Results

Cloning and sequence analysis of 13 tobacco CaM cDNAs

We cloned 13 CaM cDNAs by library screening and reverse transcriptase-polymerase chain reaction (RT-PCR).

In order to obtain tobacco CaM genes, I screened cDNA libraries prepared from; (a) TMV-infected tobacco leaves 3 h after the temperature shift from 30°C to 20°C, corresponding to 5 h before the synchronous HR lesion formation (See Methods and Figure 6A), (b) TMV-infected leaves 12 h after the temperature shift, corresponding to 4 h after the lesion appearance, (c) tobacco leaves 30 min after wounding, or (d) tobacco stems 15 min after cutting. After conventional hybridization screening of 6×10^4 plaques from each library, I isolated 178 independent positive clones using three probes; a tobacco HR-induced CaM cDNA which had been previously isolated by our group (Seo *et al.*, 1995), and soybean *SCaM-1* and *4* genomic fragments (Lee *et al.*, 1995). Sequence analyses revealed that they encode 12 distinct CaM genes, designated *NtCaM1* to *12* (Figure 1). An alignment of these *NtCaM* genes based on the deduced amino-acid sequences is shown in Figure 2. The 12 *NtCaM* genes encode three distinct proteins, NtCaM1/2, 3/4/5/6/7/8/11/12, and 9/10. The latter two types differ in only one amino acid. All the NtCaM proteins have four well-conserved Ca^{2+}-binding motif EF-hands, as indicated by asterisks, and are divided into two groups, I and II (Figure 2). NtCaM1/2 belongs to group I and has high similarity to touch-stimulated potato PCM1 (Takezawa *et al.*, 1995) with only one amino-acid substitution. NtCaM3/4/5/6/7/8/11/12 and 9/10 belong to group II, which contains the ordinary-type plant CaMs such as *Arabidopsis* ACaM-2/3/5 (Perera and Zielinski, 1992).

21

To isolate *SCaM-4*-type CaM genes (Heo *et al.*, 1999; Lee *et al.*, 1995), I conducted RT-PCR with degenerated primers for *SCaM-4* gene, using mRNA from TMV-infected leaves 3 h after the temperature shift as a template. A 327-bp fragment for a putative *SCaM-4* homolog was amplified. By subsequent rapid amplification of cDNA ends (RACE) PCR, I obtained a full-length cDNA for a possible tobacco *SCaM-4* ortholog, *NtCaM13* (Figure 1), whose gene product is substituted by 19 amino-acid residues with most of the SCaM-4-specific amino acids conserved (Figure 2). NtCaM13 is classified to group III with SCaM-4. A phylogenetic tree by the neighbor-joining method (Figure 3) showed that plant CaM isoforms identified to date are diverged into three major clusters, while vertebrate CaMs converge to one small cluster. Expectedly, the obtained NtCaM isoforms (group I, II, and III) were sorted into three clusters, I, II and III, respectively. Further, the nucleotide sequence homology among 13 *NtCaM* genes is compared in Table 1. The homology is more than 73% in the coding regions, and 47% or more even in the 3' untranslated regions (UTRs). Table 2 shows the homology of amino-acid sequences, indicating that NtCaM13 has the most unique structure among NtCaMs isolated here.

Organ-specific expression of NtCaM *genes*

For the study of organ-specific expression of individual *NtCaM* genes, I used each 3' UTR as a specific probe. The homology of the coding regions is more than 73% among the *NtCaM* genes, however that of 3' UTR is relatively low (Table 1). For instance, the homology of *NtCaM1* for *NtCaM3* to *13* is 46 to 67%, indicating the 3' UTR of *NtCaM1* could distinguish its own transcript from others except that of *NtCaM2* whose 3' UTR has 93%

homology to that of *NtCaM1*. The profile of DNA gel blot analysis of the *NtCaM1* with 3' UTR probes was almost the same as that of *NtCaM2* (data not shown), indicating that *NtCaM1* and *2* in tobacco plant would be originally the ortholog of the ancestral plants, *N. tomentosiformis* and *N. sylvestris*, respectively. Similarly, the sets of *NtCaM3* and *4*, *5* and *6*, *7* and *8*, *9* and *10*, *11* and *12*, have a high homology in their 3' UTR more than 90%, indicating similar relation to *NtCaM1* and *2*.

RNA gel blot analysis of healthy tobacco plants using individual 3' UTR showed almost similar organ-specific expression profiles in the *NtCaM1* to *12* genes (Figure 4); constitutive expression in stems, roots, flowers and shoot apices. For leaves, *NtCaM1* to *4*, *11* and *12* were expressed strongly in older leaves at lower position. Interestingly, the *NtCaM13* gene was expressed considerably in roots and slightly in shoot apices but not in other organs. However the expression level of *NtCaM13* was far lower than those of *NtCaM1* to *12*.

Preparation of antibodies specific to individual type of tobacco CaMs

Recombinant NtCaM1, 3 and 13 proteins were expressed in *E. coli* and purified by boiling and subsequent Ca^{2+}-dependent hydrophobic chromatography (Figure 5A). Antibodies that recognize the CaM proteins in groups I, II and III, were raised by immunizing rabbits with the recombinant NtCaM1, 3 and 13 proteins, respectively, and purified by immunoabsorption affinity chromatography. Figure 5B shows the result of protein gel blot analysis of the purified *NtCaM1*, *3* and *13* proteins using each affinity-purified antibody. Every antibody recognized the corresponding recombinant CaM protein strongly. In the presence of Ca^{2+}, the electrophoretic mobility of all three types of NtCaM protein was shifted

23

from the upper (indicated by open arrow heads) to the lower positions (closed arrow heads) as previously shown with bovine brain CaM (Burgess *et al.*, 1980). Purified anti-NtCaM1 and anti-NtCaM3 antibodies recognized strongly their own antigens but not NtCaM13. However, they weakly cross-reacted with NtCaM3 and NtCaM1 proteins, respectively. In contrast, the unpurified crude antiserum against NtCaM1 reacted strongly to NtCaM1, and cross-reacted moderately with NtCaM3 and slightly with NtCaM13 (Figure 5B, bottom panel), indicating affinity purification increased the specificity to NtCaM1. However, the titer was remarkably reduced. The purified anti-NtCaM3 antibody might recognize NtCaM9/10 protein as well as NtCaM3/4/5/6/7/8/11/12 protein because of their structural similarity. Anti-NtCaM13 antibody was successfully purified by affinity chromatography, and recognized specifically the NtCaM13 protein but not NtCaM1 nor NtCaM3 proteins. Thus, I could obtain almost specific antibodies for three individual types of tobacco CaM proteins.

Various expression profiles of tobacco CaM genes and predominant accumulation of NtCaM13 protein during synchronous HR in TMV-infected leaves

Using affinity-purified antibodies and DNA probes for each *NtCaM* gene, the change in the NtCaM mRNA and protein levels during synchronous HR was studied. When Samsun NN tobacco plants are infected with TMV, it can multiply without any visible HR lesion formed under the high temperature, 30°C. When the temperature is shifted down to 20°C, which is a permissive temperature for the *N* gene, synchronous hypersensitive cell death is triggered in the TMV-infected area and visible lesions appear approximately 8 h after the shift (Figure 6A). After the temperature shift, a considerable increase in the transcript level for *NtCaM1, 2,* and

13 were found at 3 h, preceding the appearance of lesions and the expression of the *PR-1a*

defense gene (Figure 6B, right panel). In mock-inoculated leaves (left panel), no remarkable

increase was found in the expression of any *NtCaM* genes after the shift. *NtCaM7* to *12*

transcripts accumulated gradually to some extent following the shift in TMV-infected leaves.

On the other hand, levels of *NtCaM3* and *4* transcripts did not change by the temperature shift

(Figure 6B).

After the temperature shift, NtCaM1-type protein decreased after a transient increment

(Figure 6C, arrow head) as shown in Figure 6D (closed circle) which outlined the change of

individual CaM level by quantification of signal intensity. The level of NtCaM3-type

decreased more gradually. However, NtCaM13-type was maintained at a certain level, while it

decreased to the half level in mock-inoculated leaves. The antibody for NtCaM3 protein

recognized two or three bands as indicated by arrow heads, all of which accumulated in the

transgenic plants overexpressing *NtCaM3* gene (data not shown), suggesting the presence of

several NtCaM3-like isoforms or more likely, modified forms of NtCaM3 protein. Actually,

modification such as carboxyl-methylation of a prenylated CaM has been reported recently

(Rodríguez-Concepción, 2000).

Wound-induced expression of NtCaM1, 2, 3, 4, *and* 13 *genes and accumulation of NtCaM1*

protein

Detached mature leaves were wounded by cutting them into pieces with a razor blade (0

time of wounding), and mRNA and CaM proteins were isolated from the incubated samples.

Wounding induced a rapid accumulation of transcripts for *NtCaM1* to *4* and *wipk* which is a

wound-inducible mitogen-activated protein (MAP) kinase gene (Seo *et al.*, 1995; Seo *et al.*, 1999), within 10 min (Figure 7A). *NtCaM13* showed an unique profile among these *NtCaM* genes, exhibiting a sharp transient increase at 1 h. Transcripts for *NtCaM5* to *12* were kept at certain levels. At the protein level, only NtCaM1-type protein increased to two-fold at 6 h after wounding (Figures 7B, 7C). The level of NtCaM3-type proteins remained unchanged, and NtCaM13-type protein rapidly decreased after wounding.

Response of NtCaM *genes to treatment with SA and MeJA*

Results in Figures 6 and 7 showed differential responses of *NtCaM* transcripts and proteins to HR or wounding. Then I examined the effects of two defense-related signal compounds, salicylic acid (SA) and methyl jasmonic acid (MeJA). When leaf pieces were incubated with SA for 48 h, accumulation of transcript was induced remarkably in *NtCaM13* and slightly in *NtCaM1*, *2*, and *5* to *12* (Figure 8A). The transcripts of *NtCaM3* and *4* were not affected by SA treatment. On the other hand, application of MeJA caused no obvious induction on any *NtCaM* genes, and *NtCaM1* and *2* rather decreased slightly. Accumulation of the transcripts for acidic-type *PR-1a*, a marker gene for HR, and basic-type *PI-II*, a marker gene for HR and wounding, obviously responded to SA and MeJA, respectively, as described by Niki *et al.* (1998). At the protein level, isoforms of PR-1 were induced by SA or MeJA (Figure 8B, closed or open arrow heads, respectively). However, similar amounts of NtCaM1-type and NtCaM3-type proteins were found 48 h after both SA and MeJA treatment. For NtCaM13-type protein, the level was obviously decreased at 48 h incubation after wounding in the presence and absence of SA or MeJA. SA application enhanced the wound-induced

decrease in the amount of protein.

Enhanced accumulation of NtCaM proteins by the treatment with a proteasome inhibitor

The results in Figures 6 and 7 revealed dynamic changes of mRNA and protein levels of individual NtCaM isoforms during HR or after wounding. However, the change in the protein levels did not simply reflect the transcript levels of corresponding *NtCaM* genes (Figure 9). Particularly, the amount of NtCaM13-type protein decreased rapidly after wounding despite enhanced transcription (Figure 7). The discrepancy suggests a complex mechanism underlying the regulation of CaM protein levels not only at the transcriptional but also at the post-transcriptional level. Thus, I examined the effect on protein degradation by proteasome, whose involvement in wound response had already been reported (Ito *et al.*, 1999). Lactacystin, an irreversible-type proteasome inhibitor, was fed through the petiole of detached leaves (100 nmol per g fresh leaf) within 30 min and the leaves were cut into pieces (0 time), and incubated for the indicated time (Figure 10A). Pretreatment with lactacystin inhibited 26S proteasome activity remarkably for at least the first 9 h (Figure 10A, bottom). Meanwhile, wound-stimulated accumulation of *PI-II* and *wipk* transcripts was repressed. In contrast, none of the *NtCaM* transcripts were affected by lactacystin. *NtCaM1, 2, 3, 4* and *13* transcripts accumulated similarly as control. For protein gel blot analysis, each tobacco leaf disc (21 mm in diameter) was incubated with 0.5 ml of 120 μM lactacystin in a hole (23 mm in diameter) in a microtiter plate (Figure 10B). All three types of NtCaM proteins increased in different kinetics (Figure 10C). Accumulation of NtCaM1-type protein was transiently increased by wounding, and the increment was enhanced by the application of lactacystin. NtCaM3-type

27

proteins transiently increased from 1 to 3 h after wounding, but the increase was inhibited by

lactacystin treatment. Then 7 to 12 h after wounding, the NtCaM3-type proteins decreased,

but oppositely lactacystin treatment induced the increased accumulation of the protein. The

NtCaM13 protein level decreased at first and recovered later after wounding, but lactacystin

treatment inhibited the decrease and the protein level gradually increased. These CaM

induction profiles by wounding are similar to those in Figure 7, but not the same probably

because of different methods of wounding and incubation. Thus, application of lactacystin

enhanced the accumulation of NtCaM1-type and 13-type proteins induced by wounding.

Furthermore, wound-induced accumulation of NtCaM3 was quite differently affected by the

proteasome inhibitor. These findings suggest the involvement of protein degradation by

proteasome in the regulation of these NtCaM protein levels.

NADK was activated by NtCaM1 and NtCaM3 but not by NtCaM13

In order to estimate the downstream signalings of the respective CaM isoforms, I

investigated their specificities in activation of known CaM-targeted enzymes. Since plant

NADK is known to be activated by CaMs (Anderson and Cormier, 1978; Roberts and

Harmon, 1992), I purified NADK from pea seedlings and elucidated which type of CaMs is

the most potent activator. NADK activity was evaluated by quantification of the product,

$NADP^+$, via a reaction with an excess of $NADP^+$-dependent glucose 6-phosphate

dehydrogenase and oxidation-reduction indicator dyes (Figure 11A). As shown in Figure 11C,

pea NADK was highly activated by NtCaM1 and NtCaM3 in the presence of Ca^{2+}, but

NtCaM13 did not activate the enzyme. In the absence of Ca^{2+}, no activity was detected (data

not shown). The V_{max}, the maximal activity compared to that of bovine brain CaM, and the K_{act}, the concentration of CaM required for half-maximal activity, were 207, 235, 9% and 16, 9, 26 nM for NtCaM1, NtCaM3, and NtCaM13, respectively (Table 3).

NOS was activated by NtCaM13 most effectively

NO has been suggested to be involved in the expression of defense-related genes in TMV-infected tobacco (Durner *et al.*, 1998). NOS-like activity was detected in a Ca^{2+}-dependent manner (Delledonne *et al.*, 1998). Since neither plant-derived NOS genes nor enzymes have ever been isolated, recombinant rat NOS was subjected to a CaM activation assay as a reference. NOS activity was determined by the citrulline method coupled with TLC separation (Figures 12A, 12B). Under my experimental conditions, NOS was activated by all of the tobacco CaM isoforms isolated (Figure 12C). However, NtCaM13 was the most potent activator, while NtCaM1 and NtCaM3 were moderate and weak activators, respectively. Without Ca^{2+}, no activity of NOS was found (data not shown). The V_{max} compared to that of bovine CaM and the K_{act} for NtCaM1, NtCaM3, and NtCaM13 were 98, 67, 119% and 56, 89, 67 nM, respectively (Table 3).

CaN was activated strongly by NtCaM3 and moderately by NtCaM1 and NtCaM13

CaN, a CaM-dependent protein phosphatase, is also a well-known target of CaM. The activity was easily determined by a fluorometric method with 4MUP as the substrate (Figure

29

13A). In the absence of Ca^{2+}, bovine brain CaN exhibited 35 to 45% of the activity with Ca^{2+},

whether any CaM was added or not (data not shown). In the presence of Ca^{2+}, NtCaM3

increased the activity of bovine brain CaN several-fold, and NtCaM1 and NtCaM13 had a

weak effect (Figure 13B). The V_{max} compared to that of bovine CaM and the K_{act} for NtCaM1,

NtCaM3, and NtCaM13 were 81, 130, 74% and 4.6, 4.0, 7.5 nM, respectively (Table 3).

Expression of PR and wipk *genes was affected in CaM transgenic plants*

I generated transgenic tobacco plants in which individual type CaMs were overexpressed

or suppressed, and analyzed the effect on expression of defense-related genes with at least 20

independent lines for each construct (Figure 15). At first, the transgenic plants overexpressing

three types of CaM were subjected to RNA and protein gel blot analyses using the same 3'

UTR specific probes and affinity-purified, individual type CaM-specific antibodies as

described above. Remarkably, often only HR-dominant type *NtCaM13*-overexpressors

showed the constitutive expression of both acidic (*PR-1a*) and basic (*PI-II*) types of PR genes

(lines 21, 32, 60, and 63 in Figure 16). A wound-inducible peroxidase gene, *tpoxN1*, whose

transcript was scarcely found in healthy control plants carrying the *luc* gene, was expressed at

considerable levels without wounding in all sense *NtCaM13* plants tested. Little correlation

between the transcript level of *PI-II* and *tpoxN1* genes shown in the sense *NtCaM13* lines

indicates that the expression of these genes would be conferred by NtCaM13 via different

signaling pathways. This phenomenon is consistent with our previous observation that the

expression of *tpoxN1* was not induced by JA and ethylene which activate many wound-

inducible genes such as *PI-II* (Hiraga *et al.*, 2000a). Intriguingly, among sense *NtCaM13*

plants, lines 10, 21, 30, 60 and 63 also accumulated NtCaM1-type protein at considerable levels. However, no transgenic plants accumulated the transcript of *tpoxC1*, another HR-inducible peroxidase gene, suggesting that *tpoxC1* gene expression is controlled by signal mediators other than NtCaM13. Heo *et al.* (1999) reported the spontaneous formation of HR-like lesions on the leaves of transgenic plants overproducing SCaM-4, an ortholog of NtCaM13. However, I found no such lesions in my NtCaM13-overproducing tobacco plants, although I did find a similar constitutive expression of PR genes. In antisense *NtCaM13* plants, the two most suppressed lines expressed less *PR-1a* during HR than the control *luc* plants. In other lines, however, the repression of *PR-1a* expression was not clear (data not shown).

Another observation in transgenic plants was enhanced accumulation of the *wipk* transcript in sense *NtCaM1* lines 9, 40, 52 and 54 without wounding (Figure 17). In contrast, the antisense *NtCaM1* lines 49 and 68 did not accumulate the *NtCaM1* and *wipk* transcripts in response to TMV-induced HR (lane T) compared to healthy leaves (lane H), while an HR-induced increase in the level of *wipk* transcript was obvious in the *luc* control lines. Thus, in most sense *NtCaM1* lines, transcript levels of both *NtCaM1* and *wipk* were correlated, and in well-suppressed antisense *NtCaM1* plants, neither transcript was induced by HR. These findings suggest that NtCaM1 is likely involved in the regulation of *wipk* gene expression.