and in most tissues, although with different efficiency according to plants species. As expected in this study, *OsCam1-1* was overexpressed and highly accumulated at the mRNA level in transgenic rice leaves under the control of this promoter as same as some examples including *OsCDPK7* transgene in transgenic rice and *OsBIERF3* rice transgene in tobacco reported by Saijo *et al.*, (2000) and Cao *et al.*, (2006), respectively

In previous study, Cao *et al.*, (2006) reported that the OsBIERF3-overexpressing transgenic tobacco which showed enhanced deseases resistance and increased tolerance to salt stress. Saijo *et al.*, (2000) reported the overexpression of *OsCDPK7* gene, which is a rice gene encoding a calcium-dependent protein kinase, in transgenic rice. The extent of tolerance to cold and salt/drought stresses of these plants correlated well with the level of *OsCDPK7* expression. Moreover, Phean-o-pas *et al.*, (2005) reported the expression of *OsCam1* mRNA level which strongly increased in response to NaCl, manitol and wounding treatment. Therefore our transgenic rice plants, which contain the *OsCam1-1* transgene and show overexpression at mRNA level, may be further used to study the extent of tolerance to those stresses especially salt stress tolerance.

3.6.5 Detection of OsCaM1-1 protein in transformed plants by western blot analysis

To determine whether the *OsCam1-1* gene which was over-expressed at the

mRNA level in transgenic plants was expressed at the protein level, Western blot analysis was performed. Total protein was extracted from leaves grown *in vitro* from wild type plants, control plants transformed with pCAMBIA1301 alone, transgenic rice and tobacco plants harboring 35SCaMV-*OsCam1-1-nos*. The concentration of each protein sample was determined by Bradford's method. Approximately 80 µg total protein of each transgenic plant was analyzed on 12.5% SDS-PAGE (Figure 3.25 and 3.26) followed by transferring to nitrocellulose membrane and incubation with anti-His antibody (Amersham Biosciencea Inc., USA) since the 35SCaMV promoter-*OsCam1-1-nos* gene cassette was constructed to encode the *OsCam1-1* fused with a His tag at its carboxyl terminal. A 16 kDa recombinant His-tagged purified from *E. coli* was used as a positive control in all blots.

The expected band of OsCaM 1-1 protein was not detected in both transgenic rice plants (Figure 3.27) and transgenic tobacco plants (Figure 3.28) as same as their control plants while protein of the 16 kDa recombinant His-tagged protein from *E. coli* which was used as a positive control in all blots displayed a positive band. Although, *gus* gene which was fused in-frame with a His tag showed GUS activity by histochemical analysis in these transgenic plants, the His tag fused with *gus* gene cannot be detected using anti-His antibodies. Even though, a northern blot analysis could detect the transcript encoding the OsCaM1-1 protein, the protein was not detected by western blot analysis. This may be attributed to a not enough level of crude protein loaded for SDS-PAGE or a low level of OsCaM1-1 protein that was beyond the sensitivity of the method of detection used or to a lack of stability of its transcript. Additional strategies for stabilizing cytosolically expressed antibodies have been reported. Schouten *et al.*, (1996, 1997) have demonstrated that the addition of a C-terminal peptide increased the cytosolic expression level of that protein fragment.

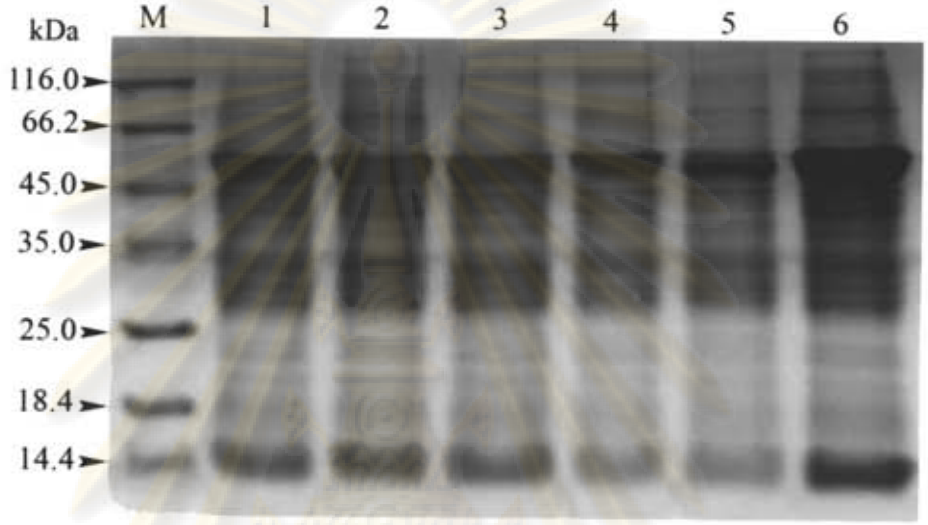

Figure 3.25 SDS-PAGE analysis of protein extract from leaves of transgenic rice plants. Total soluble protein extract (80 μg) was fractionated by SDS-PAGE. Molecular masses of protein marker are indicated at left.

Lane M	Protein marker
Lane 1	Protein extract from non-transgenic plants
Lane 2, 3	Protein extract from control plants line 1 and 2, respectively transformed with pCAMBIA1301 alone
Lane 4, 5, 6	Protein extract from transgenic rice plants

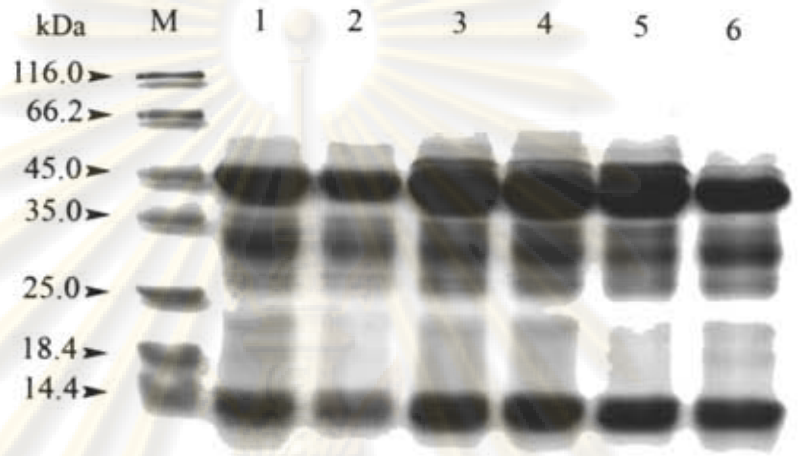

Figure 3.26 SDS-PAGE analysis of protein extract from leaves of transgenic tobacco plants. Total soluble protein extract (80 μg) was fractionated by SDS-PAGE. Molecular masses of protein marker are indicated at left.

Lane M	Protein marker
Lane 1	Protein extract from non-transgenic plants
Lane 2, 3	Protein extract from control plants transformed with pCAMBIA1301 alone
Lane 4, 5, 6	Protein extract from transgenic tobacco plants line 1, 2, 3 and 4, respectively

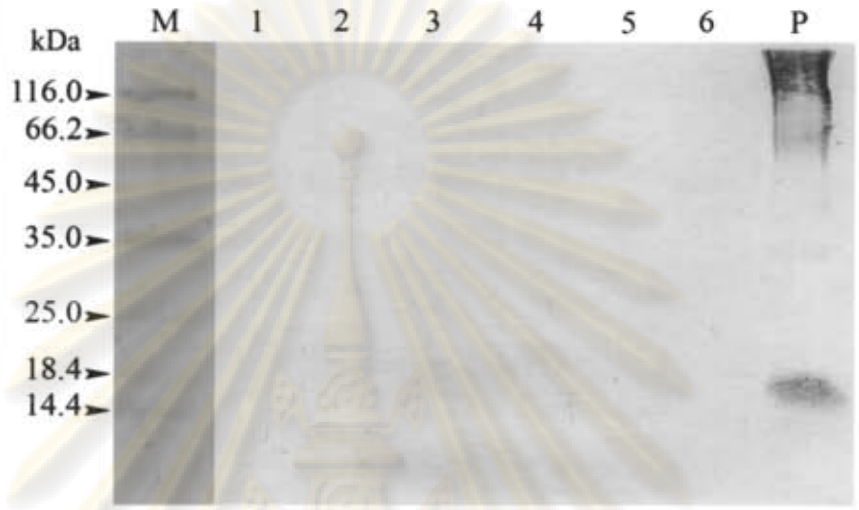

Figure 3.27 Western blot analysis of OsCaM1-1 protein from leaves extract of the transgenic rice plants. Total protein extract (80 μg) was fractionated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with anti-His-antibody at a dilution of 1:3,000. Molecular masses of protein marker are indicated at the left.

Lane M	Protein marker
Lane P	Protein recombinant His-tagged purified from *E. coli* (positive control)
Lane 1	Protein extract from non-transgenic plants
Lane 2, 3	Protein extract from control plants line 1 and 2, respectively transformed with pCAMBIA1301 alone
Lane 4, 5, 6	Protein extract from transgenic rice plants

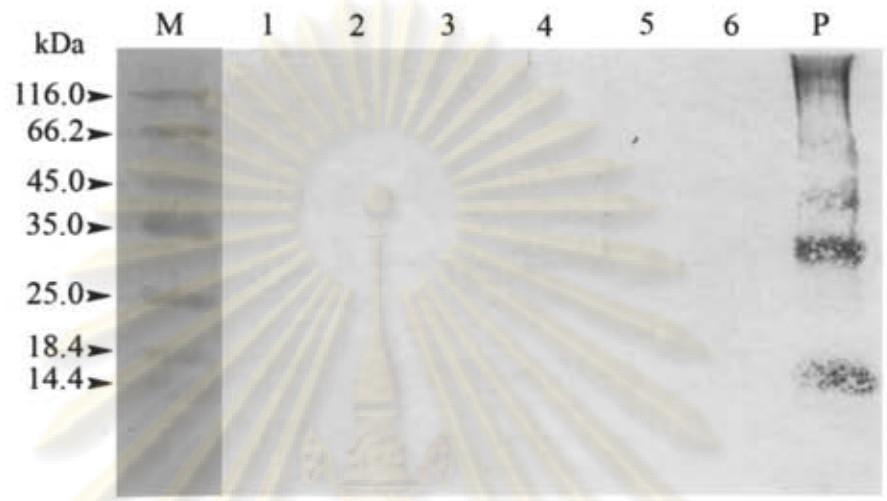

Figure 3.28 Western blot analysis of OsCaM1-1 protein from leaves extract of the transgenic tobacco plants. Total protein extract (80 μg) was fractionated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with anti-His-antibody at a dilution of 1:3,000. Molecular masses of protein marker are indicated at the left.

Lane M Protein marker

Lane P Protein recombinant His-tagged purified from *E. coli* (positive control)

Lane 1 Protein extract from non-transgenic plants

Lane 2 Protein extract from control plants transformed with pCAMBIA1301 alone

Lane 3, 4, 5, 6 Protein extract from transgenic tobacco plants line 1, 2, 3 and 4, respectively

They suggested that short polypeptides may protect the protein antibody from proteolytic degradation. Because of this, it is possible that the OsCaM1-1 protein was produced but hydrolytically degraded. Alternately, 6X His which was linked to OsCaM1-1 protein at its C-terminus may be degraded His tag resulting in the disappearance of the protein band when detected by anti-His-antibody.

Moreover, even though the *OsCam1-1* mRNA was present, it might not be properly used for translation. This may be due to the fact that the construct of 35S CaMV-*OsCam1-1-nos* does not have the 5′UTR region of the *OsCam1-1* gene. In future study, a recombinant plasmid with the 5′UTR region of the *OsCam1-1* gene may be constructed and used for overexpressing the gene in transgenic rice.

CHAPTER IV

CONCLUSIONS

1. The *OsCam1-1* gene was obtained from an *OsCam1-1* cDNA clone given from the International Institute of Agrobiological Science, Japan by PCR. The *OsCam1-1* gene was placed downstream of a 35SCaMV promoter in pCAMBIA1301 using *Nco*I and *Nhe*I sites engineered by PCR amplification so that the coding region was fused in frame with a His tag sequence at its 3' end.
2. *Agrobacterium*-mediated transformation was able to be used to introduce a gene sequence consisting of the *OsCam1-1* gene driven by 35SCaMV promoter into rice (*Oryza sativa* L. cv. *KDML105*) and tobacco (*Nicotiana tobacum* L. cv. *Virginia Coker*). Transformants were selected on antibiotic containing media.
3. Transformation was confirmed in transgenic rice and transgenic tobacco plants by the assay of GUS activity using X-Gluc as a substrate and the insertion of the *Oscam1-1* transgene was confirmed in all transgenic plants by PCR amplification of 35SCaMV promoter-*OsCam1-1* gene cassette using their genomic DNA as a template.
4. At least one transgenic rice line was obtained from the regeneration of seven hygromycin resistant rice calli while control rice plants transformed with pCAMBIA1301 alone were obtained as two independent lines by Southern blot analysis.
5. The expression of *OsCam1-1* mRNAs was detected in all transgenic plants by RT-PCR using oligonucleotide primers designed base on its transcript but not found in control transgenic plants harboring the pCAMBIA1301 vector used for the introduction of *OsCam1-1* gene alone.

6. Transgenic rice plants show the overexpression of *OsCam1-1* RNA under *in vitro* growth by northern blot analysis using a specific probe designed based on its transcript.
7. In both rice and tobacco transgenic plants, bands of the expected OsCaM1-1 protein and GUS protein fused with a His tag were not detected by western blot analysis using anti-His antibody.

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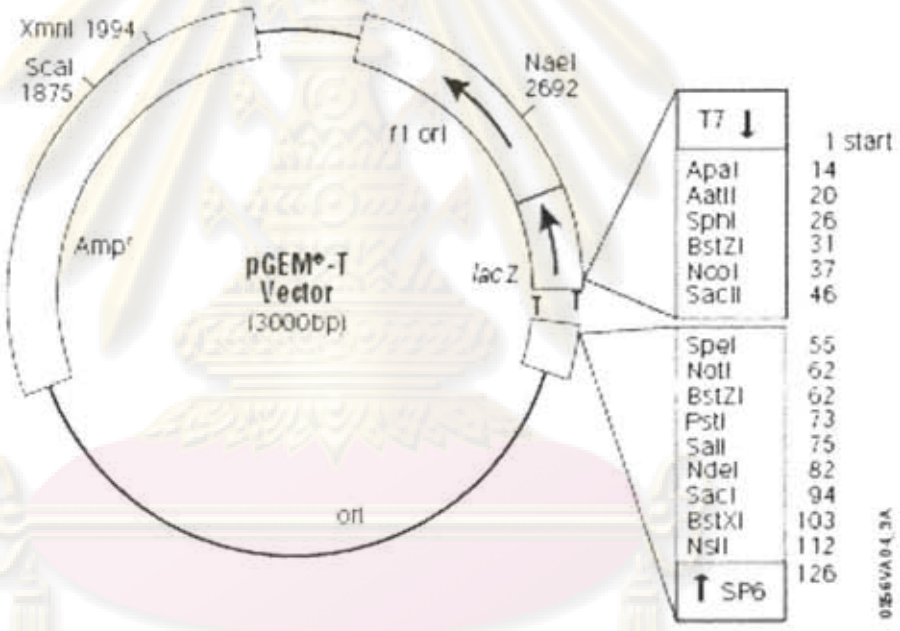
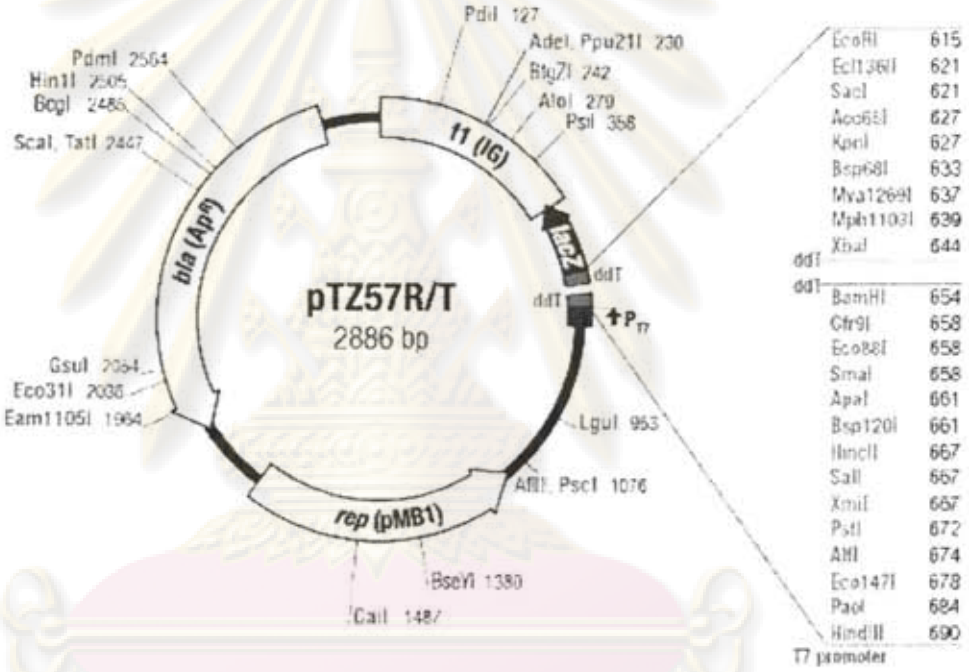
APPENDICES

APPENDIX A

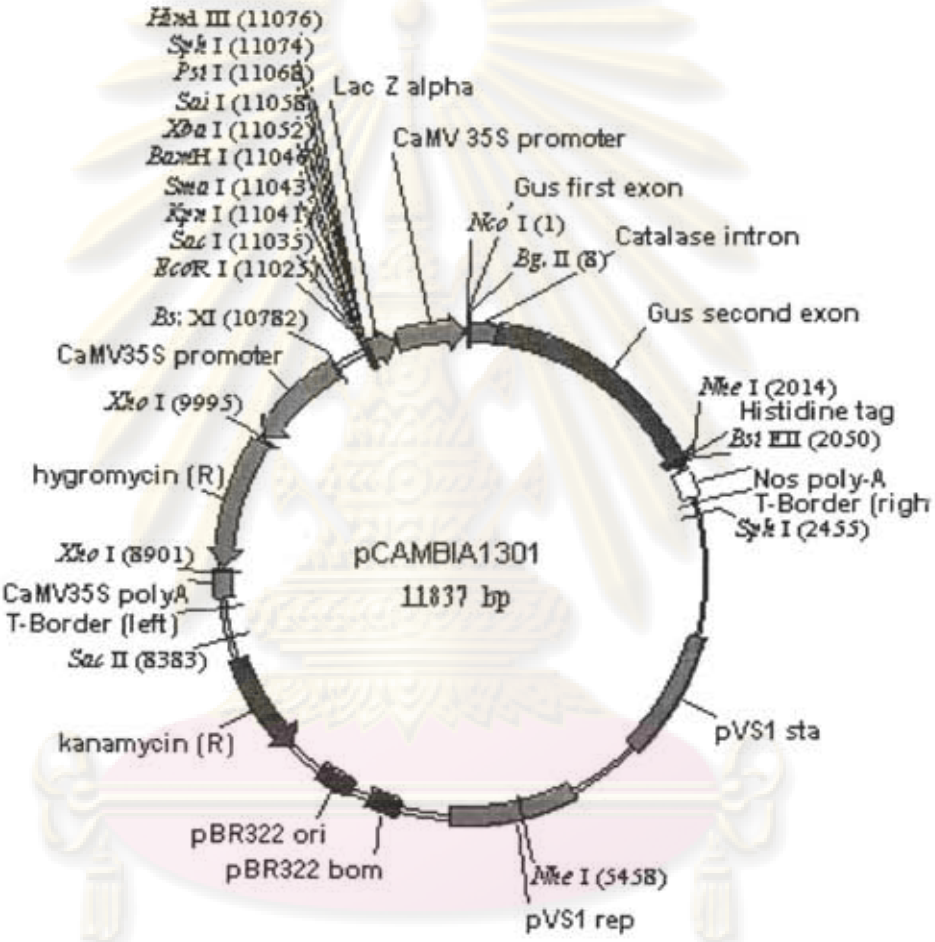
Restriction map of pGEM®-T Vector



Restriction map of pTZ57R/T



Restriction map of pCAMBIA1301



APPENDIX B

The composition of rice tissue culture and *Agrobacterium tumefaciens* medium

Table 1 The composition of NB medium (Li et al. 1993)

Solution	Chemicals	Concentration (mg/l)
N6 Macronutrients	KNO_3	2,830
	$(NH4)_2SO_4$	463
	$CaCl_2.2\ H_2O$	166
	$Mg_2SO_4.7H_2O$	185
	KH_2PO_4	460
B5 micronutrients	KI	0.75
	H_3BO_3	3
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.7H_2O$	10
	$ZnSO_4.7H_2O$	2
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
B5 vitamins	Myo-inosital	100
	Nicotinic acid	1
	Pyridoxine HCl	1
	Thiamine HCl	10
	Casein hydrolysate	300
	L-Proline	500
	L-Glutamine	500
	Sucrose	30,000
	Agar	8,000

pH 5.8

Table 2 The composition of 2NB medium (callus induction medium)

Solution	Chemicals	Concentration (mg/l)
N6 Macronutrients	KNO_3	2,830
	$(NH4)_2SO_4$	463
	$CaCl_2.2\ H_2O$	166
	$Mg_2SO_4.7H_2O$	185
	KH_2PO_4	460
B5 micronutrients	KI	0.75
	H_3BO_3	3
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.7H_2O$	10
	$ZnSO_4.7H_2O$	2
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
B5 vitamins	Myo-inosital	100
	Nicotinic acid	1
	Pyridoxine HCl	1
	Thiamine HCl	10
	2,4-D	2
	Casein hydrolysate	300
	L-Proline	500
	L-Glutamine	500
	Sucrose	30,000
	Agar	8,000
pH 5.8		

Table 3 The composition of 2NB-AS medium (co-cultivation medium)

2NB media supplemented with

Chemicals	Concentration (g/l)
Sucrose	30
Glucose	10

Acetosyringone 300 μM (add after autoclave)

pH 5.2

Table 4 The composition of 2NB-CH medium (selection medium)

2NB medium autoclave cool to room temperature and add

Chemicals	Concentration (mg/l)
Cefotaxime	500
Hygromycin	50

pH 5.2

Table 5 The composition of NB-RE medium (regeneration medium)

Solution	Chemicals	Concentration (mg/l)
N6 Macronutrients	KNO_3	2,830
	$(NH4)_2SO_4$	463
	$CaCl_2.2\ H_2O$	166
	$Mg_2SO_4.7H_2O$	185
	KH_2PO_4	460
B5 micronutrients	KI	0.75
	H_3BO_3	3
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.7H_2O$	10
	$ZnSO_4.7H_2O$	2
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
B5 vitamins	Myo-inosital	100
	Nicotinic acid	1
	Pyridoxine HCl	1
	Thiamine HCl	10
	BAP	4
	L-Proline	500
	L-Glutamine	500
	Sucrose	30,000
	Yeast extract	1,000
	Casein hydrolysate	300
	Agar	8,000

Coconut water 15%

pH 5.8

Table 6 The composition of AB medium (Chilton et al., 1974)

Solution	Chemicals	Concentration (mg/l)
AB buffer	K_2HPO_4	1,500
	NaH_2PO_4	200
AB salt	NH_4Cl	1,000
	Mg_2SO_4	300
	KCl	150
	$CaCl_2.2H_2O$	150
	$FeSO_4.7H_2O$	2.5
	Glucose	5,000
	Agar	15,000

Table 7 The composition of AAM medium (Toriyama and Hinata, 1985)

Solution	Chemicals	Concentration (mg/l)
AA macronutrients	Na_2HPO_4	169.6
	$MgSO_4.7H_2O$	500
	KCl	150
	$CaCl_2.2H_2O$	150
AA micronutrients	$MnSO_4.4H_2O$	10
	$Na_2MoO_4.2H_2O$	0.25
	H_3BO_3	3
	$ZnSO_4.7H_2O$	2
	$CuSO_4.5H_2O$	0.0387
	$CoCl_2.6H_2O$	0.025
	KI	0.75
AA iron	$FeSO_4.7H_2O$	28
MS vitamin	Inosital	100
	Nicotinic acid	0.5
	Pyridoxine HCL	0.5
	Thiamine	0.5
AA amino acid	Glycine	7.5
	Arginine	174
	Glutamine	876
	Casamino acid	500
	Sucrose	68,500
	Glucose	35,000

Acetosyrigone 300 μM (add after autoclave)

pH 5.2

Table 8 The preparation of chemicals used in tissue culture

Chemicals	Preparation
2,4-Dichlorophenoxyacetic acid (2,4-D) (1 mg/ml)	Dissolve 100 mg of 2,4-D in 1 ml absolute ethanol, add 3 ml of 1 N KOH, adjust to pH 6.0 with 1N HCl (very sensitive adjust carefully), and make upto 100 ml with sterilize deionized water.
6-Benzylaminopurine (BAP) (2 mg/ml)	Weigh 100 mg BAP, add 1N KOH dropwise until powder is dissolved, make upto 50 ml with sterilize deionized water.
1-Naphtalene acetic acid NAA (0.1 mg/ml)	Weigh 10 mg NAA, add 1N NaOH dropwise until powder is dissolved, make upto 100 ml with sterilize deionized water.
AS-Acetosyringone (100 mM)	Add 19.62 mg of acetosyringone to 1 ml of Dimethyl sulphoxide (DMSO) or methanol. Protect from light and add to media after autoclaving.

Table 9 The composition of MS medium (Murashige and Skoog, 1962)

Solution	Chemicals	Concentration (mg/l)
Macronutrients	NH_4NO_3	1,650
	KNO_3	1,900
	$CaCl_2.2H_2O$	440
	KH_2PO_4	170
	$MgSO_4.7H_2O$	370
Micronutrients	KI	0.83
	H_3BO_3	6.2
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.7H_2O$	16.9
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
B5 vitamins	Myo-inosital	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
	Glycine	2
	Sucrose	30,000
	Agar	8,000

pH 5.8

Table 10 The composition of CM medium (selection medium)

MS media supplemented with

Chemicals	concentration (mg/l)
BAP	1 mg/l
NAA	1 mg/l

pH 5.8

Table 11 The composition of MS-selection medium

CM media autoclave cool to room temperature and add

Chemicals	Concentration (mg/l)
Cefotaxime	250
Hygromycin	50

pH 5.8

Table 11 The composition of MS-shoot induction medium

MS media supplemented with

Chemicals	Concentration (mg/l)
BAP	1
Cefotaxime	250

pH 5.8

APPENDIX C

Chemical solution

1. β-Glucoronidase (GUS) assays

Staining Solution

100 mM NaH_2PO_4/Na_2HPO_4 pH7.5	10 ml of 0.2 M
0.1% Triton X-100	200 µl of 10% (v/v)
10 mM EDTA	400 µl of 0.5 M
1 mM X-Gluc	1 ml of 20 mM in DMF (0.104 g/10 ml)
0.5 mM K Ferricyanide	1 ml of 10 mM (33 mg/10 ml H_2O)
0.5 mM K Ferrocyanide	1 ml of 10 mM (44 mg/10 ml H_2O)
H_2O	6.4 ml

A time-saving alternative

Mix phosphate buffer, EDTA, Triton X-100, and H_2O in the propotions given above and store at room temperature. Just before using, prepare the K-Ferricyanide and K-Ferrocyanide stocks. Mix the components in the following proportions.

Component	For 5 ml
Buffer Stock	4.25
K-Ferro Stock	0.25
K-Ferri Stock	0.25
X-Gluc Stock	0.25

2. Southern bloting

Denaturation Solution

NaCl	21.9g (1.5 M)
NaOH	5 g (0.5N)
ddH_2O	200 ml

Adjust the volume to 250 ml with distilled water.

Neutralization Solution

Trizma base	30.3 g (0.5M)
NaCl	87.6 g (3M)
ddH_2O	400 ml

Adjust pH to 7.2 (± 0.3) with HCl and adjust the volume to 500 ml with distilled water.

20X SSPE:

NaCl	350.4 g (3M)
$NaH_2PO_4.H_2O$	55.2 g (0.2M)
$Na_2EDTA.2H_2O$	14.8 g (20mM)
NaOH	20 g
ddH_2O	1.5 liters

Adjust pH to 7.4 with NaOH and adjust the volume to 2 liters with distilled water.

3. Southern blot hybridization, Northern blot hybridization

1 M Tris/HCl pH8:

Tris base (Trizma)	60.5 g
H_2O	400 ml

Stir the solution to dissolve Tris completely, adjust pH to 8.0 with 6 N HCl and adjust the volume to 500 ml with distilled water. Filter through a sterile, 0.22 μm nitrocellulose filter and autoclave for 20 minutes at 121 °C

100X Denhardt' Solution:

Prepare and store in a sterilized container

Bovine serum albumin (BSA)	2 g
(Sigma# A 6003-fatty acid free)	
Polyvinyl pyrrolidone (PVP)	2 g
(Sigma# PVP40, avg.mol.wt. 40,000)	
Ficoll(type 400, Sigma# F4375)	
Sterile ddH_2O	80 ml

Dissolve with rapid stirring and adjust the volume to 100 ml with sterile distilled water. Filter through a sterile, 0.22 μm nitrocellulose filter and store at 4 °C

Denatured DNA stock (5mg/ml):

DNA (*calf thymus- Sigma# D1501)	500 mg
TE buffer pH 8	75 ml

Dissolve by vortexing, shaking, and then by sonicating. Sonicate 5 to 10 times in 30 second bursts at a power setting that gibes cavitations, but no forming. Chill the DNA solution in an ice H_2O bath between each sonication. Shake the DNA at

moderate speed on a shaker at room temperature until the DNA is completely in solution. Dialyze 4 times for 4 hours each at 4 °C against 20 volumes of TE pH 8. Take 10 µl and dilute to 1 ml for A_{260} (A_{260}=50 µg/ml). Dilute to 5 mg/ml with TE pH 8. Store at 4 °C.

20% (w/v) SDS:

Sodium dodecylsulfate	20 g
Sterile ddH_2O	100 ml

Stir rapidly to dissolve completely and filter through a sterile, 0.22 µm nitrocellulose filter.

Prehybridization Solution:

Formamide	50 ml (50% v/v)
20X SSPE	25 ml (0.75M Na^+)
100X Denhardt's	5 ml (5X)
Denatured DNA stock	2 ml (100 µg/ml)
H_2O	17 ml
20% (w/v) SDS	1 ml (0.2%w/v)

Add components, in the order given, and mix well. Filter through a 5 µm Teflon filter.

Hybridization Solution:

Formamide	50 ml (50% v/v)
20X SSPE	25 ml (0.75M Na^+)
100X Denhardt's	1 ml (5X)

H_2O	23 ml
20% (w/v) SDS	1 ml (0.2%w/v)

4. Oligolabeling DNA fragment

10 X buffer

500 mM Tris-HCl pH 6.9	0.5 ml of 1 M stock
1000 mM $MgSO_4$	100 µl of 1M stock
1 mM Dithiothreitol	1µl of 1M stock
600 µM each of dGTP, dATP and dTTP	6 µl of 100 mM stock

Adjust the volume to 1 ml with sterile distilled water.

Scintillation Fluid (500 ml):

PPO	2 g
POPOP	0.025 g
Toluene	333.5 ml
Triton	166.5 ml

5. Formadehyde gels for fractionating RNA and northern blotting

Gel Buffer 40X:

Triethanolamine	23.6 g (1.6 M)
$Na_2EDTA.2H_2O$	29.8 g (80 mM)
ddH_2O	500 ml

Adjust pH to 7.5 with 85% H_3PO_4, bring the volume to 1 liter with distilled water and autoclave.

Sample Buffer 1.25X:

Gel buffer 40X	25 µl
Formadehyde	165 µl
Formamide	500 µl
50% (v/v) glycerol	110 µl
Bromophenol blue	a few crystals to give
Xylene cyanal	a strong blue color

Adjust the volume to 1 ml with sterile distilled water.

Diethylpyrocarbonate Solution:

In a 250 ml flask with a stir bar, in the hood.

ddH_2O	100 ml
20% (w/v) SDS	5 ml
Diethyl pyrocarbonate	100 µl

Stir rapidly until diethyl pyrocarbonate has dissolved. Use within 10 minutes to clean gel box, casting stand, glass plates, slot-forming combs.

Reservior Buffer:

Gel Buffer 40X	12.5 ml
Formadehyde	42 µl

Bring the volume to 500 ml distilled water.

0.2 M Phosphate Buffer Stock:

A. $Na_2 HPO_4.7H_2O$	26.8 g

Dissolve in a final volume of 500 ml ddH_2O

B. $NaHPO_4.H_2O$	13.8 g

Dissolve in a final volume of 500 ml ddH_2O

Place solution A in a 1000 ml beaker with a stir bar on a magnetic stirrer. Add solution B with stirring until the pH reaches 7.0.

Ethidium Bromide Stock:

Ethidium Bromide	1 g
H_2O	100 ml

Stir several hours with a magnetic stirrer and store in a dark bottle at 4 °C.

DEPC-treated H_2O:

H_2O	100 ml
DEPC	0.5 ml

Shake vigorously and leave open under a hood overnight and autoclave.

6. SDS Gel

Resolving gel buffer 8X

Tris base	181.7 g
H_2O	250 ml

Adjust pH to 8.8 with conc. HCl, bring the volume to 500 ml with distilled water. Filter through a 0.22 μm nitrocellulose. Store at room temperature.

Stacking gel buffer 4X

Tris base	15.1 g
H_2O	150 ml

Adjust pH to 6.8 with conc. HCl, bring the volume to 250 ml with distilled water. Filter through a 0.22 μm nitrocellulose. Store at room temperature.

Acrylamide, 30% a: 0.8% B

Acrylamide	150 g
Bisacrylamide	4 g

Dissolve in final volume of 500 ml in distilled water. Filter through a 0.22 μm nitrocellulose. Store at 4 °C in a dark bottle.

Reservoir buffer 10X

Tris base	60.5 g
Glycine	288 g

Dissolve in final volume of 2 L in distilled water. Store at room temperature. Before use, add 2 g of SDS (0.1% SDS).

Sample buffer 5X

Tris base	375 mg
20% (w/v) SDS	5 ml
Glycerol	5 ml
2-mercaptoethanol	2.5 ml
H_2O	12.5 ml
Bromophenol blue	50 mg

Store at -20 °C in 1 ml aliquots.

10% (w/v) Ammonium Persulfate

Ammonium persulfate	0.5 g

Dissolve in a final volume of 5 ml in H_2O. Store at 4 °C. Prepare fresh every two weeks.

7. Protein staining solution

Staining solution, 1 litre

Coomassie Brilliant Blue R250	2.5 g
Methanol	500 ml
Glacial acetic acid	70 ml
H_2O	430 ml

Dissolve with rapid stirring and store at room temperature.

Destaining Solution

Methanol	100 ml
Glacial acetc acid	70 ml
H_2O	830 ml

Dissolve with rapid stirring and store at room temperature.

8. Western blotting

5X Transfer Buffer

Tris	5.82 g
Glycine	2.93 g
ddH_2O	700 ml

Adjust pH to 8.3, bring the volume to 1 liter with distilled water. Store at room temperature.

5X TBS (Tris buffer saline)

Tris	12.1 g
NaCl	146.1 g

Adjust pH to 7.5 with conc. HCl, bring the volume to1000 ml with distilled water.

BIOGRAPHY

Miss Warintra Takpirom was born on November 1, 1981 in Chachoengsao. After graduating with degree of Bachelor of Science from the department of Botany with genetic major at Chulalongkorn University in 2003, she furthered her education to obtain the Master of Science at the Biotechnology Program, Faculty of Science at Chulalongkorn University in that year.