Discussion

In plants, several divergent types of CaMs have been cloned and shown to have distinct induction profiles in response to various stimuli including wounding and pathogen attack (Takezawa et al., 1995; Heo et al., 1999) in clear contrast to the mammal system in which only one form of CaM works. The fact would indicate that individual CaM isoforms have divergent physiological roles in plants to assure flexible responses. Thus plants which lack a complicated immune system might have developed well-tuned systems for the regulation of downstream responses by the specialized use of individual CaMs. In the present study, I isolated three divergent types of CaMs from tobacco plants and revealed dynamic regulation of their amounts during pathogen-induced HR and after wounding (Figure 9).

Involvement of NtCaM13-type CaMs in HR signaling

The 3' UTR specific probes and affinity-purified antibodies allowed me to analyze quantitatively the individual transcripts and isoform proteins. Interestingly, CaM protein levels differentially changed during HR. Among three groups of CaMs, the NtCaM1-type and 3-type proteins decreased after a transient accumulation, whereas the NtCaM13-type maintained a certain level (Figure 6). The ratio of each type of protein to total CaM protein amount (Table 4) clearly shows such a contrasting change; the NtCaM1-type decreased from 42% to 19% and the NtCaM13-type increased from less than 13% to 39% within 48 h after the HR-inducing treatment. Meanwhile, NtCaM3-type proteins were kept at a certain ratio. At the transcript level, a clear increase was found in NtCaMI, 2 and 13 during HR, suggesting
supplement of the CaMs by increased transcription. Salicylic acid (SA), a major defense-related signal compound of HR, induced accumulation of the transcript for \textit{NtCaM}13, and slightly 1 and 2, whereas the amount of NtCaM13-type protein decreased by application of SA (Figure 8). At present, I do not know the mechanism underlying this discrepancy of response to SA. However the high ratio of protein amount suggests that NtCaM13-type (group III) protein would have important roles in HR. This is consistent with the results from soybean plant, in which SCaM-4 and SCaM-5 proteins, also belonging to cluster III (Figure 3), reportedly accumulate after pathogen infection (Heo \textit{et al.}, 1999).

\textit{Involvement of NtCaM1-type CaMs in wound signaling}

Upon wounding, the NtCaM1-type CaMs increased rapidly at both the mRNA and protein level in contrast to the decrease in the NtCaM13-type protein level (Figure 7). A marked wound-induced accumulation of NtCaM1-type proteins was obvious in the ratio to total CaM amount; an increase from 21\% at time 0 to 40\% at 6 h after wounding (Table 4). A decrease in the ratio of NtCaM13-type protein was also remarkable; 10\% at time 0 and 0.9\% at 24 h. Among the \textit{NtCaM} genes in group II, transcripts for \textit{NtCaM}3 and 4 accumulated considerably after wounding. However, at the protein level, the NtCaM3-type, which accounted for more than 50\% of the total CaMs, remained at almost the same level after wounding (Figure 7 and Table 4). Thus, in response to wounding, NtCaM1-type proteins (group I) were induced to the largest extent, suggesting that group I-type CaMs might be important in wound response.
Involvement of regulated degradation of NiCaM proteins by proteasome in wound response

The wound-induced responses for all three groups of CaM proteins were obviously affected by the treatment with lactacystin (Figure 10), which binds to a proteasome regulatory subunit irreversibly and inhibits its activity (Fenteany et al., 1995), suggesting degradation of CaM isoforms by the protease activity of 26S proteasome. Ito et al. (1999) showed that lactacystin treatment suppressed the expression of wound-inducible genes such as PI-II and wipk genes. Proteasome activity also has been reported to be indispensable for defense response in cucumber hypocotyls (Becker et al., 2000). The activity of a vertebrate CaM has been reported to be regulated by ubiquitination of Lys21 (Laub et al., 1998). As the 21st residue of the NtCaM1 and 3 proteins is Lys, these proteins might be orientated to the 26S proteasome complex after ubiquitination of Lys21. However, that of NtCaM13 is Arg, suggesting that this isoform is destroyed in a different manner. A key enzyme for polyamine biosynthesis in mammals, ornithine decarboxylase is known to have an extremely short life, and has been shown to be degraded by proteasome without ubiquitination (Murakami et al., 1992). Also in vitro-damaged CaM that had undergone a long period of incubation was reportedly destroyed by proteasome without ubiquitination (Tarsa et al., 2000). NtCaM13-type protein might be destroyed by unknown machinery without ubiquitination or via ubiquitination of Lys residues other than Lys21. To my knowledge, my data provide the first evidence to suggest involvement of the proteasome system in the regulation of CaM protein amounts in vivo. However, at this point, I can not exclude the alternative possibility that these CaMs are down-regulated due to repressed translation of their cognate mRNAs. The association between the mRNAs and ribosomes might be inhibited as proposed previously.
(Gallie, 1996; Gallie, 1998). Otherwise, the elongation of their peptide strands might be repressed, as reported for a pathogenesis-induced ascorbate peroxidase (Mittler et al., 1998).

**Possible target proteins of NtCaMs**

I am interested in the functions of individual types of CaMs, including the newly-isolated type, NtCaM1. I studied the possible downstream response of tobacco pathogen- and/or wound-induced CaMs by two approaches; in vitro target enzyme assay and in vivo analyses using transgenic plants. The first approach is to identify the target enzymes of CaMs by in vitro enzyme assays. I successfully detected the activation of three major CaM-dependent enzymes; NADK, NOS and CaN, by using purified proteins of three typical plant CaMs. The results clearly showed the divergent character of each type of CaM, as summarized in Figure 14. Pea NADK was activated by wound-induced NtCaM1 and a constitutive type, NtCaM3 (Figure 11). The production of ROS is believed to be evoked by the putative NADPH oxidase activity, which requires NADPH as substrate (Harding et al., 1997; Harding and Roberts, 1998). Then, these CaMs might enhance the ROS production by promoting NADP⁺/NADPH production. In tobacco suspension-cultured cells treated with cryptogein, a phytopathogenic fungus-derived elicitor, the pentose phosphate pathway is known to be activated and enhance NADP⁺/NADPH conversion (Pugin et al., 1997). Recently, the production of O₂⁻ by tobacco homologs of NADPH oxidase has been reported to be stimulated by TMV infection and Ca²⁺ (Sagi and Fluhr, 2001). In clear contrast, mammalian NOS was markedly induced by HR-dominant NtCaM13 (Figure 12). Although neither cDNA nor protein of NOS has been isolated from plants, Ca²⁺-dependent NOS-like activity was shown to be necessary for the
expression of defense-related genes following TMV infection in tobacco plants (Durner et al., 1998). Therefore, NtCaM13 might be involved in the production of NO and promote the expression of PR genes, especially SA-activated acidic-type PR genes. Cho and his colleagues reported activation of these enzymes by soybean SCAm isoforms in vitro, and elucidated that Lys30 and Glu40 are required for activation of NADK (Lee et al., 1997) and Met144 is important for that of NOS (Cho et al., 1998; Kondo et al., 1999). Based on amino-acid sequence analogy, I could predict which NtCaM isoforms activate NADK or NOS effectively. My experimental data was almost consistent with the estimations; NtCaM3 and 13 activated NADK and NOS most effectively, respectively, while NtCaM1 could activate both enzymes moderately.

Mammalian CaN, a CaM-dependent protein phosphatase, was activated by NtCaM3 most effectively (Figure 13). Several reports have indicated that CaN might be involved in salt tolerance in plants. Ectopic simultaneous expression of yeast catalytic A and regulatory B subunits of CaN enhanced salt tolerance in transgenic tobacco (Pardo et al., 1998). Several genes encoding the CaN B subunit have been isolated from Arabidopsis, among which SOS3 is the causative gene for the mutant impaired in salt tolerance (Liu and Zhu, 1998). However, this subunit reportedly interacted with and activated a protein kinase but not protein phosphatases (Halfter et al., 2000). The physiological meaning of the potential of each NtCaM isoform in the activation of CaN remains to be elucidated.

As a second approach to study the downstream function of individual CaM isoforms, I generated transgenic plants with altered amounts of each type of CaM. Using a series of transgenic tobacco plants carrying three types of CaM genes in the sense and antisense orientation, a total of 120 lines, I found two notable tendencies in the expression of defense-related genes. At first, the overexressor of NtCaM13 frequently showed constitutive
expression of defense-related genes, such as an acidic-type PR gene (PRIa), a basic-type PR gene (PI-II), and a wound-inducible peroxidase gene (tpoxN1), but not an HR-inducible peroxidase gene (tpoxCl) (Figure 16). In these plants, NtCaM1-type CaM protein also accumulated at high levels. Therefore I can not simply conclude that NtCaM13 directly affects the expression of these genes. However, since neither sense NtCaM1 nor NtCaM3 plants showed high level of expression for these genes (data not shown), this phenomenon might be the unique consequence of an accumulation of NtCaM13-type CaM. A similar result has been reported by Heo et al. (1999); tobacco plants overexpressing soybean SCaM-4, which is the ortholog of NtCaM13, showed the constitutive expression of PR genes.

Secondly, I found that the expression of a wound- and HR-inducible MAP kinase gene, wipk, was altered by overexpression or suppression of NtCaM1. WIPK, a tobacco wound-induced protein kinase, has been reported to be involved in the production of JA, and both its transcription and activity are rapidly induced after wounding and during HR (Seo et al., 1995; Seo et al., 1999; Seo et al., 2001). The wipk gene was found to be constitutively expressed in tobacco plants overexpressing NtCaM1 (Figure 17). The expression of wipk induced during TMV-triggered HR was repressed in NtCaM1 antisense plants, suggesting that NtCaM1-type CaM controls the expression. In tobacco cells, the activation of MAP kinases in response to cryptogein has been reported to require an influx of Ca\(^{2+}\) (Lebrun-Garcia et al., 1998). At present, I do not know whether CaMs activate MAP kinases directly, indirectly, or not at all. Studies on the effect of the overexpression or suppression of NtCaM1 on the activation of WIPK are in progress.

The NtCaM1 and NtCaM13 proteins have been shown to accumulate dominantly 6 h after wounding or more than 24 h after the temperature shift for HR induction (Table 4). From the results in Figure 14 on the target enzyme specificity and Figures 16 and 17 on the phenotypes
of the CaM transgenic plants, I could predict the possible downstream signaling pathways of each type of CaM, as shown in Figure 18. Wound stress results in an accumulation of NtCaM1- and NtCaM3-types, which might activate NADK leading to an increase in ROS production via enhancement of NADP⁺/NADPH production and the NADPH oxidase reaction. NtCaM1 might enhance the accumulation of a wound signal compound, JA, by activating transcription of the wipk gene. In the case of TMV-induced HR, the dominant types are NtCaM3 and NtCaM13. NtCaM13 could increase the putative NOS-like activity, resulting in expression of the acidic-type PR genes via extensive production of NO. Actually, the overproduction of NtCaM13 induced the accumulation of a basic-type PR transcript as well as the acidic type, as shown in the experiment with transgenic plants, although the underlying mechanism for the induction remains to be determined. During HR, NtCaM3-type CaMs, which accumulate constitutively at a considerable level, might enhance ROS production in the same manner as in the wound response. The other targets of NtCaM isoforms might be protein kinases or phosphatases. In tobacco plants, expression of acidic-type PR-1 and PR-2 (β-1,3-glucanase) genes has been reported to be mediated by protein dephosphorylation (Conrath et al., 1997). Conversely, expression of the basic-type PR-2 and PR-3 (chitinase) genes, and the ERF2 gene which encodes a possible transcription factor for these basic genes, has been suggested to require protein phosphorylation in tobacco suspension-cultured cells (Suzuki et al., 1995; Yamamoto et al., 1999). CaMs are known to activate many protein kinases and phosphatases. Thus, unknown CaM-dependent protein kinases and/or phosphatases regulating these PR gene expression might be activated by wound- or HR-induced CaMs.
Potentiation of defense responses by the dynamic change in CaM balance

NO has been reported to be generated at an early stage, such as 4 to 10 h after the temperature shift for TMV-induced HR in tobacco (Durner et al., 1998). In cryptogein-treated tobacco cells, rapid production of ROS and NO has been also observed (Foissner et al., 2000; Tavernier et al., 1995). The dynamic change in the CaM content of wounded cells or cells undergoing HR, which occurs gradually (Table 4), might take place to protect against secondary wounding, insect chewing, or microbe attack. The altered CaM balance could facilitate more rapid defense responses such as ROS/NO production and PR gene expression. In pathogen-infected tobacco plants, enhanced expression of defense-related genes such as \textit{PAL} and \textit{PR10} has been reported after secondary wounding or infection (Mur et al., 1996). In tomato plants, pre-irradiation with ultraviolet light UV-A/UV-B stimulated a more rapid accumulation of basic PR-6 (proteinase inhibitor) proteins in response to subsequent wounding (Stratmann et al., 2000). Pre-treatment with SA potentiates the faster elicitor-induced defense responses such as H$_2$O$_2$ production, \textit{PAL} gene expression, and phytoalexin accumulation (Kauss et al., 1992; Kauss and Jeblick, 1995). The CaM response reported here might be a so-called 'potentiation' phenomenon.

At present, the real \textit{in vivo} targets of CaMs in tobacco plants are not known. Further studies such as the screening of CaM-interacting proteins will help our understanding of the importance of individual CaM isoforms and the reason why plants have evolved a divergent CaM family in a well-tuned regulation.