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Differential expression of rice calmodulin promoters in response to stimuli and developmental tissue in transgenic tobacco plants

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The promoters of *OsCaM1* and *OsCaM3* were characterized after sequencing and fused to the reporter gene, GUS. The constructs were then transformed into the tobacco plant. Histochemical analysis of GUS showed different expression patterns in pOsCaM1::GUS and pOsCaM3:: GUS transgenic plants. The expression of pOsCaM1::GUS in 4- to 15-day-old seedlings in particular was observed only in the root, while the expression of pOsCaM3::GUS was detected in both the cotyledons and root. Also, pRCaM1::GUS was detected in all the tissues surrounding the root system, while the presence of pOsCaM3::GUS was observed in the root, except in the root meristem. However, in mature transgenic plants, the expression of pOsCaM1::GUS and OsRCaM3::GUS was scarcely detected. Under wounding stress, the GUS activity of pOsCaM1 and pOsCaM3 was strongly induced, and the activity of pOsCaM3 especially, was retained for long periods. In the phloem, pOsCaM3 activity induced by hormone treatments and abiotic stresses was also identified. [BMB reports 2010; 43(1): 9-16]

INTRODUCTION

Calcium (Ca^{2+}), regarded as a universal second messenger, acts as a mediator responding to stimulus in the regulation of diverse cellular functions. Cytosolic Ca^{2+} concentration rapidly rises via an increased Ca^{2+} influx in response to stimuli including light, gravity, abiotic and biotic stresses and hormones, and then quickly reverts to the basal level by Ca^{2+} efflux in plant (1, 2). Transient Ca^{2+} elevations are sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins, which most often contain the 'EF-hand' motif(s) and a helix-loop-helix structure (1, 3). Based on the number and organization of EF-hands and the similarity of the amino acid sequences, three major classes of Ca^{2+} sensors including EF-hand have been characterized in the plant, distinguishable as calcium-dependent protein kinase (CDPK) (4, 5), calcineurin B-like protein (CBL) (6), and calmodulin (CaM) (1, 3). Among those, CaM has been identified as the highly conserved Ca^{2+}-binding protein in plants. In addition, CaM isoforms have been described in higher plants (7, 8). The diversity observed in CaM isoforms has opened up avenues for investigation concerning their various functions, regulation, and evolution. CaM isoforms function differently on Ca^{2+}-binding ability and activation of CaM-dependent enzyme in plants (9, 10). It has been reported that CaM isoforms were related to salicylic acid (SA)-dependent signal transduction, producing disease resistance in soybean (9). Further research has revealed novel CaM-like proteins, which contain an extended C-terminal basic domain and CaaV-box motifs required for efficient prenylation of protein (11). To the recent, CaMs have been isolated, characterized, and reported upon, following biochemical studies. However, CaMs warrant active investigation, as detailed information is still unavailable.

CaM isoforms express themselves in variety of ways as they are differentially regulated by multiple signals such as hormones and chemicals, and stimuli such as the external abiotic (e.g. light, gravity, heat, touch, cold, salinity and drought) and biotic (e.g. phytohormones and pathogens) factors (10). CaM gene expression also varied with developmental stages specific to each organ during plant growth (12). CaM protein and mRNA are normally observed in high concentrations in proliferating populations of cultured cells and in the meristematic regions of shoot apex, stolon tip, and vascular tissues in plants (12). However, CaM mRNA expression can be induced by mechanical stresses such as touching and wounding in plants (8), though the mechanisms by which they recognize and respond to most environmental stimuli is still poorly understood. Particularly, the molecular mechanisms regulating wound-inducible expression of CaM genes are yet to be fully studied.

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In this investigation, a study of the expression of pOsCaMs revealed how plants sense and respond to extra-cellular signals and wounding. The pOsCaMs were characterized using transgenic plants carrying two different rice CaM promoters fused to the GUS reporter gene. In this study, transgenic tobacco plants were studied to investigate the regulation in the expression of the two types of rice CaM promoters induced by developmental stages and abiotic stresses, including wounding and hormone treatments. Tissue-specific expression was studied in the root tip, stem, and petiole of young transgenic tobacco plants.

RESULTS AND DISCUSSION

Analysis of *OsCaM* promoter

Promoters are short regulatory DNA sequences located upstream of a gene. The structural analysis of promoter sequences plays a significant role in successful gene prediction. To analyze the *OsCaM* promoter, two genomic clones for CaM named *OsCaM1* (OS01g16240) and *OsCaM3* (OS01g59530) were isolated from the rice genomic library and sequenced. In the 5′-flanking region, a number of potential regulatory motifs were found, quite similar to the cis-regulatory elements from eukaryotic promoters (Fig. 1). *OsCaM1* TATA box was observed at position -124 to -118 upstream from the transcription initiation site (Fig. 1A). *OsCaM3* TATA box was observed at positions -115 to -109 upstream from the transcription initiation site, while the CAAT box was found at position -137 to -134 (Fig. 1B); these boxes serve as the basal promoter elements for transcription. Significantly, sequence elements like W-box, earlier identified in plant genes, were found in the *OsCaM* promoter region. Seven W-boxes (TTGAC) were discovered in *OsCaM1*, though only one in *OsCaM3*. Three WRKY elements (TGAC) were also found in *pOsCaM1* and *pOsCaM3*, respectively. W-boxes and WRKY elements were involved in the sugar and GA_3 signaling pathway, and expression induced by wounding (13, 14). Seven GT-1 (GRWAAW; R = A/G; W = A/ T), that were reported in response to light and SA- induced expression, were discovered in *pOsCaM3* (15). Two GARE, recognized to be involved in GA_3 induction, were discovered at the -173 to -168 and -529 to -524 positions, respectively, in *pOsCaM3* (16). Their presence suggests the possibility of SA and GA_3-induced RCaM3 expression. On further examination, the nucleotide composition of *OsCaM1* and *OsCaM3* in the 5′-UTR revealed many stretches rich in C/T sequences. The C/T repeat sequences significantly control gene expression (17). The sequence analysis of the *OsCaM* promoters therefore, suggests that *OsCaM* promoters may potentially be related to induce tolerance against stresses into plants. According to these results and previous report, we selected the re-

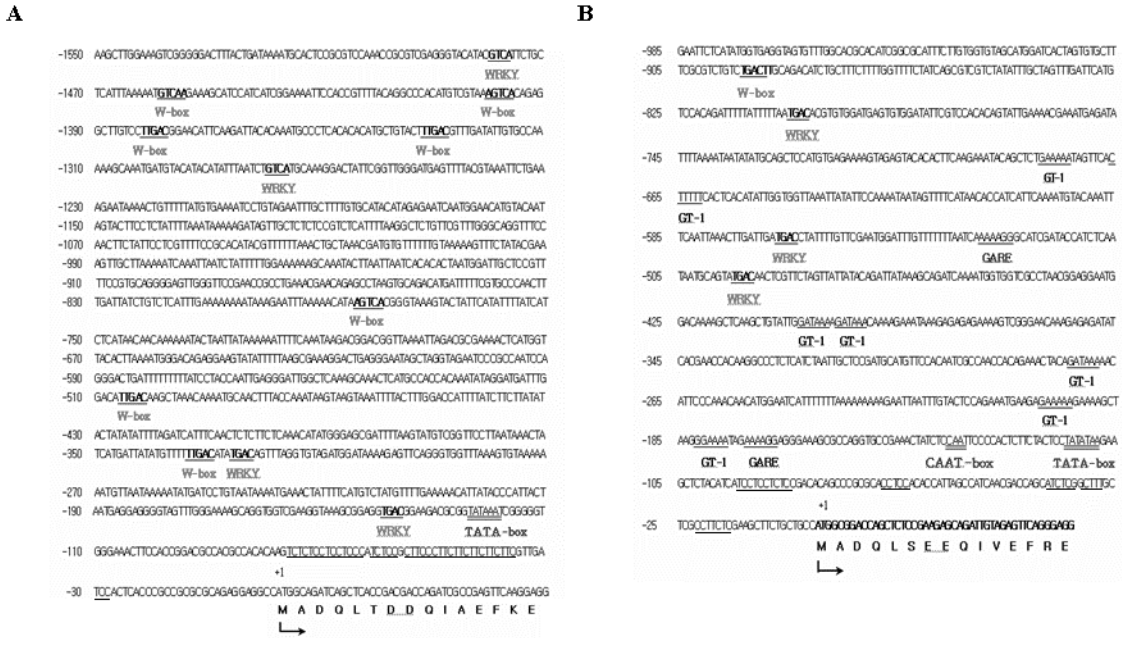

Fig. 1. Sequence analysis of *OsCaM1* (A) and *OsCaM3* (B). (A) Nucleotide sequence of the promoter region of *OsCaM1* gene. Sequences of approximately 1.5 kb of 5'-flanking region are shown together with the first exon of the *OsCaM1* gene. (B) Nucleotide sequence of the promoter region of *OsCaM3* gene. Sequences of approximately 1 kb 5'-flanking region are shown together with the first exon of the *OsCaM3* gene. The putative TATA box and CAAT box is double underlined. The number of nucleotides relative to the translation initiation site (+1) is shown. Potential W-box and WRKY element are shown in bold and are underlined. The hormone response motif is shown underlined.

gions indicated in Fig. 1 and constructed transgenic plants with the regions (18).

Expression of pOsCaMs in transgenic tobacco plants

Two *OsCaM* promoter-GUS fusion constructs were generated using a promoter expression vector system. The expression of the pOsCaM1-GUS or pOsCaM2-GUS fusion constructs in the various developmental stages of transgenic tobacco was then analyzed. Next, using histochemical staining, the GUS expression patterns were determined. Strong GUS staining was observed in almost all parts of the seedlings containing *pOsCaM1* (Fig. 2A), in the germination stage. In 3-day-old seedlings, only a weak GUS expression was observed in the cotyledon and radicle (Fig. 2A). As the seedlings grew, GUS staining was observed only in the root (Fig. 2A). In seedlings containing *pOsCaM3*, GUS expression was detected only in the cotyledon, in the germination stage (Fig. 2B). In 3-day-old seedlings, a strong GUS expression was observed in the cotyledon and root cap (Fig. 2B). As the seedlings grew, GUS staining was noted in the cotyledon and root (Fig. 2B). However, no GUS expression was observed in the leaves at the adult stage, and was only weakly discernible in the upper side of the petal and sepal (data not shown). These results indicated that *OsCaM1* and *OsCaM3* are differently expressed at each of the germination stages, and the expression is highly induced in the early developmental stages.

In transgenic tobacco for pOsCaM1-GUS or pOsCaM3-GUS fusion constructs, tissue-specific expression of *OsCaM1* and *OsCaM3* was also observed. In seedlings containing *pOsCaM1*, GUS staining was observed in the root meristem, root cap, and zone of cell elongation, and was especially high in the meristematic tissues associated with the activation of cell division

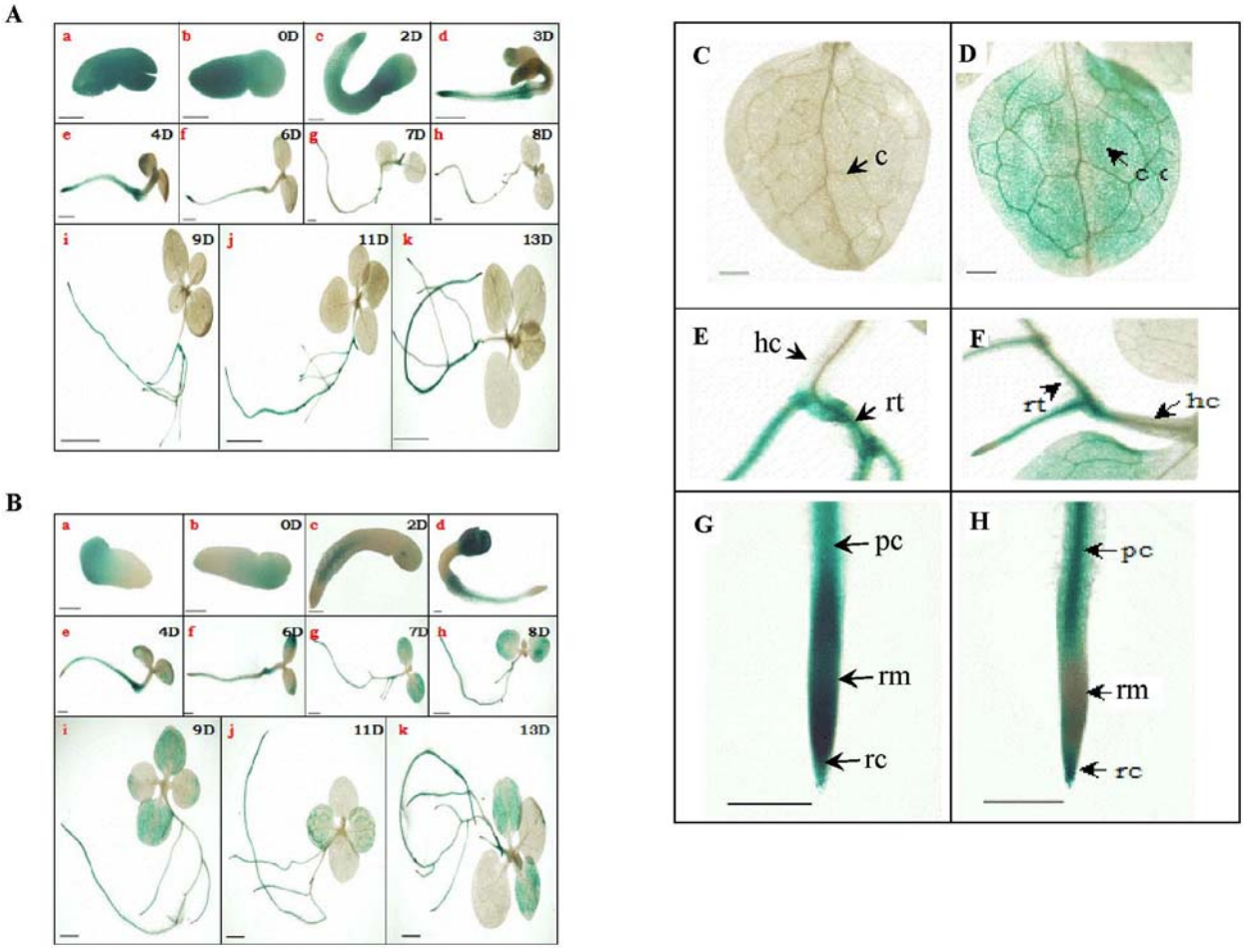

Fig. 2. Localization of GUS activity according to developmental stages in whole seedlings and organs of transgenic tobacco plants harboring pOsCaM1::GUS (A, C, E, G) or pOsCaM3::GUS (B, D, F, H). (A) Bar = 200 μm (a, b, c), 1 mm (d, e, f, g, h), 5 mm (i, j, k). (B) Bar = 200 μm (a, b, c), 500 μm (d, e), 1 mm (f), 2 mm (g, h, i, j, k). c, cotyledon; rt, root; hc, hypercotyl; pc, procambium; rm, root meristem; rc, root cap. Bars = 2 mm.

(Fig. 2E, G). However, no GUS signal was detected in the hypocotyl (Fig. 2C). In *pOsCaM3*-containing seedlings, GUS staining was observed in almost all the plant parts (Fig. 2D, F, H). Although GUS staining was not detected in the root meristem, a strong expression was observed in the root cap and procambium (Fig. 2H).

During the post-germination growth period, the degradation of bulk storage protein was reported to be induced in the cotyledon, where most of the storage nutrients are located (19). Although the evidence was not enough to demonstrate that OsCaM3 is involved in transporting the peptides, the expression of OsCaM3 in Fig. 2 indicates that OsCaM3 is possibly involved in transporting the materials derived from the degradation of storage materials in the growing and differentiating cells, and subsequently in mobilizing them from the cotyledon to the embryonic axis during the post-germinative growth period. Interestingly, OsCaM1 and OsCaM3 expression was detected in root tissues, which mobilized the protein reserves during germination (20). Both CaMs may potentially be related to signal transduction process and cell growth.

Induction of pOsCaMs::GUS expression in response to wounding

The induction of the pOsCaMs::GUS expression in response to wounding was observed by histochemical staining, in two-month old transgenic tobacco plants. As shown in Fig. 3, pOsCaMs were strongly induced by wounding in young leaves. pOsCaM1 was dramatically induced within 1 min after wounding, but disappeared after 30 min (Fig. 3A). pOsCaM3 was induced within 1 min after wounding and its activity was retained until 3 hours (Fig. 3B). From these results, it was found that the GUS expression was not detected in the leaves

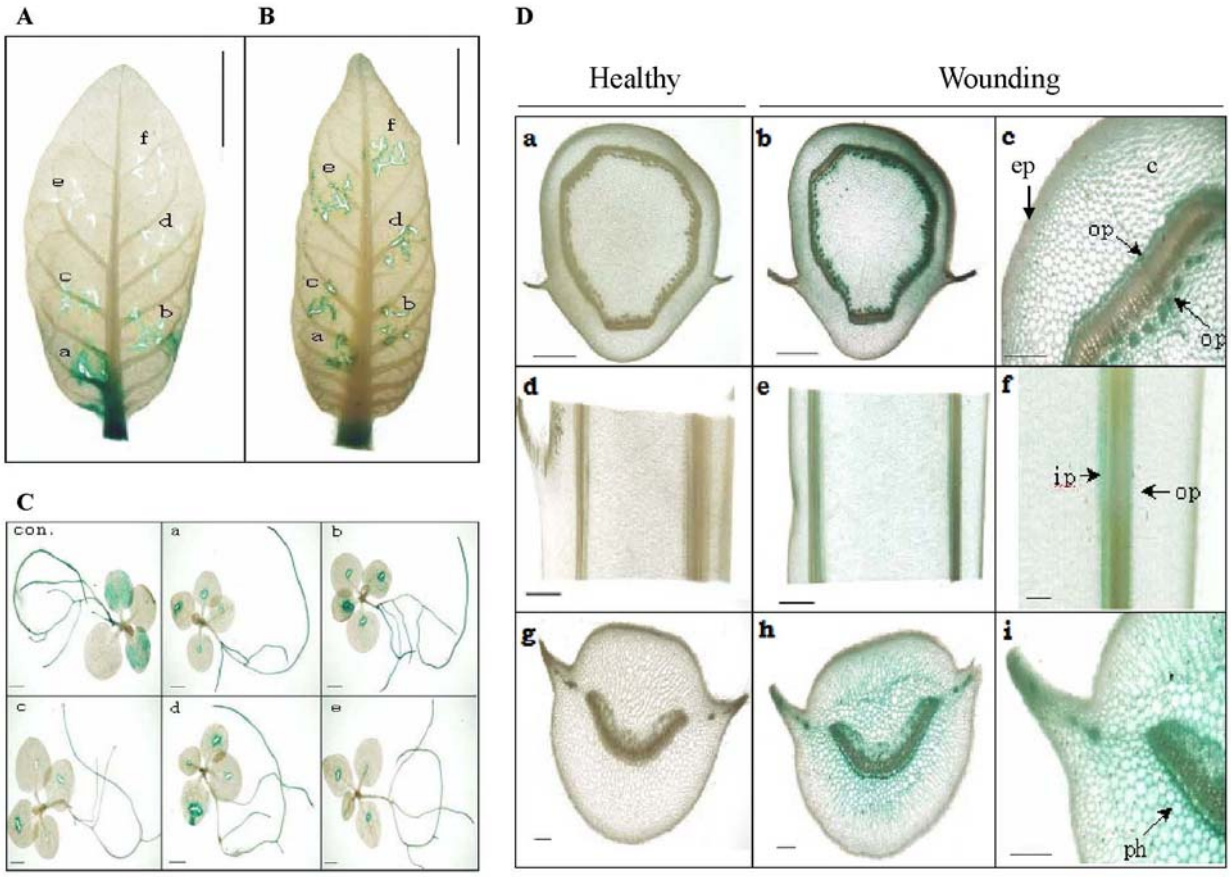

Fig. 3. Wounding response in organs of transgenic tobacco plants harboring pOsCaM1::GUS or pOsCaM3::GUS after wounding stress. In leaves and young seedlings harboring pOsCaM1::GUS (A) or pOsCaM3::GUS (B, C) after wounding stress, the responses were investigated until 1 (a), 5 (b), 10 (c), 30 (d), 60 (e) and 180 (f) min. In organs of transgenic tobacco plants harboring pOsCaM3::GUS after wounding stress, the responses were indicated (D). Stem and petiole in transgenic tobacco plants harboring pOsCaM3::GUS were prepared. a, transverse section of stem; d, longitudinal section of stem; g, transverse section of petiole; b, c, transverse section of stem at 10 min; e, f, longitudinal section of stem; h, i, transverse section of petiole at 10 min; c, cortex; ep, epidermis; ip, internal phloem; op, outer phloem; ph, phloem; con, control.

at the adult stage, but was induced within 1 min by wounding. In addition, pOsCaM3::GUS expression was retained for quite a long time by wounding. This reveals that OsCaM is highly sensitive to wounding, and that RCaM3 may be specifically involved in the recovering system or in interacting with other proteins to be alive. GUS expression induced by wounding was also examined in 15-day-old transgenic tobacco seedlings containing *pOsCaM3* (Fig. 3C). In GUS staining, OsCaM3 was induced within 1 min after wounding and its activity was retained until 1 hour (Fig. 3C). In addition, GUS expression in protein levels remained for 6 hours (data not shown).

To examine the tissue-specific expression of OsCaM in response to wounding, two-month-old transgenic *pOsCaM3*-containing tobacco plants were used. At 10 min after wounding, a significant induction of GUS expression in the vascular tissue, particularly in the phloem (Fig. 3D) was observed, mainly in the transverse and longitudinal sections of the stems and petioles. In addition, wounding-induced GUS expression was weakly detected in the cortex, though not in the epidermis.

In the stems and petioles, pOsCaM3 induction was observed in response to wounding (Fig. 3D). GUS expression particularly, was prominent in young stems and petioles. As the stem contains major vascular systems necessary for survival the negative phloem pressure leading to water absorption from the soil via the root occurs during the daytime. However, it disappears by a quick change in the membrane potential of the cells surrounding the phloem observed on a cross-section of the stem (21). The rapid and systemic change in hydraulic pressure was reported to have happened in response to wounding (22). These reports indicate that the wounded vascular system should immediately heal to recover the normal phloem pressure to sustain life. It is thus evident that the expression of the wound-induced gene is highly detectable in vascular systems. Our results demonstrated CaM's role in survival adaptability by responding to wounding.

Expression patterns of pOsCaM3 in response to various stresses and hormone treatments

To study the expression of pOsCaM in response to various stresses and hormone treatments 15-day-old transgenic *pOsCaM3*-containing tobacco seedlings were used. The protein accumulation in the seedlings, after stress or hormone treatment was analyzed using fluorometric GUS assay. Under MeJA, GA_3 or SA treatment, pOsCaM3 expression was enhanced approximately two- to three-fold relative to that of non-treated seedlings (Fig. 4). Its expression induced by SA and GA_3 was predicted from OsCaM promoter analysis. Under ABA treatment, pOsCaM3 expression was enhanced by about 1.5-fold relative to that of non-treated seedlings. However, the expression was inhibited by 2, 4-D (Fig. 4). Abiotic stresses, like drought, heat, and flooding enhanced pOsCaM3 expression, but no expression was induced in NaCl-treated seedlings. Under drought, heat, and flooding stresses, pOsCaM3 expression was enhanced by about 2.5, 3.0, and 2.0-fold relative to that of non-treated seedlings, respectively (Fig. 4). These results indicated CaM's involvement in mediating plant responses to biotic and abiotic stimuli.

In pOsCaM3 analysis, the presence of seven GT1-box binding core sequences suggested the possibility of salicylic acid effect on OsCaM3 expression. The GARE motif also indicated its expression by GA_3. These predictions are in concord with the results shown in Fig. 4. OsCaM3 was responsive to SA and GA_3. Plant growth and development have been reported to be regulated by SA and GA_3 (7, 9). This report along with our results demonstrated that CaM is involved in regulating plant development. OsCaM3 particularly, is more likely involved in plant developmental induction. In Fig. 4, it is evident that 2, 4-D, a common systemic herbicide, inhibited its expression. OsCaM3 was induced by MeJA, which are assumed to play a role in plant wounding (22, 23). The expression of OsCaM3 by wounding was confirmed by the earlier results (Fig. 3). OsCaM3 was responsive to drought and ABA, but no significant induction was detected by NaCl treatment (Fig. 4), suggesting that OsCaM3 could possibly be related to the ABA-dependent pathway during drought (24). In this study, the role of OsCaM3 was enhanced by heat and flooding. These results were in accordance with the earlier reports of CaM adapting to heat shock and anoxia (25, 26). Thus, CaM has been determined to demonstrate an important role in mediating plant responses to biotic and abiotic stimuli. Although more experiments are warranted in this study, it may be suggested that the 5'-flanking region of OsCaM genes is related to responses induced by hormone treatments and abiotic stresses and that OsCaM1 and OsCaM3 are differentially expressed in developmental stage and tissue and OsCaM3 does especially play a very important

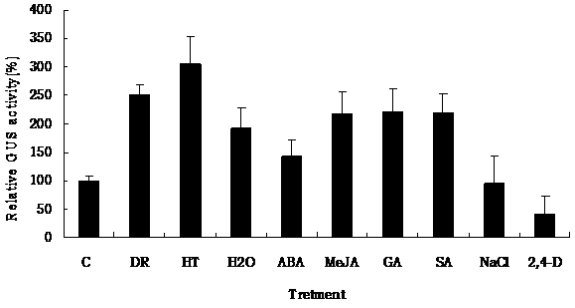

Fig. 4. GUS activity determined by the quantitative MUG assay in pOsCaM3::GUS transgenic tobacco plant under phytohormones and abiotic stresses. Whole plants were treated with abiotic stresses such as dehydration (DR), high temperature (HT), and flooding (H_2O), hormones such as abscisic acid (ABA), methyl jasmonic acid (MeJA), gibberellic acid (GA_3), salicylic acid (SA), and chemicals such as NaCl and 2,4-D, respectively. The transgenic plants harboring a pBI101.2 construct were used as the control plant (C). H_2O solution was used as the control solution for phytohormone. The error bars are the standard deviations. The GUS assay has been described under Materials and Methods.

part during early developmental stages.

MATERIALS AND METHODS

Analysis of promoter sequences

DNA sequences were analyzed using TRANSFAC (27) and Matinspector (28). TRANSFAC is a database containing eukaryotic transcription-regulating DNA sequence elements and the transcription factors binding to and acting through them. Matinspector is an internationally recognized tool for the analysis of the transcription factor, while PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, 29) and PLACE (http://www.dna.affrc.go.jp/PLACE/, 30) were used to determine the *cis*-acting regulatory elements in plant and to analyze the promoter sequences.

Construction of promoter-GUS fusions

The cellular expression of *OsCaM* genes was examined by fusing the GUS coding region to the *pOsCaMs*. The pOsCaM-GUS fusions were constructed using the pBI101.2 promoter expression vector with the β-glucuronidase (GUS) reporter gene. The *OsCaM1* and *OsCaM3* promoter regions (*pOsCaM1* and *pOsCaM3*) were digested with *Stu*I and treated with Klenow fragment to generate a blunt end. Next, pOsCaM1 was cut with *Hin*dIII, while pOsCaM-3 was cut with *Eco*RI. The pOsCaM1 and pOsCaM3 fragments were then ligated into the *Hin*dIII and *Sma*I sites of the pBI101.2. The resulting constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404.

Plant transformation and growth conditions

The pBI101.2 vector carrying the *pOsCaM*-GUS fusion constructs was transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method, and later used to transform the tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants. The transgenic seeds were surface-sterilized in 70% ethanol solution before being rinsed in bleach plus 0.1% Triton X-100 for 3 min, and then washed five times with sterilized water. After stratification for four days, the seeds were placed in a growth chamber (24-25°C, 16-h photoperiod, and 65% relative humidity), germinated, and grown on the selection media plate (1×Murashige and Skoog media containing 3% sucrose, 50 μg/ml kanamycin, and 250 μg/ml carbenicillin, 0.25% phyta-agar, pH 5.7 in KOH). The 15-day-old seedlings were then transferred into soil and grown in a culture chamber for further experiments. The transgenic plants, approximately two months old, were grown in the greenhouse to study the response to wounding and tissue specificity. In these experiments, the R2 progeny of the transgenic tobacco plants were used.

GUS histochemical analysis

Tissue-specific and wound-inducible CaM promoters were studied in the transgenic tobacco plants using several organs and tissues at different stages of development. Histochemical analysis of GUS activity was performed on a modified version of the earlier method. Briefly, plant materials were fixed in a fix solution (0.1 M Na_2SO_4 pH 7.0, 0.1% formaldehyde, 0.1% Triton X-100, and 0.1% β-mercaptoethanol) for 30 min and rinsed twice with 0.1 M Na_2SO_4 (pH 7.0). Then the samples were vacuum infiltrated in GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl glucuronide, 50 mM potassium phosphate buffer pH 7.0, 2 mM of potassium ferricyanide, 2 mM potassium ferrocyanide, and 0.2% Triton X-100) and incubated overnight at 37°C in darkness. To remove the chlorophyll, the stained tissues were then rinsed five times with 70% (v/v) ethanol at 37°C. Images were obtained using Olympus SZX12 stereo microscope (Olympus Corp., Tokyo, Japan) fitted with a Sony SSC-DC38P digital camera (Sony Corp. Tokyo, Japan).

GUS fluorometric analysis

The behavior of *OsCaM* promoters induced by various stresses was studied using transgenic tobacco seedlings. Fluorometric analysis of GUS activity was performed using 4-methylumbelliferyl-b-glucuronide (4-MUG). First, soluble proteins were extracted from the 15-day-old transgenic tobacco seedlings carrying each transgenic construct. The extracted proteins were mixed with GUS assay buffer (2 mM 4-MUG, 50 mM sodium phosphate buffer pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauroyl sarcosine, and 0.1% Triton X-100). The addition of the stop buffer (0.2M Na_2CO_3) halted the reaction. Next, 4-MUG was hydrolyzed by GUS to produce the fluorochrome of 4-methylumbelliferone (4-MU). GUS activity was determined in triplicate with a microplate spectrofluorometer for each time point (Wallac Victor 1420 Multilabel Counter, USA). GUS values were expressed as pmol 4-MU min^{-1} μg $protein^{-1}$ and data represented the mean ± S.D. The concentration of protein in the samples was determined using Bradford assay, according to the manufacturer's instructions.

Wounding stress and other treatments

Two-month-old transgenic tobacco plants were grown in the greenhouse at 24-26°C. Fully expanded leaves of healthy plants were wounded in several places on the leaf blade using a syringe, and GUS activity was observed from 1 min to 3 h. The expression of pOsCaM1::GUS and pOsCaM3::GUS induced by the wound were then compared. Stems and petioles were wounded with a scalpel, and GUS activity was detected in the transverse and longitudinal sections up to 10 min. Using a syringe, the leaves of the seedlings grown in MS medium in Petri dishes were wounded in several places on the leaf blade, and GUS activity was observed from 1 min to 6 h.

To study the effect of the stress of hormone treatments, 15-day-old seedlings were placed in MS liquid medium including 2 mM SA, 200 μM MeJA, 100 μM ABA, 20 μM GA_3, or 1 mM 2, 4-D. For the salt or flooding stress, transgenic seedlings were subjected to soaking in Petri dishes filled with 250 mM NaCl solution or sterilized water for 30 min. For the drought stress, the transgenic seedlings were placed in dry dishes and

exposed to air for 30 min. Heat stress was carried out at 37°C in darkness. Most treatments were performed at room temperature in darkness, unless otherwise mentioned. Following each stress treatment, the plant samples were immediately frozen in liquid nitrogen for further study.

REFERENCES

1. Reddy, A. S. (2001) Calcium: silver bullet in signaling. *Plant Sci.* **160,** 381-404.
2. Sanders, D., Pelloux, J., Brownlee, C. and Harper, J. F. (2002) Calcium at the crossroads of signaling. *Plant Cell* **14(Suppl 1),** S401-417.
3. Snedden, W. and Fromm, H. (2001) Calmodulin as a versatile calcium signal transducer in plants. *New Physiol.* **151,** 35-66.
4. Harmon, A. C., Gribskov, M. and Harper, J. F. (2000) CDPKs - a kinase for every Ca^{2+} signal? *Trends Plant Sci.* **5,** 154-159.
5. Cheng, S. H., Willmann, M. R., Chen, H. C. and Sheen, J. (2002) Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. *Plant Physiol.* **129,** 469-485.
6. Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S. and Wilhelm Gruissem, W. (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *Plant Cell* **14,** S389-400.
7. Khan, M., Yang, S., Iwasaki, Y., Fujisawa, Y., Fukuda, H. and Komatsu, S. (2005) A gibberellin-regulated protein phosphorylated by a putative Ca^{2+}-dependent protein kinase is G-protein mediated in rice root. *Plant Cell Environ.* **28,** 679-687.
8. Nakagawa, Y., Katagiri, T., Shinozaki, K., Qi, Z., Tatsumi, H., Furuichi, T., Kishigami, A., Sokabe, M., Kojima, I., Sato, S., Kato, T., Tabata, S., Iida, K., Terashima, A., Nakano, M., Ikeda, M., Yamanaka, T. and Iida, H. (2007) Arabidopsis plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 3639-3644.
9. Heo, W. D., Lee, S. H., Kim, M. C., Kim, J. C., Chung, W. S., Chun, H. J., Lee, K. J., Park, C. Y., Park, H. C., Choi, Y. J. and Cho, M. J. (1999) Involvement of specific calmodulin isoforms in salicylic acid-dependent activation of plant disease resistance responses. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 766-771.
10. Lee, S. H., Johnson, J. D., Walsh, M. P., van Lierop, J. E., Sutherland, C., Xu, A., Snedden, W. A., Kosk-Kosicka, D., Fromm, H., Narayanan, N. and Cho, M. J. (2000) Differential regulation of Ca^{2+}/calmodulin-dependent enzymes by plant calmodulin isoforms and free Ca^{2+} concentration. *Biochem. J.* **350,** 299-306.
11. Xiao, C., Xin, H., Dong, A. W., Sun, C. R. and Cao, K. M. (1999) A novel calmodulin-like protein gene in rice which has an unusual prolonged C-terminal sequence carrying a putative prenylation site. *DNA Res.* **6,** 179-181.
12. Yamakawa, H., Mitsuhara, I., Ito, N., Seo, S., Kamada, H. and Ohashi, Y. (2001) Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plant. *Eur. J. Biochem.* **268,** 3916-3929.
13. Sun, C., Palmqvist, S., Olsson, H., Boren, M., Ahlandsberg, S. and Jansson, C. (2003) A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. *Plant Cell* **15,** 2076-2092.
14. Zhang, Z. L., Xie, Z., Zou, X., Casaretto, J., Ho, T. H. and Shen, Q. J. (2004) A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. *Plant Physiol.* **134,** 1500-1513.
15. Buchel, A. S., Brederode, F. T., Bol, J. F. and Linthorst, H. J. M. (1999) Mutation of GT-1 binding sites in the Pr-1A promoter influences the level of inducible gene expression *in vivo*. *Plant Mol. Biol.* **40,** 387-396.
16. Mena, M., Cejudo, F. J., Isabel-Lamoneda, I. and Carbonero, P. A. (2002) Role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in Barley Aleurone. *Plant Physiol.* **130,** 111-119.
17. Huang, J., Teyton, L. and Harper, J. F. (1996) Activation of a Ca^{2+}-dependent protein kinase involves intramolecular binding of a calmodulin-like regulatory domain. *Biochem.* **35,** 13222-13230.
18. Phean-o-pas, S., Limpaseni, T. and Buaboocha T. (2008) Structure and expression analysis of the OsCam1-1 calmodulin gene from Oryza sativa L. *BMB Rep.* **41,** 771-777.
19. Muntz, K., Belozersky, M. A., Dunaevsky, Y. E., Schlereth, A. and Tiedemann, J. (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *J. Exp. Bot.* **52,** 1741-1752.
20. Katsutomo, S., Susumu, H., Hiroyuki, I., Shigemi, S., Hirokazu, M. and Yuko, O. (2002) A wound-inducible tobacco peroxidase gene expresses preferentially in the vascular system. *Plant Cell Physiol.* **43,** 108-117.
21. Lavine, A. R., Pennell, I., Alvarez, M. E., Palmer, R. and Lamb, C. (1996) Calcium mediated apoptosis in a plant hypersensitive disease resistance response. *Current Biol.* **6,** 427-436.
22. Leon, J., Rojo, E. and Sánchez-Serrano, J. J. (2001) Wound signalling in plants. *J. Exp. Bot.* **52,** 1-9.
23. Leon, J., Rojo, E., Titarenko, E. and Sánchez-Serrano, J. J. (1998) Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca^{2+}/calmodulin in Arabidopsis thaliana. *Mol. Gen. Genet.* **258,** 412-419.
24. Hu, X., Jiang, M., Zhang, J., Zhang, A., Lin, F. and Tan, M. (2007) Calcium-calmodulin is required for abscisic acid-induced antioxidant defense and functions both upstream and downstream of H_2O_2 production in leaves of maize (*Zea mays*) plants. *New Phytol.* **173,** 27-38.
25. Aurisano, N., Bertani, A. and Reggiani, R. (1995). Involvement of calcium and calmodulin in protein and amino acids metabolism in rice roots under anoxia. *Plant Cell Physiol.* **36,** 1525-1529.
26. Gong, M., van der Luit, A. H., Knight, M. R. and Trewavas, A. J. (1998) Heat-shock induced changed in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. *Plant Physiol.* **116,** 429-437.
27. Wingender, E., Dietze, P., Karas, H. and Knüppel, R.

(1996) TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic. Acids Res.* **24,** 238-241.
28. Kerstin, Q., Kornelie, F., Holger, K., Edgar, W. and Thomas, W. (1995) Matind and Matinspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic. Acids Res.* **23,** 4878-4884.
29. Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouze, P. and Rombauts, S. (2002) Plant CARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic. Acids Res.* **30,** 325-327.
30. Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA-elements (PLACE). *Nucleic. Acids Res.* **27,** 297-300.