Materials and Methods

Plant materials, synchronous HR induction by TMV, wounding and chemical treatments

Tobacco plants containing the *N* resistance gene (*Nicotiana tabacum* L. cv Samsun NN) were grown in a greenhouse at 20°C to 30°C. Well-expanded upper leaves of 6- to 8-week-old plants, unless otherwise described, were detached and used for the experiments.

Detached leaves were inoculated with or without (mock) 25 μg/ml of purified tobacco mosaic virus (TMV) suspension using carborundum #600. Inoculated leaves were incubated in a moisture box for 40 h at 30°C (a nonpermissive temperature for the *N* gene), allowing virus multiplication without lesion formation. Then these leaves were transferred to 20°C (a permissive temperature for the *N* gene), inducing synchronous lesion formation approximately 8 h after the shift (Figure 6A). For wounding treatment, tobacco leaves were cut into approximately 3-cm squares with a razor blade and incubated at 22°C. For chemical application, tobacco leaf pieces were floated in a solution containing 500 μM salicylic acid (SA) or 50 μM methyl jasmonate (MeJA) plus 0.1% (v/v) dimethyl sulfoxide while control leaves were floated in distilled water or 0.1% dimethyl sulfoxide solution, respectively, and incubated at 22°C for 48 h. For treatment with a proteasome inhibitor, tobacco leaf discs (21 mm in diameter) were incubated with 0.5 ml of 120 μM lactacystin solution at 22°C and used for protein gel blot analysis. For RNA gel blot analysis, lactacystin (100 nmol per g fresh leaf weight) was supplied through the petiole of whole leaves 30 min before the leaves were cut to small pieces as described previously (Ito *et al.*, 1999). All samples were incubated under continuous illumination at an intensity of 120 μmol of photons m^{-2} sec^{-1}. After an appropriate period of incubation, the leaves were frozen in liquid nitrogen and stored at -80°C until use.

Complementary DNA library screening

RNA was isolated from TMV-infected leaves at 3 h and 12 h after temperature shift to 20°C, and from the leaves 30 min after wounding and the stems 15 min after cutting, respectively, by guanidine thiocyanate/cesium chloride ultracentrifugation (Kingston *et al.*, 1996). Poly(A)$^+$ RNA was isolated with Oligotex-dT30, Super (Roche Diagnostics, Tokyo, Japan). Complementary DNA libraries were prepared from each poly(A)$^+$ RNA using a cDNA synthesis kit (Stratagene, La Jolla, CA). The cDNA was inserted into the Uni-ZAP XR vector. Approximately 6×10^4 unamplified recombinant phages from each library were screened using labeled probes with [α-^{32}P]dCTP (ICN Biomedicals, Costa Mesa, CA) by the random priming method with a labeling kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The used probes were a tobacco CaM cDNA which had been isolated as a HR-induced clone (Seo *et al.*, 1995), and soybean 1.4-kbp *SCaM-1* and 1.4-kbp *SCaM-4* genomic fragments (Lee *et al.*, 1995) which were obtained by PCR. Hybridization was performed at 45°C for 16 h in 5×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS containing 0.1% Ficoll 400 (Amersham Pharmacia Biotech), 0.1% BSA, 0.1% polyvinylpyrrolydone, and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice for 15 min each in 2×SSC, 0.1% SDS at 25°C and three times for 20 min each in 2×SSC, 0.1% SDS at 55°C. The filters were exposed to XAR film (Kodak, Rochester, NY) with an intensifying screen at -80°C for 20 h. Individual positive plaques were purified by several rounds of plating and hybridization (Sambrook *et al.*, 1989), and the cDNAs were excised with helper phage and recircularized to generate subclones in the pBluescript SK plasmid according to the manufacturer's instructions

(Stratagene). In total, 187 clones were selected and subjected to sequencing.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

Poly(A)$^+$ RNA was prepared from TMV-infected leaves 3 h after the temperature shift as described above. Complimentary DNA for PCR was synthesized with a cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) following the manufacturer's instructions. Degenerated primers were synthesized for SCaM-4-specific amino acid sequences as the sense primer; 5'-AYGGIGATGGNTGYATYACNGTNGA-3', and the antisense primer; 5'-CCAARTCNGCTTCYTYRATCATYTGY-3', where I is inosine, N is A, C, G or T, R is A or G, and Y is C or T, corresponding to the 22nd to 30th (DGDGCITVE) and 122nd to 131st (EQMIKEADLD) amino acids of the SCaM-4 sequence, respectively. Amplification was carried out with a profile of 9-min denaturation at 96°C, followed by 40 cycles of 96°C for 40 sec, 56°C for 1 min, and 72°C for 2 min using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The resulting 327-bp PCR products were fractionated on a 3.5% agarose gel, eluted, subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and subjected to sequencing.

The full-length cDNA for the tobacco SCaM-4 homolog was obtained by both 3' and 5' RACE, and subsequent end-to-end PCR using the same cDNA as the template according to the instructions of the cDNA amplification kit (Clontech Laboratories). The full-length sequence was verified by sequencing individual subclones generated by three independent PCRs.

Sequencing and sequence analyses

The *NtCaM* cDNAs subcloned into the pBluescript SK or pCR 2.1 vectors were sequenced on both strands by the dideoxy chain termination method using a DNA-sequencing kit and a model 373A DNA sequencer (Applied Biosystems). Nucleotide and deduced amino-acid sequences were analyzed with the Genetyx-Mac software system (Software Development Co., Tokyo, Japan). Amino-acid sequences of CaM from various organisms were obtained by GenBank searches, and a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

DNA gel blot analysis

Ten micrograms each of genomic DNA isolated from *N. tabacum, N. sylvestris*, and *N. tomentosiformis* by the cetyltrimethylammonium bromide method (Rogers and Bendich, 1985) was digested with *Eco*RI, *Hin*dIII, or *Xba*I, separated on a 1.0% agarose gel, then transferred to a Hybond-N+ nylon membrane filter (Amersham Pharmacia Biotech). Blots were prehybridized at 42°C for 6 h in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3 × SSC, 50% (v/v) formamide, 0.02% Ficoll 400, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. This was followed by overnight hybridization at 42°C in the same solution supplemented with ^{32}P-labeled probe and 5 mg/ml Blocking Reagent (Roche Diagnostics). After hybridization, blots were routinely

washed twice for 15 min each in 2×SSC, 0.1% SDS at 25°C and three times for 20 min each in 0.5×SSC, 0.1% SDS at 55°C, and imaged by a PhosphorImager SI (Amersham Pharmacia Biotech). The cDNA probes for *NtCaM* genes were prepared by PCR using synthetic primers to yield the 145- to 351-bp fragments corresponding to each 3' untranslated region (UTR).

RNA gel blot analysis

Two micrograms per slot of poly(A)$^+$ RNA was fractionated by formaldehyde-denaturing agarose gel electrophoresis and blotted onto a Hybond-N membrane (Amersham Pharmacia Biotech), which was then subjected to hybridization with the ^{32}P-labeled probe (Sambrook *et al.*, 1989). Hybridization and washing were performed as for the DNA gel blot analysis, and the membrane was imaged by a PhosphorImager SI. For detection of *NtCaM* gene transcripts, the same probes as those for DNA gel blot analysis were used. The regions used for probing PR genes, which are specific for each fragment, were; +507 to +737 bp for the acidic *PR-1a* gene (Matsuoka *et al.*, 1987), and +941 to +1328 for the basic *PI-II* gene (Balandin *et al.*, 1995). The *wipk* cDNA probe was a 1156-bp fragment, corresponding to positions +147 to +1302 (Seo *et al.*, 1995). The transcripts for *tpoxC1* (Hiraga *et al.*, 2000b) and *tpoxN1* (Hiraga *et al.*, 2000a) genes were detected with specific probes corresponding to positions +911 to +1356 and +811 to +1172 bp, respectively.

Production of recombinant CaM proteins in E. coli

To construct plasmids for recombinant NtCaM1, 3, and 13 proteins, individual coding

regions were amplified by PCR using synthetic primers with the translational start codon included in the *Nco*I site (CCATGG), and subcloned between the *Nco*I and *Bam*HI sites of pET15b vector (Novagen, Madison, WI), which allows production of CaM proteins without any tags. The resulting constructs, pET-NtCaM1, 3, and 13, the integrity of which was verified by nucleotide sequencing, were expressed in *Escherichia coli* BL21(DE3) by incubation in 1 mM isopropyl β-D-thiogalactopyranoside for 2.5 h at 37°C. The cells were collected by centrifugation at 5,000*g* for 5 min at 4°C and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and one tablet of protease inhibitor cocktail (Complete; Roche Diagnostics) per 50 ml. The recombinant proteins were purified by Ca^{2+}-dependent hydrophobic chromatography using Phenyl Sepharose CL-4B (Amersham Pharmacia Biotech) by the method of Fromm and Chua (1992). The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA) with BSA as the standard. For the control of the enzyme assays described below, bovine brain CaM was obtained from Wako (Osaka, Japan).

Production and affinity purification of anti-(tobacco CaM) antibodies

The polyclonal anti-(NtCaM1, 3, and 13) antibodies were raised by immunizing rabbits with the corresponding recombinant proteins. To remove cross-reactivities among these CaM isoforms, the antisera were purified by antigen-affinity column chromatography as described previously (Dedman *et al.*, 1978; Lee *et al.*, 1995). NtCaM-Sepharose columns were prepared by conjugating each recombinant NtCaM protein to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). Anti-NtCaM13 serum was

purified by passage through a Sepharose column harboring both NtCaM1 and NtCaM3 proteins as the ligand, subsequent loading onto a NtCaM13-Sepharose column, and collection of the bound fraction. Similarly, anti-NtCaM1 and anti-NtCaM3 sera were purified. Because their titers decreased remarkably, these antisera were purified by passage through a Sepharose column harboring only NtCaM13 protein and binding to the corresponding NtCaM-Sepharose column, resulting in minor cross-reactivities between NtCaM1 and NtCaM3 proteins remained (Figure 5B).

Protein extraction and protein gel blot analysis

All the extraction procedures were performed on ice or at 4°C. Leaf materials were macerated in three volumes of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 14 mM (v/v) 2-mercaptoethanol and one tablet of protease inhibitor cocktail (Complete) per 50 ml using a Polytron homogenizer (Kinematica, Luzern, Switzerland), and an aliquot of the supernatant after centrifugation at 12,000*g* for 15 min at 4°C was subjected to protein gel blot detection for PR proteins. For detection of CaM proteins, the supernatant was further purified by heating to 90°C for 10 min. After clarification of denatured protein by recentrifugation, the supernatant was concentrated by ethanol precipitation and subjected to protein gel blot analysis.

For detection of tobacco CaM isoforms, an aliquot of heat-stable protein was separated by Tricine-SDS gel electrophoresis (Schägger and von Jagow, 1987) on a 12% T, 5% C SDS-polyacrylamide gel, where T and C denote the percentage of both acrylamide and bisacrylamide, and the percentage of bisacrylamide relative to the total concentration,

respectively. For the detection of PR proteins, total soluble protein was fractionated by SDS-PAGE on 15% polyacrylamide gels (Laemmli, 1970). For Ca^{2+}-dependent electrophoretic mobility shift assay, 10 ng each of purified recombinant NtCaM1, 3, and 13 protein was separated on a 15% SDS-polyacrylamide gel in the presence of 7.5 mM of $CaCl_2$ or EGTA (pH 7.0) in the SDS sample buffer. The proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semidry electroblotting (Kyhse-Andersen, 1984).

After transfer, the polyvinylidene difluoride membranes were stained with Ponceau S (Sigma, St. Louis, MO) to verify transfer efficiency, according to Ausubel *et al.* (1987). The Ponceau S stain of the membranes revealed no differences in the intensity of the band corresponding to the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (the most abundant protein in leaf tissue) in each lane, demonstrating that an equal amount of protein had been loaded on to the gel. After the removal of Ponceau S, the membranes were used for immunological detection of CaM proteins, as described below.

The membranes were rinsed briefly with PBST [10 mM sodium phosphate buffer (pH 7.2) supplemented with 0.9% NaCl and 0.05% Tween 20], blocked for 1 h with 10% skim milk in PBST and for 30 min with 1% BSA in PBST, then incubated overnight at 4°C with respective primary antibodies. The dilution of the primary antibodies used was 1:10, 1:20, and 1:50 for the affinity-purified anti-NtCaM1, anti-NtCaM3, and anti-NtCaM13 antibodies, and 1:500 for the unpurified anti-NtCaM1 serum, respectively. Specific antibodies against tobacco PR-1 and PR-3 proteins (Niki *et al.*, 1998; Yamakawa *et al.*, 1998), respectively, were used at a dilution of 1:1000. After being washed with PBST extensively and treated with 1% BSA in PBST, the membranes were incubated with an alkaline phosphatase-conjugated anti-(rabbit IgG) antibody (ICN Pharmaceuticals, Costa Mesa, CA) as a secondary antibody at a dilution

of 1:1000 for 40 min at room temperature. Then, the membranes were washed as described above, and the antibody-antigen complexes were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Quantitation of the relative protein level was carried out with the ImageQuant 1.1 program (Amersham Pharmacia Biotech). Each of three NtCaM proteins was quantified by comparison with appropriate amount of the recombinant protein, and the ratio of individual CaM protein isoform types to total CaM amount was determined.

Measurement of proteasome activity

26S proteasome activity was determined as described by Ito *et al.* (1999). Leaves were homogenized in 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 2 mM ATP, and 10% (w/v) polyclar AT. Aliquots of the crude extracts (4 mg protein equivalent) were fractionated by 15 to 35% (v/v) glycerol density gradient centrifugation. Concentration of liberated 7-amino-4-methyl-coumarin was measured in the absence of SDS using succinyl-Leu-Leu-Val-Tyr-4-methylcoumalyl-7-amide as a substrate. The total value of activity of all separated fractions was defined as the proteasome activity.

NAD^+ kinase assay

Pea NAD^+ kinase (NADK) was partially purified from pea seedlings (*Pisum sativam* L. cv. Akabana-tsurunashi-endo) by the method of Muto and Miyachi (1977). Peas were

germinated in moistened vermiculite and grown under artificial light without additional nutrients. The following purification procedures were carried out at 4 °C. The aerial parts of 12-day-old seedlings (627 g) were macerated into fine powder with liquid nitrogen and extracted with 3-volumes of 25 mM triethanolamine-acetate (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 0.5 M sucrose, and 1 mM dithiothreitol. The homogenate was squeezed through double layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 27,000*g* for 30 min. A one tenth-volume of 0.7% protamine sulfate solution in 10 mM triethanolamine-acetate buffer (pH 7.5) was added to the supernatant (2,000 ml). After continuous stirring for 15 min, the precipitate was collected by centrifugation at 27,000*g* for 15 min. From the precipitate, the enzyme was extracted with 250 ml of 0.2 M Na-acetate buffer (pH 6.0), and 1 μg/ml pepstatin A. To the extract, the same volume of 50% (w/w) polyethylene glycol 6,000 solution was added and the mixture was stirred for 30 min. After centrifugation at 39,000*g* for 30 min, the precipitate was resuspended in 100 ml of 50 mM Tris-HCl (pH 7.0), 100 mM KCl, 3 mM $MgCl_2$, and 1mM EGTA. The insoluble materials were removed by centrifugation at 27,000*g* for 15 min. The supernatant solution was passed through a DEAE-Sephacel (Amersham Pharmacia Biotech) column (1.6 × 30 cm) pre-equilibrated with the same buffer as mentioned above, allowing CaM to be completely adsorbed on the column. The effluent was stocked at –80°C in small portions of 5% glycerol solution and used for NADK assays.

The NADK assay was conducted as described previously (Harmon *et al.*, 1984) in a 0.5-ml reaction mixture containing 50 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 2 mM NAD^+, 3 mM ATP, 1 mM $CaCl_2$ or EGTA, and a various amount of CaM. The reaction was initiated by the addition of 10 μl of freshly-thawed, purified NADK stock solution. After incubation for 60 min at 37 °C, the reaction was terminated by placing the tubes in boiling water for 3 min. The

tubes were then cooled to the ambient temperature, and 0.5 ml of 50 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 1 mM EGTA, 0.8 mM glucose 6-phosphate, 0.1 mg/ml phenazine methosulfate, and 0.15 mg/ml 2,6-dichlorophenolindophenol was added. The mixture was transferred to a cuvette pre-warmed at 30 °C, and 20 µl of glucose 6-phosphate dehydrogenase (6 U/ml) was added. The decrease in A_{600} per minute was monitored with a Beckman spectrophotometer (Model DU-7400; Fullerton, CA) equipped with a temperature controller set at 30 °C. The amount of $NADP^+$ produced by the NADK reaction was calculated from a standard curve of $NADP^+$ amount versus descending rate of A_{600} (Figure 11B). No activation by Ca^{2+} was found in the absence of exogenous CaM, confirming that the preparation of NADK was free from contamination with pea endogenous CaM (data not shown).

NO synthase assay

NOS activity was determined by the citrulline assay followed by thin-layer chromatography (TLC), as described by Kumar *et al.* (1999). The freshly-prepared reaction mixture (20 µl) consisted of 30 mM Hepes-NaOH (pH 7.0), 2 mM NADPH, 100 µM FAD, 100 µM tetrahydrobiopterin, 1 mM $CaCl_2$ or EGTA, a various concentration of CaM, 0.25 µl of L-[U-^{14}C]arginine (272 mCi/mmol, 100 µCi/ml; Moravek Biochemicals, Brea, CA), and 100 mU of recombinant rat neuronal NOS (Calbiochem). The reaction was carried out at 30°C for 60 min. The NOS reaction was terminated by adding 50 µl of cold methanol. The samples were left on ice for 20 min and centrifuged at 20,000*g* for 10 min. An aliquot (10 µl) of the supernatant was spotted onto a silica gel TLC plate (Merck, Darmstadt, Germany), air-dried, and subjected to chromatography. The solvent was ammonium

hydroxide:chloroform:methanol:water (4:1:9:2). The plate was imaged by a PhosphorImager SI (Amersham Pharmacia Biotech) after exposure for 48 h and the radioactivity of the product, L-[^{14}C]citrulline, was quantified by the ImageQuant 1.1 program (Amersham Pharmacia Biotech). The R_f values for L-arginine and L-citrulline were 0.44 and 0.90, which were confirmed to be the same as those of the standard amino acids stained with ninhydrin (data not shown).

Calcineurin assay

The activity of a CaM-dependent protein phosphatase, CaN, was determined by a fluorescent assay with 4-methyl umbelliferyl phosphate (4MUP; ICN, Costa Mesa, CA) as the substrate, as reported previously (Anthony *et al.*, 1986). Each assay was conducted in 200 µl of 50 mM Tris-HCl (pH 8.0), 1 mg/ml BSA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$ or EGTA, 12.5 nM bovine brain CaN (Upstate Biotechnology, Lake Placid, NY), a various concentration of CaM, and 200 µM 4MUP, which was added to start the reaction. After incubation at 37°C for 60 min, the reaction was terminated by addition of 1 ml of 0.2 N Na_2CO_3. Fluorescence was monitored in a quartz cuvette (1 cm light path) using a Hitachi fluorescence spectrophotometer (Model F-2500; Tokyo, Japan) with an excitation wavelength of 365 nm and an emission wavelength of 446 nm. To correlate the amount of product, 4-methyl umbelliferone (4MU), with the amount of fluorescence, a standard curve was made by monitoring the fluorescence intensity as a function of the concentration of 4MU. All assays were corrected for the nonenzymatic hydrolysis of 4MUP. Specific activity was defined as nmol 4MU/mg/min.

Generation of transgenic tobacco plants

For transformation of tobacco (*Nicotiana tabacum* L. cv. Samsun NN), I prepared binary vectors for the overexpression of individual CaM genes, designated p35SΩ-S1, S3, S13, and *luc*, which contain respectively, *NtCaM1*, *3*, *13* and a firefly luciferase (for control; from pT3/T7-*luc*; Clontech) cDNA in the sense orientation under the control of an efficient promoter for the cauliflower mosaic virus 35S transcript and a translational enhancer of the TMV Ω sequence (Figure 15). For suppression vectors (namely p35SΩ-A1, A3 and A13), *NtCaM1*, *3* and *13* cDNAs were placed in the antisense orientation downstream of the same promoter and translational enhancer, respectively. I first made a modified binary vector p35SΩ-GUS by insertion of the Ω sequence (Nishiguchi *et al.*, 1985) into the *Xba*I–*Bam*HI site of the pBI121 vector (Clontech) and substitution of the nopaline synthase terminator with the terminator for the cauliflower mosaic virus 35S transcript (Timmermans *et al.*, 1990) plus a 5'-flanking additional *Kpn*I site, which facilitates separate detection of the transcript derived from the introduced gene with the cDNA of this 35S terminator region as a probe. For the sense construct, the coding region of the *NtCaM1*, *NtCaM3*, and *NtCaM13* cDNA was amplified by PCR with *Bam*HI and *Kpn*I sites attached at the 5' and 3' ends, respectively, and verified by DNA sequencing. For the antisense construct, the coding region was amplified with *Kpn*I and *Bam*HI sites at the 5' and 3' ends, respectively. The *luc* fragment was excised from pT3/T7-*luc* by digestion with *Bam*HI and *Sac*I. These fragments were subcloned into the p35SΩ-GUS vector which had been digested with the corresponding restriction enzymes. The resulting sense and antisense *NtCaM* expression constructs were introduced into

Agrobacterium tumefaciens LBA4404 by electroporation (Wen-jun and Forde, 1989).

The transformation of tobacco was performed by the leaf disc cocultivation method (Horsch *et al.*, 1985). Leaf discs were immersed for 7 min in a Luria-Bertani medium (0.1% Bacto tryptone, 0.05% Bacto yeast extract and 0.1% NaCl) containing the transformed *Agrobacterium* cells, placed on an incubation medium [basal Murashige-Skoog medium (Nihon Seiyaku Co., Tokyo, Japan) with 3% sucrose and B5 vitamins] containing naphthaleneacetic acid (100 μg/l) and benzyl amino purine (1 mg/l) for 2 days at 25°C under continuous illumination from a white fluorescence lamp at an intensity of 10 μmol of photons m^{-2} sec^{-1}, and transferred to the same medium with 200 μg/ml cefotaxime (Chugai Pharmaceutical Co., Tokyo, Japan). After 2 days, the leaf discs were transferred to selection medium (incubation medium containing 200 μg/ml cefotaxime and 100 μg/ml kanamycin). Plates containing leaf discs on selection medium were incubated at 25°C under a cycle of 16 h of light and 8 h of darkness at an intensity of 120 μmol of photons m^{-2} sec^{-1}. The leaf discs were transferred to new selection medium every 10 days until green shoots were generated. The shoots were transferred to a phytohormone-free selection medium to allow rooting. Plantlets were transplanted to pots containing moistened vermiculite and grown under a cycle of 16 h of light and 8 h of darkness at an intensity of 120 μmol of photons m^{-2} sec^{-1}.

The transgenic tobacco plants were inoculated with 15 μg/ml of TMV suspension, as described above. For RNA gel blot analyses, the transcripts derived from transgenes were detected with cDNA for the region of the 35S terminator.