Introduction

Plants are immobile and facing various stresses including pathogen infection and wounding in the nature. So they are believed to have evolved well-tuned adopting systems for surviving in the severe environment. Tobacco plants are often infected by tobacco mosaic virus (TMV) in the field, resulting in mosaic and dwarf symptoms. However, tobacco plants carrying the *N* gene, a resistance gene to TMV, restrict the spread of the invading virus by killing the infected tissue themselves. Such a hypersensitive cell death of tissue results in the formation of necrotic lesions at the site of infection, which is a hallmark of a defense response of plants known as the hypersensitive reaction (HR) (Goodman and Novacky, 1994). Simultaneously, tobacco plants undergoing HR accumulate antimicrobial compounds such as pathogenesis-related (PR) proteins locally and systemically to defend against further infection (Van Loon, 1999; Ohashi and Ohshima, 1992). In tobacco plants, members of PR protein families 1 to 5 (PR-1 to -5) are classified as either acidic or basic. Both types are induced by a TMV-triggered HR, while wounding induces only the expression of the basic type. PR-2 and -3 proteins were shown to have β-1,3-glucanase and chitinase activity, respectively, and PR-6 proteins act as proteinase inhibitors, which are of the basic type. They can serve for degrading the cell wall of invading pathogenic fungi or abolishing digestion by chewing insects, respectively. The accumulation of acidic and basic PR proteins is induced by defense-related signal compounds, salicylic acid (SA) and jasmonic acid (JA), respectively (Niki *et al.*, 1998). Furthermore reactive oxygen species (ROS) such as O_2^- and H_2O_2 accumulate immediately after infection (Doke and Ohashi, 1988; Fodor *et al.*, 2001; Lamb and Dixon, 1997) and are important for defense responses such as the reinforcement of degraded plant cell wall by cross-linking of structural proteins (Bradley *et al.*, 1992; Brisson *et al.*, 1994) and

lignification (Hammerschmidt and Kuc, 1982; Köhle *et al.*, 1984) with the help of peroxidases. Recently nitrogen monoxide (NO), which is also a reactive molecule, has been reported to accumulate during HR and induce the expression of sets of PR proteins via the production of SA in tobacco plants (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Klessig *et al.*, 2000).

TMV-infected tobacco plants carrying the *N* resistance gene are a good material to study the mechanisms of HR. In the system, a nearly synchronous HR can be induced by controlling incubation temperature. TMV-inoculated leaves can not undergo HR at temperatures above 28°C allowing a non-restricted multiplication of virus. However, shifted down to low temperature such as 20°C, a permissive temperature for the *N* gene, the infected tissues immediately exhibit hypersensitive cell death resulting in visible lesions 8 h after the shift in temperature. The synchronized cell death has been confirmed by the rapid leakage of ion from the dying leaf tissue (Ohashi and Shimomura, 1976). With this system, Ohashi *et al.* have isolated novel HR-responsive genes whose transcript level increased or decreased rapidly (within 3 h) after the temperature shift, that is, 5 h before the lesions appeared. Among these genes, those encoding calmodulin (CaM), ubiquitin (Ito *et al.*, 1999) and mitogen-activated protein (MAP) kinase were isolated. The MAP kinase gene expressed immediately after wounding, namely *wipk*, has been also induced by TMV-triggered HR and suggested to control the production of JA (Seo *et al.*, 1995; Seo *et al.*, 1999; Seo *et al.*, 2001). I am interested in the CaM gene, which was expressed in the early period of HR, for the following reasons.

In plant defense systems, Ca^{2+} signaling would be indispensable as well as in the animal defense systems. For instance, external stimuli such as fungal elicitor treatment, and oxidative, osmotic and heat-shock stresses are known to trigger a rapid and transient elevation of

cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in plant cells (Sanders *et al.*, 1999). At the site of fungal infection, transient $[Ca^{2+}]_i$ increase has been observed in the response of hypersensitive cell death in cowpea plants (Xu and Heath, 1998). Plant suspension-cultured cells respond to fungal infection or elicitors from phytopathogen-infected tissues, exerting defense responses such as production of H_2O_2, induction of PR proteins (Nürnberger *et al.*, 1994; Hahlbrock *et al.*, 1995; Piedras *et al.*, 1998; Park *et al.*, 1998; Grant *et al.*, 2000), and production of NO (Delledonne *et al.*, 1998) in Ca^{2+}-dependent manner. CaM is a major Ca^{2+} receptor which functions in numerous developmental processes and stress responses by activating an increasing number of target enzymes in the presence of Ca^{2+} (Klee and Vanaman, 1982). The involvement of CaMs in plant stress responses has been reported. A transgenic tobacco plant harboring a mutated CaM which hyperactivates NAD^+ kinase (NADK) showed enhanced H_2O_2 production in response to elicitor treatment or incompatible pathogen infection (Harding *et al.*, 1997; Harding and Roberts, 1998). Since H_2O_2 is believed to be produced by putative NADPH oxidase activity, this CaM might promote the production of its substrate, $NADP^+$/NADPH, by activating NADK to convert NAD^+ to $NADP^+$. On the other hand, NO is produced in mammalian cells by NO synthase (NOS) which is also a CaM-dependent enzyme (Marletta, 1993). Furthermore, pharmacological experiments revealed that expression of the PR genes might be regulated by protein kinases and/or phosphorylases (Conrath *et al.*, 1997; Yamamoto *et al.*, 1999). In mammal cells, several protein kinases and phosphorylases are known to be regulated by CaM.

As well as the $[Ca^{2+}]_i$ level, expression of many plant CaM genes has been reported to be induced by physical stresses, and CaM and CaM-related genes have been isolated as major touch-stimulated genes from *Arabidopsis* plants (Braam and Davis, 1990). Potato plants have eight CaM genes encoding at least two distinct CaM isoforms, among which the *PCM1* gene

showed an increase in expression upon touching and *PCM6* during tuberization (Takezawa *et al.*, 1995). Among the five divergent soybean CaM genes isolated to date, *SCaM-4* and *SCaM-5* were transcriptionally activated by phytopathogenic bacterial infection or fungal elicitor application, suggesting their involvement in defense against pathogen attack (Heo *et al.*, 1999). These data suggest that each type of CaM has distinct physiological roles in plants.

However, the role of Ca^{2+}-CaM signaling in defense responses after pathogen infection or wounding is little understood. In this study, I have isolated and characterized a family of CaM genes from tobacco plants, and I describe the involvement of the proteasome protein degradation system in the regulation of CaM isoform levels. Furthermore, I aimed to explore the downstream roles of each CaM isoform in defense response against pathogen attack and wounding by two approaches; *in vitro* enzyme activation assay and analyses of transgenic plants whose CaM levels had been altered. Finally I discuss the possible physiological roles of each CaM.