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Tissue Specific Expression of Wound-Inducible RCaM-2 Promoter in Transgenic Tobacco Plants

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To study calmodulin (CaM) gene expression and its regulation, rice CaM promoter (RCaM-2) was isolated and fused to β-glucuronidase (GUS), reporter gene. X-Gluc staining patterns revealed that GUS localization is high in meristemic tissues such as the stem apex, stolen tip, and vascular regions. GUS staining in the transverse sections of stem and petiole was restricted to the inside of the vascular system, and cortex and epidermis located outside of the vascular system usually did not show GUS staining even a plant that expressed strong activity. GUS activity was found to be tissue specific expressed and exhibited a dramatic transient increase in response to wounding. These results suggest that the 5'-flanking region of RCaM gene regulates wound-inducible expression.

Key words – calmodulin promoter, wounding, histochemical localization

The ability to respond to extracellular signals is essential for the development and survival, of all living organism, to adapt to changing and adverse environmental conditions. One of the severest environmental stresses to which plants may be subjected is wounding, which may come about through such diverse causes as mechanical injury or herbivore attack. Thus, plant cells perceive a various signals from their surrounding, and convert them into a cellular response via signal transduction pathways.

Calmodulin known as multifunctional regulatory protein is a highly conserved protein and it interacts with other proteins in the cell and regulate their activity. In plants, the activities of several enzymes such as protein kinase, NAD-kinase, glutamate decarboxylase, and Ca^{2+}-ATPase are known to be regulated by calmodulin and calmodulin related protein[24,29,36].

Several CaM and CaM-related genes have been described in high plants such as *Arabidopsis thaliana*[3,9,22], potato[30], soybean[18], wheat[34], tobacco[33] and rice[6]. The diversity of CaM or CaM-related genes raised questions concerning their function, regulation and evolution. Plant CaM isoforms showed different functions for calcium-binding abilities and activation of CaM-dependent enzymes [11,18,19] Recently, it has been proposed that specific soybean CaM isoforms are components of SA-independent signal transduction chain leading to disease resistance[11]. The calmodulin-like proteins were also identified novel calmodulins with an extended C-terminal basic domain and CaaV-box motifs which are required for efficient prenylation of the protein[27,32].

The expression of calmodulin isoforms is known to be differentially regulated by a variety of signals such as hormones and chemicals[4,7,14] CaM gene expression was also regulated in developmental and an organ-specific manner during the plant growth[7,30,35]. The high level of CaM protein and mRNA generally are observed in proliferating populations of cultured cells and in plant meristematic regions including the shoot apex, stolen tip, and vascular tissues[30,32]. The expression of calmodulin mRNA is also induced by mechanical stresses such as touching and wounding in plants[1,2,3,30]. However, how plants recognize most environmental stimuli is poorly understood. The molecular mechanisms regulating wounding inducible expression of calmodulin genes have not been understood.

To investigate how plants sense and respond to wounding, we focus on the expression of the rice calmodulin promoters. We have characterized using transgenic plants carrying two different calmodulin promoters fused to the GUS reporter gene. The regulation of developmental and wound-induced calmodulin gene expression was studied in transgenic tobacco plants during the plant growth. Furthermore, tissue specific expression was studied in the root tip, stem, and petiole of young transgenic tobacco plants.

Materials and Methods

Bacterial strains and materials

The *Escherichia coli* host strain used for the gene mani-

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pulation of RCaM-2 promoters was XlL1-Blue (Stratagene). *Agrobacterium tumefaciens* strain LBA4404, which carries helper Ti plasmid pAL4404[12], was used for maintenance of plasmids and transformation of tobacco leaves. The plasmid pAL4404 is an avirulent derivative of the octopine Ti plasmid Ach5 obtained by deletion of the entire transfer DNA (T-DNA) that carries the tumor genes. RCaM-2 promoter-GUS fusion gene was constructed using a Ti-binary vector plasmid PBI101.2 containing the β-glucuronidase (GUS) reporter gene. Tobacco plants *(Nicotiana tabacum* L. cv. Xanthi) were maintained on MS[23] agar medium as sterile shoot cultures.

Construction of the Plant expression Vector

RCaM promoter-GUS fusion gene was constructed using a promoter exoression vector pBI101.2 the β-glucuronidase (GUS) reporter gene as described in Fig. 1. The RCaM-2 promoter region was digested with *Nco*I and treated with Klenow fragment to give a blunt end, and cut with *Hin*dIII. RCaM promoter fragment was ligated into the *Hin*dIII-*Sma*I digested pBI101.2 to generate pMB 302. Nucleic acid manipulation was done as described by Maniatis *et al.*[28].

Plant transformation

The RCaM promoter-GUS fusion gene was transferred by the directed DNA transfer method to the *Agrobacterium tumefaciens* strain LBA4404, which was used for the transformation of tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants by the cocultivation method. Transformed cells and regenerated plantlets were selected on MS agar medium supplemented with 3% sucrose, 50 μg/ml kanamycin, and 250 μg/ ml carbenicillin.

Transgenic tobacco plants were grown in the green house at 25℃/20℃ days/night, under a 16 h photoperiod, and 65% relative humidity. About 2-month-old transgenic plants grown in the greenhouse were used to study wound response and tissue specificity.

Histochemical analysis of β-glucuronidase activity

The expression of tissue specific and wound-inducible of CaM promoters was performed utilizing several organs and tissues at different stages of plant development in transgenic tobacco plants. Histochemical staining for GUS activity was performed according to the procedure of Jefferson with the modifications proposed by Koltunow[16]. Whole Leaves and different plant organ samples were fixed for 30 min in a fix solution (0.1 M Na_2SO_4, pH 7.0, 0.1% formaldehyde, 0.1% Triton X-100, and 0.1% 2-mercaptoethanol) and rinsed twice with 0.1 M Na_2SO_4 (pH 7.0). The tissue samples were incubated in a staining solution of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (x-Glu) and 50 mM Na_2SO_4, (pH 7.0) at 37℃ overnight. For chlorophyll removal, the tissues were soaked in 70% ethanol and the blue staining was scored visually.

Transverse and longitudinal sections of the petioles and stem are cut by hand with a scalpel. For wound induction studies, leaves were wounded with the syringe in several places on the leaf blade to 5 to 30 mm, without crushing. Wound-induced expressions of RCaM-2 promoter-GUS fusion gene is compared. Stems and petioles were wounded by the syringe in a place on the tissues after cutting.

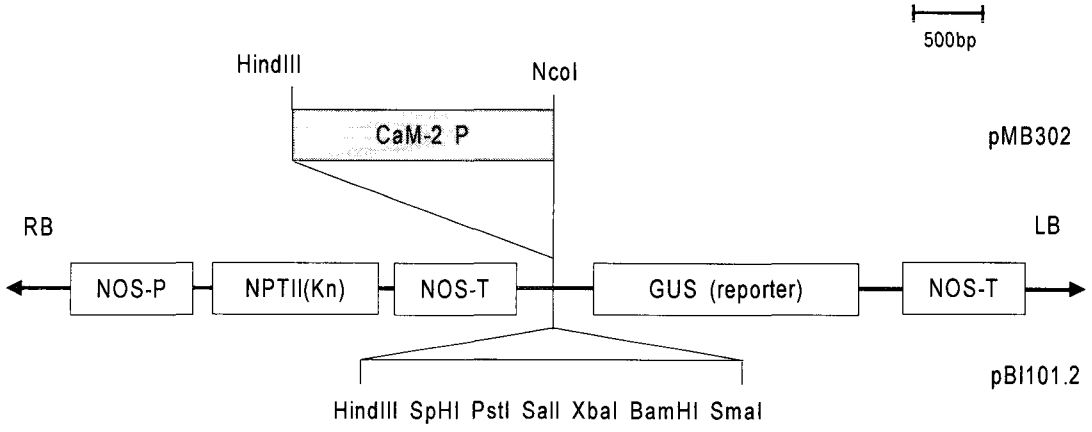

Fig. 1. Structure of plasmid used this study. Ti binary vector pBI101.2 containing the β-glucuronidase (GUS) reporter gene was digested with *Hin*dIII-*Sma*I, which is located at the upstream region of the GUS gene. The RCaM-2 promoter was digested with *Hin*dIII-*Nco*I that cut at start site the *exon*-1 of the rice CaM-2. DNA fragments was ligated to generate pMB 302 in which the RCaM-2 promoter gene linked to the GUS gene in the same reading frame to form a fusion protein.

Results and Discussion

Construction of the RCAM-2 promoter GUS fusion gene

The CaM genes from the rice genomic library have been isolated and sequenced[6]. RCAM-2 promoter-GUS fusion gene was constructed using a promoter expression vector system (Fig. 1). Ti binary vector pBI101.2 containing the β -glucuronidase (GUS) reporter gene was digested with *Hin*dIII-*Sma*I, which is located at the upstream region of the GUS gene. The RCaM-2 promoter was digested with *Hin*dIII-*Nco*I that cut at start site the *exon*-1 of the rice CaM-2. The DNA fragment was ligated to generate pMB 302 in which the RCaM-2 promoter gene linked to the GUS gene in the same reading frame to form a fusion protein.

Tissue specific expression of RCaM promoters-GUS fusion gene in transgenic plants

We have previously reported that calmodulin gene expression in transgenic tobacco plants was developmentally regulated using RCaM promoter-CAT fusion molecules[7]. To further investigate the expression of calmodulin promoter, GUS activity in transgenic tobacco plants with the rice RCaM-2 promoter-GUS fusion gene was carried out with X-glucuronidase as the chromogenic substrate. RCaM-2 promoter was preferentially expressed in specific tissues of organ, including stem apex, root tip and vascular tissues of young plants (Fig. 2). The RCaM-2 promoter activity was differentially expressed in various organs during development. During plant growth, the activity of RCaM-2 promoter was significantly reduced.

Histochemical staining for GUS activity in transgenic tobacco with the RCaM-2 promoter-GUS fusion gene in the transverse (Fig. 2A, D) and longitudinal sections (Fig. 2B, C) of stem and petioles was restricted to the inside of the vascular system, and the cortex and epidermis located of the vascular system usually did not show GUS staining even a plant that expressed strong activity.

Transverse sections of the root tip in transgenic tobacco with the RCaM-2 promoter-GUS fusion gene taken at two zone along the root were prepared (Fig. 3). Histochemical localization with the RCaM-2 promoter-GUS fusion gene revealed that in the region where the metaxylem vessel members began to differentiate expression was apparent in epidermis and meristem cortex. In more proximal parts of the root expression gradually decreased.

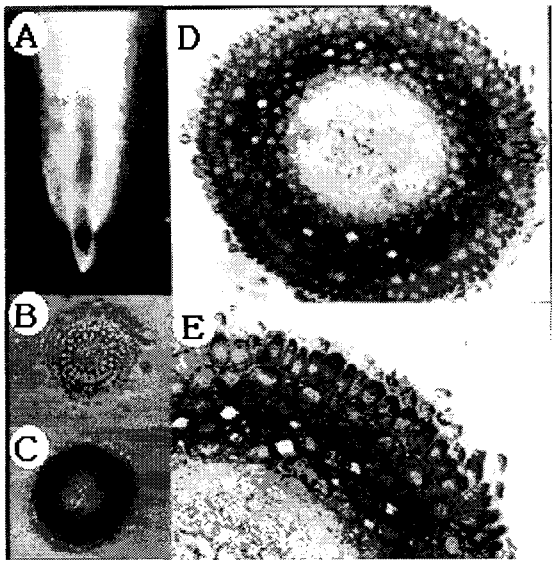

Fig. 3. Histochemical localization of expression of GUS activity in root tip of tobacco plants transformed with RCaM-GUS fusion gene. A, root tip; B, transverse section of root; C, transverse section of root tip; D, E, expended picture of transverse section of root tip (3X, 5X) respectively.

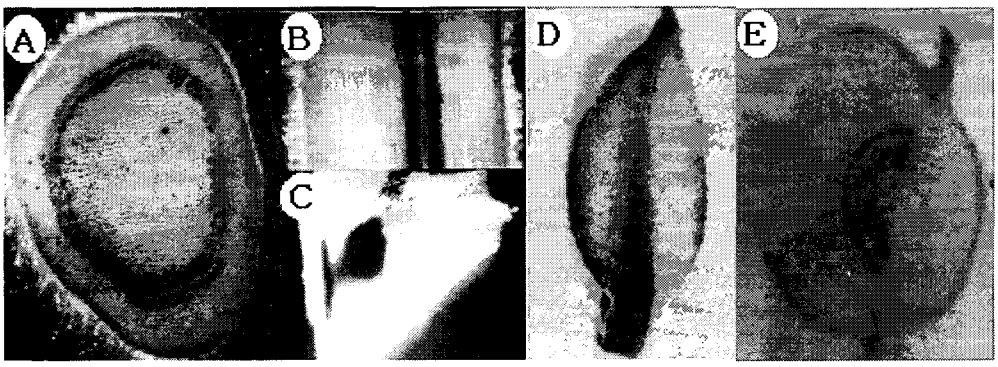

Fig. 2. Histochemical localization of expression of GUS activity in stems and petioles of transgenic tobacco plants harboring RCaM promoter-GUS fusion gene. A, transverse section of stem; B-C, longitudinal section of stem and axillary bud. D, young leave; E, transverse section of petioles.

The promoter regions of the different CaM genes have been analyzed (Table 1). Several putative transcription factor sites such as a GC box, a TATA-like box, a cyclic AMP response element CRE) and several AGGGA elements have been identified, however the elements responsible for their strong co-expression, and even those providing different spatial and temporal control, remain to be elucidated.

The effect of wounding on activation of RCaM-2 promoter

The induction of the RCaM-2 promoter-GUS reporter gene in response to wound was confirmed by histochemical staining of the transgenic tobacco plants. As shown in Fig. 4, RCaM-2 promoter in young (upper) leaves was strongly induced by wounding, however little was induced in matured (lower) leaves. GUS activity was dramatically induced within 5 min after wounding and reduced after 2h. In petal, stamina and petioles, the induction of RCaM-2 promoter was observed in response to wound (Fig. 4C-F). High GUS activity was detected young stem and petioles, whereas old leaves was dramatically reduced. An interesting result was found that GUS activity did not detected in leaves blade but the activity was highly induced 5 min after wounding. Especially, GUS activity is high in meristemic tissues correlated with active s cell division[26,30]. These results suggest that 5'flanking region of RCaM-2 controls developmental, tissue specific and wound-induced expression.

In view of the role Ca^{2+} plays in mediating plant responses to biotic[17] and abiotic[15] stimuli, it is not surprising that CaM, as an important cellular Ca^{2+} modulator, is involved in mediating these responses. Table 2 indicates the evidence for the involvement of CaM in plant

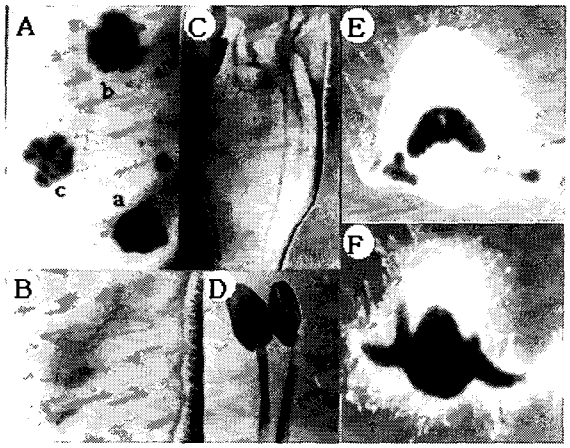

Fig. 4. Expression of RCaM promoter-GUS fusion gene in response to wound. A, young (upper) leaf ; B, old (lower) leaf; C, petal; D, stamina; E, F, petioles. Young leaf was wounded *in vivo* conditions for 5(a), 30(b), and 120(c) min. Other organs were wounded *in vivo* conditions for 5min.

Table 1. Comparison of putative promoter sequences of rice CaM genes (RCaM-1 and RCaM-2) with those of other CaMs

Gene	TATA box	CAAT box	GC rich region	CREB/AP-2	HSE	AGGA
Rice CaM-1	+	-	+	-	-	+
Rice CaM-2	-	+	+	+/+	-	+
Soybean CaM-1	+	+	+	-	-	+
Soybean CaM-4	+	+	+	-	-	+
Arabidopsis CaM-3	+	+	-	+/+	+	-
Chlamidomonas	+	-	+	-	+	-
Human CaM-III	-	+	+	-	-	+
Rat CaM I	+	-	+	-	-	+
Rat CaM II	-	-	+	+/+	-	+
Drosophila CaM	+	-	+	-	-	+

Table 2. Involvement of calmodulin in plant responses to touch and wound

CaM, CaM-like genes	Type of evidence	Reference
RCaM-2(rice)	gene expression[1)]	This research
PCM1(potato)	gene expression[2)]	[30]
TCH CaM-like genes (*Arabidopsis*)	gene expression[2)]	[5]
NtCaM-1,2,3 and 4(tobacco)	gene expression[1,2)]	[21]
MBCaM-1(Mung bean)	gene expression[2)]	[2]
NpCaM-1(*Nicotiana plumbaginifolia*)	gene expression[2)]	[31]

[1)]Promoter activity analysis, [2)]mRNA analysis

responses to wound signal. RCaM-2 promoter was developmentally regulated and expressed in response to stresses such as hormones and wound signal.

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초록 : 상처에 의해서 유도되는 벼 calmodulin promoter의 transgenic 담배에서조직 특이적 발현

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Calmodulin 유전자의 발현 조절을 연구하기 위해, 벼 calmodulin promoter (RCaM-2)를 분리하여 GUS (report 유전자)에 융합하였다. GUS 활성은 정단조직, 근단 및 관다발 영역과 같은 성장조직에서 높게 발현되었다. 줄기와 페티올의 transverse 절단부위 GUS 활성은 관다발계의 안쪽에 제한되었으며 관다발계의 외부에 위치한 피층과 표피에서는 강하게 발현된 식물에서도 GUS 활성이 나타나지 않았다. GUS 활성은 어린 조직에서 특이적으로 발현되었으며 상처에 의해서 신속하게 증가하였다. RCaM-2 promoter는 세포분열이 왕성한 어린조직이나 생장점에서 강하게 발현되며 mechanical 신호에 의해서 현저히 유도되었다. 이러한 결과는 RCaM-2 유전자의 5'-flanking 영역이 상처에 의해서 유도되는 발현을 조절하는 것으로 추정된다.