Plant Physiology, November 1999, Vol. 121, pp. 705–714, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Distinct Calcium Signaling Pathways Regulate Calmodulin Gene Expression in Tobacco[1]

Arnold H. van der Luit*, Claudio Olivari, Ann Haley, Marc R. Knight, and Anthony J. Trewavas

Institute for Molecular Cell Biology, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands (A.H.v.d.L.); Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, United Kingdom (A.H., A.J.T.); Dipartimento di Biologia, Sezione Biochimica e Fisiologia delle Piante, University of Milan, Via Celoria 26, 20133 Milano, Italy (C.O.); and Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom (M.R.K.)

Cold shock and wind stimuli initiate Ca^{2+} transients in transgenic tobacco (*Nicotiana plumbaginifolia)* seedlings (named MAQ 2.4) containing cytoplasmic aequorin. To investigate whether these stimuli initiate Ca^{2+} pathways that are spatially distinct, stress-induced nuclear and cytoplasmic Ca^{2+} transients and the expression of a stress-induced calmodulin gene were compared. Tobacco seedlings were transformed with a construct that encodes a fusion protein between nucleoplasmin (a major oocyte nuclear protein) and aequorin. Immunocytochemical evidence indicated targeting of the fusion protein to the nucleus in these plants, which were named MAQ 7.11. Comparison between MAQ 7.11 and MAQ 2.4 seedlings confirmed that wind stimuli and cold shock invoke separate Ca^{2+} signaling pathways. Partial cDNAs encoding two tobacco calmodulin genes, *NpCaM-1* and *NpCaM-2*, were identified and shown to have distinct nucleotide sequences that encode identical polypeptides. Expression of *NpCaM-1*, but not *NpCaM-2*, responded to wind and cold shock stimulation. Comparison of the Ca^{2+} dynamics with *NpCaM-1* expression after stimulation suggested that wind-induced *NpCaM-1* expression is regulated by a Ca^{2+} signaling pathway operational predominantly in the nucleus. In contrast, expression of *NpCaM-1* in response to cold shock is regulated by a pathway operational predominantly in the cytoplasm.

Calmodulin is highly conserved in eukaryotes and is considered to be a multifunctional protein because of its ability to interact and regulate the activity of a number of other proteins (Hepler and Wayne, 1985; Gilroy et al., 1993; Poovaiah and Reddy, 1993; Trewavas and Knight, 1994). In plant cells, calmodulin is considered to be the primary sensor for changes in cellular free Ca^{2+} levels ($[Ca^{2+}]_i$) (Roberts and Harmon, 1992). As $[Ca^{2+}]_i$ rises transiently after signaling, the combination of Ca^{2+} with calmodulin leads to the activation of numerous target proteins initiating the physiological response.

Calmodulin has been purified and characterized from a number of plant species. Genomic and/or cDNA clones encoding calmodulin have been isolated and characterized from Arabidopsis (Ling et al., 1991; Perera and Zielinski, 1992), potato (Takezawa et al., 1995), and wheat (Yang et al., 1996). In all multicellular organisms in which it has been examined, genes encoding the different calmodulin isoforms are under the control of different promoters that exhibit distinct temporal and spatial expression (Ling et al., 1991; Gannon and McEwen, 1994; Shimoda et al., 1995; Solà et al., 1996). In plant cells, stimuli such as touch, wind, or temperature shocks induce the rapid accumulation of mRNA levels encoding calmodulin and calmodulin-related proteins (Jena et al., 1989; Braam, 1992; Perera and Zielinski, 1992; Watillon et al., 1992; Takezawa et al., 1995). Since many of these signals also elevate $[Ca^{2+}]_i$ (Knight et al., 1991, 1992, 1997), and artificial elevation of $[Ca^{2+}]_i$ in cultured cells increases calmodulin mRNA accumulation (Braam, 1992), it has been suggested that the transduction of environmental signals regulating calmodulin gene expression are in part regulated by $[Ca^{2+}]_i$ levels (Braam and Davis, 1990; Braam, 1992).

Calmodulin has been detected in several plant cell compartments (Biro et al., 1984; Collinge and Trewavas, 1989). In particular, a substantial amount of calmodulin has been found in both plant and animal nuclei and in combination with nuclear Ca^{2+} signals, gene expression is thought to be regulated via Ca^{2+}/calmodulin interaction with transcription factors or via specific protein kinases (Bachs et al., 1992; Gilchrist et al., 1994; Kocsis et al., 1994; Zimprich et al., 1995; Szymanski et al., 1996).

Plants transformed with a cDNA encoding the Ca^{2+}-sensitive luminescent protein aequorin provides a simple, non-invasive means of measuring $[Ca^{2+}]_i$ in whole plants. Many new signals initiating rapid changes in $[Ca^{2+}]_i$ have subsequently been detected with this technology, including the mechanical signals of touch and wind, salt/drought, heat shock, and osmotic pressure (Trewavas and Knight, 1994; Haley et al., 1995; Knight et al., 1996, 1997; Takahashi et al., 1997; Gong et al., 1998). Furthermore, aequorin targeted to chloroplasts (Johnson et al., 1995) and the vacuole membrane (Knight et al., 1996) clearly indicated that the $[Ca^{2+}]_i$ signal is strictly compartmentalized within the cell.

In a previous paper (Knight et al., 1992), wind and cold shock stimulation were investigated in tobacco (*Nicotiana plumbaginifolia*) seedlings. Mechanical stimulation induced by puffs of air blown over the seedling resulted in a slight movement of the seedling around the hypocotyl/root junc-

[1] This work was funded by the Research Training Grant Body of the European Commission and the Biotechnology and Biological Sciences Research Council.

* Corresponding author; e-mail luit@bio.uva.nl; fax 31–20–5257934.

tion lasting about 0.02 to 0.3 s. Temperature shocks can be induced by irrigating the plant briefly with cold water at 0°C to 5°C. Both signals induce $[Ca^{2+}]_i$ spikes in aequorin transgenic tobacco seedlings. However, careful titration with different inhibitors suggested specific spatial organization of the Ca^{2+} signal depending on the type of stimulation. Ruthenium red at low concentrations specifically blocked the transient induced by wind or touch and did not affect the cold shock $[Ca^{2+}]_i$ transient; lanthanum and gadolinium chlorides, which are Ca^{2+}-channel blockers, specifically blocked the cold shock signal without influencing the wind-induced $[Ca^{2+}]_i$ transient. It was concluded that the two signals were mobilizing separate pools of $[Ca^{2+}]_i$.

At present, no direct evidence is available to indicate whether compartmentalization of the Ca^{2+} signal is significant for other downstream responses such as calmodulin gene expression. To address this question, we created a fusion protein between nucleoplasmin (a major oocyte nuclear protein) and aequorin, which was then used to transform tobacco seedlings. Transfection of animal cells with this construct allowed measurement of nuclear Ca^{2+} concentrations and indicated the presence of compartmentalized regulation of Ca^{2+} signaling pathways (Badminton et al., 1995, 1996, 1998). The use of the same construct in plant cells could help to clarify the Ca^{2+} signaling pathways involved in the control of calmodulin gene expression by wind stimuli and cold shock.

MATERIALS AND METHODS

All enzymes used for recombinant DNA manipulation were purchased from Promega Biotech (Southampton, UK). Plasmid DNA isolation kits were obtained from Qiagen (Dorking, UK), agar was from Difco Laboratories (Detroit), and all plant tissue culture reagents and other chemicals were from Sigma (Dorset, UK). Exceptions were 1,2-bis(o-aminophenoxy)ethane-N;N;N;N-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) from Calbiochem (Nottingham, UK) and ruthenium red from LC Laboratories (Woburn, MA). Macerozyme and cellulase used for the production of protoplasts were from Yakult Honsha (Tokyo). Native coelenterazine and *cp*-coelenterazine were purchased from Molecular Probes (Leiden, The Netherlands). Oligonucleotide primers were prepared by Genosys (Cambridge, UK).

Plant Materials and Growth Conditions

MAQ 2.4, the transgenic tobacco (*Nicotiana plumbaginifolia*) line that expresses cytosolic aequorin (Knight et al., 1991), was used to measure changes in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$). All seedlings used for experiments were grown on one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) and 0.8% (w/v) agar either in luminometer cuvettes or on plates at 25°C with a 16-h photoperiod, and used when 7 to 10 d old.

Design and Expression of Nuclear-Targeted Chimeric Aequorin

To target aequorin to plant nuclei, a chimeric construct in which the nucleoplasmin coding region was placed in frame with the coding region of apoaequorin (Badminton et al., 1995; kindly provided by Dr. M. Badminton, University of Wales, UK) was cloned into pDH51 (Pietrzak et al., 1986) as a *Sma*I-*Sal*I fragment. The entire construct, including the 35S promoter and terminator, was cloned into the *Agrobacterium tumefaciens* binary vector pBIN19. *Escherichia coli* JM101 and XL-1 Blue were used as hosts for all recombinant DNA manipulations (Sambrook et al., 1989). *A. tumefaciens* LBA4404 and *N. plumbaginifolia* were used for plant genetic transformation (Draper et al., 1988).

Immunolocalization of Aequorin

For immunolocalization using fluorescein isothiocyanate (FITC)-labeled secondary antibodies, protoplasts were washed and pelleted in 0.5% (w/v) MES (pH 5.8), 80 mM $CaCl_2$, 300 mM mannitol, and fixed for 15 min on poly-L-Lys treated slides using 4% (w/v) paraformaldehyde. Cells were permeabilized for 40 min with 0.5% (v/v) Triton X-100 in 50 mM PIPES (pH 6.9), 5 mM $MgSO_4$, 5 mM EGTA, and 300 mM mannitol. Samples were incubated for 5 min with 1% (w/v) BSA followed by a 1.5-h incubation at 37°C with mouse anti-aequorin (1:1,000) obtained as previously described (Knight et al., 1991), and then with FITC-labeled goat anti-mouse IgG from Sigma (Dorset, UK) (1:30) in PBS (pH 5.8), 1% (w/v) BSA, 20 mM NaN_3 for 45 min at 37°C. Cells were stained with DAPI, mounted in Citifluor (Citifluor Products, Kent, UK), and photographed with an epifluorescence microscope (Polyvar, Reichert-Jung, Vienna, Austria) using Ektachrome T film (ASA 64, Eastman-Kodak, Rochester, NJ).

For immunoelectron microscopy using gold-labeled secondary antibodies, protoplasts were prepared as described above and fixed for 15 min using PBS-buffered one-fourth-strength Karnovsky's fixative at pH 5.8 (Karnovsky, 1965). The fixed tissue was dehydrated by consecutive 10-min incubations in 30%, 50%, 70%, and 90% (v/v) ethanol, and for 20 min in three changes of dehydrated absolute ethanol followed by propylene oxide (twice for 15 min). The embedding of fixed and dehydrated tissue was carried out using resin from Agar Scientific (Essex, UK). Thin sections (80–90 nm) placed on gold grids were incubated in 1% (w/v) BSA in PBS for 5 min at room temperature. Sections were incubated with mouse anti-aequorin (1:200) obtained as previously described (Knight et al., 1991) for 2 h at room temperature or for 18 to 24 h at about 4°C in a moist chamber. The antisera or immunosorbent-purified antibodies were diluted in 1% (w/v) BSA-PBS (pH 7.4). The grids were placed on drops of a 20-fold dilution of the 1 nM gold-labeled goat anti-mouse IgG solution from British Biocell International (Cardiff, UK) for 1 h at room temperature in a moist chamber. The sections were stained with 5% (v/v) uranyl acetate (5–7 min), and washed thoroughly with distilled water and PBS and a subsequent Reynold's lead citrate solution (2–5 min). Gold particles were stained

with a silver enhancement kit from Sigma prior to examination using an electron microscope (model 100S, JEOL, Hartfordshire, UK).

In Vitro and in Vivo Aequorin Reconstitution, Wind and Cold Shock Stimulation, and Ca^{2+} Measurements

For in vitro reconstitution of aequorin, seedlings were homogenized in 50 mM Tris-Cl (pH 7.4), 500 mM NaCl, 5 mM β-mercaptoethanol, 10 mM EGTA, and 0.1% (w/v) BSA, incubated with 2 μM coelenterazine for at least 4 h in the dark, discharged by adding an equal volume of 100 mM $CaCl_2$ (Knight et al., 1991), and the total amount of luminescence produced was measured. Luminescence was measured using a digital chemiluminometer consisting of an photomultiplier (model 9829A, EMI, Middlesex, UK) with a cooling system (FACT50, EMI) (Badminton et al., 1995). For in vivo reconstitution of aequorin, seedlings were germinated as described above. Aequorin was reconstituted in vivo by placing a 3-μL droplet of 2 μM coelenterazine between the cotyledons and incubating at least 4 h in the dark.

For experiments with inhibitors, seedlings were submerged and incubated for 4 h. A long period is needed to allow the compounds to penetrate into the seedling. Following this treatment, the liquid was drained and a 3-μL droplet of 2 μM coelenterazine with the relevant inhibitor was placed between the cotyledons and left for at least another 4 h in the dark, after which time the liquid was removed and the luminescence measurement carried out. Wind stimulation was simulated by instantly injecting 5 mL of air into the sample housing of the luminometer. Cold shock was simulated by slowly injecting 1 mL of ice-cold water into the sample housing of the luminometer. The light emitted by the seedling is a measure of the change in the $[Ca^{2+}]_i$ and was recorded every 0.2 s using a cooled photomultiplier tube.

For measurement of changes in cytosolic Ca^{2+} native coelenterazine, we used the luminophore used in our previous experiments (Knight et al., 1991, 1992). Initial investigations showed nuclear Ca^{2+} changes to be smaller than those in the cytoplasm in response to cold shock. To reduce errors in the measurement of emitted light, the more sensitive *cp*-coelenterazine was used, which enabled approximate equality in light emission measurements between the cytosolic and nuclear compartments (Shimomura et al., 1993). Reconstituted *cp*-aequorin shows improved light emission in the lower Ca^{2+} ranges and is thus useful for detecting smaller changes in $[Ca^{2+}]_i$ (Shimomura et al., 1993). Calibration constants for *cp*-coelenterazine (and many other coelenterazines) in comparison with native coelenterazine have been published previously (Shimomura et al., 1993). The luminescent light was calibrated into Ca^{2+} concentrations by a method based on the calibration curve of Allen et al. (1977): $L/L_{max} = ([1 + K_R \times \{Ca^{2+}\}]/[1 + K_{TR} + K_R \times \{Ca^{2+}\}])^3$, where L is the amount of light per second, L_{max} is the total amount of light present in the entire sample over the course of the experiment, $[Ca^{2+}]$ is the calculated Ca^{2+} concentration, K_R is the dissociation constant for the first Ca^{2+} ion to bind, and K_{TR} is the binding constant of the second Ca^{2+} ion to bind to aequorin; $K_R = 26 \times 10^6$ M^{-1} and $K_{TR} = 57$ M^{-1} for *cp*-coelenterazine (Shimomura et al., 1993) and $K_R = 2 \times 10^6$ M^{-1} and $K_{TR} = 55$ M^{-1} for native coelenterazine.

Total RNA Extraction, RACE (3′ RACE), and Northern-Blot Analysis

Total RNA from seedlings was extracted according to the method of López-Gómez and Gómez-Lim (1992), a method designed to obtain RNA free of polysaccharide contamination. Seedlings for RNA extraction were 7 to 10 d old and grown under the same conditions as seedlings for luminometry. Inhibitors were applied for a 4-h period, after which time the solution was drained and the seedlings were allowed to recover overnight.

For 3′-RACE, total RNA was extracted from unstimulated seedlings (T_0), 1 h after wind stimulation (T_1W), and 2 h after cold shock stimulation (T_2CS). cDNA was synthesized from 5 μg of total RNA in a buffer consisting of 50 mM Tris-Cl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, and 0.5 μM of each dNTP, 10 units of RNasin (Promega Biotech), 100 ng μL^{-1} of dT_{17}-adapter primer, Q_T (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC-TT_{17}VN-3′, with V = G, C, A and N = G, C, T, A), 10 units of SuperScript II RNase H$^-$ reverse transcriptase from Life Technologies (Paisley, UK) in a total volume of 20 μL. The mixture was incubated for 5 min at room temperature, for 1 h at 42°C, for 10 min at 50°C, and for 15 min at 70°C. The RNA was then removed with 0.2 unit of RNase H from Life Technologies and the whole reaction was diluted with 1 mL of TE buffer (10 mM Tris-Cl [pH 7.6] and 1 mM EDTA) to produce the cDNA pool for amplification.

For amplification, a PCR cocktail was prepared consisting of: 5 μL of 10× PCR buffer (670 mM Tris-Cl, pH 8.8, 67 mM $MgCl_2$, 17 mg mL^{-1} BSA, 166 mM $[NH_4]_2SO_4$), 5 μL of DMSO, 5 μL of 10× dNTPs (10 mM each), and 30 μL of distilled water, 1 μL of adapter primer, Q_i (ACGAGGACTCGAGCTCAAGC, 25 pmol μL^{-1}), 1 μL of a calmodulin-specific primer, E086 (GCATCACGACTAAGGAGCTT, 25 pmol μL^{-1}), and 1 μL of cDNA pool. The cDNA was denatured 5 min at 95°C and cooled to 72°C. Then 2.5 units of *Taq* polymerase and 30 μL of mineral oil were added. Primers were annealed and extended at 52°C or 56°C for 5 min and at 72°C for 40 min to ensure correct replication, respectively, followed by a 20- to 35-times cycle: 95°C for 40 s, 52°C or 56°C for 1 min, 72°C for 3 min, and ended by a 15-min incubation at 72°C to complete the reaction. *NpCaM-1* (accession no. AJ005039) and *NpCaM-2* (accession no. AJ005040) were cloned using the pCR-Script Amp SK(+) cloning kit from Stratagene (Cambridge, UK) and used for sequence analysis.

Sequence analysis was carried out on both the DNA strands in quadruple. For each strand, sequencing reactions were performed using a dye-terminator cycle sequencing ready reaction kit (PRISM, ABI, Perkin-Elmer, Cheshire, UK) and sequenced using an automatic sequencer (Perkin-Elmer).

For northern-blot analysis, 5 to 15 μg of total RNA was size-fractionated on a 1.3% (w/v) denaturing-formaldehyde

agarose gel (Sambrook et al., 1989). To ensure that an equal amount of RNA was loaded, a picture of the ethidium bromide-stained gel was taken, scanned, and quantified with Imagequant software (Molecular Dynamics, 's-Hertogenbosch, The Netherlands). The 3′-untranslated regions (UTRs) of *NpCaM-1* and *NpCaM-2* were used as DNA hybridization probes and were labeled with [^{32}P]dCTP by random-primed labeling from Amersham (Buckinghamshire, UK), and hybridized in 4× SSC, 1% (w/v) SDS, 200 mM Tris-Cl (pH 7.6), 10% (w/v) dextran sulfate, 100 μg mL^{-1} herring-sperm DNA, and 2× Denhardt's solution, at 65°C overnight, and washed for 20 min in 2× SSC at 65°C, followed by a brief wash in 2× SSC, 1% (w/v) SDS at room temperature. Filters were either exposed to HyperfilmMP from Amersham or a phosphor plate, and imaged with a phosphor imager from Molecular Dynamics. Intensities of hybridizing bands were quantified using Imagequant software.

RESULTS

Transformation of Tobacco with a Nucleoplasmin Aequorin Construct and Localization of the Expressed Fusion Protein

Wind and cold shock stimulation initiate specific Ca^{2+} signaling pathways (Knight et al., 1992). The use of different inhibitors suggested the specific organization of the Ca^{2+} signal depending on the type of stimulation. To investigate the organization of the Ca^{2+} signal in more detail, we transformed tobacco with a nucleoplasmin/aequorin construct. This construct was used previously (Badminton et al., 1995, 1996, 1998) to investigate the putative independence of the regulation of nuclear ($[Ca^{2+}]_{nuc}$) and cytoplasmic ($[Ca^{2+}]_{cyt}$) Ca^{2+} in transfected mammalian cell lines. Nucleoplasmin is an abundant nuclear protein in *Xenopus laevis* oocytes (Philpott and Leno, 1992).

After leaf disc transformation, 7-d-old F_1 seedlings of >20 individual transformants were homogenized in 50 mM Tris-Cl (pH 7.4), 500 mM NaCl, 5 mM β-mercaptoethanol, 10 mM EGTA, and 0.1% (w/v) BSA, aequorin was reconstituted with added coelenterazine overnight as described previously (Knight et al., 1991, 1993, 1996), and aequorin levels were measured by light emission. Homogenates of all transformants were separated on SDS gels and the relative amounts of apoaequorin confirmed using western blotting and mouse anti-apoaequorin as described previously (Knight et al., 1991). The transformant containing the highest levels of expression was designated MAQ 7.11.

The cellular distribution of the nucleoplasmin/aequorin fusion protein was examined using immunocytochemistry with anti-apoaequorin and either FITC or gold-labeled secondary antibodies. Protoplasts were isolated from mature leaves of untransformed tobacco and from MAQ 2.4 and MAQ 7.11 containing the nucleoplasmin/aequorin construct, and stained for apoaequorin distribution. Figure 1, A to C, shows protoplasts stained first with 4′,6-diamidino-2-phenylindole dihydrochloride or DAPI (to highlight DNA) and then stained with anti-apoaequorin followed by fluorescent secondary antibody (Fig. 1, D–F). The distribution of staining between the MAQ 2.4 and the MAQ 7.11 construct is clearly very different. The aequorin is distributed throughout the cytoplasm of the highly vacuolated protoplasts of MAQ 2.4 (Fig. 1, B and E), while the nucleoplasmin/aequorin construct is predominantly concentrated in the nuclear region of the protoplasts for MAQ 7.11 (Fig. 1, C and F).

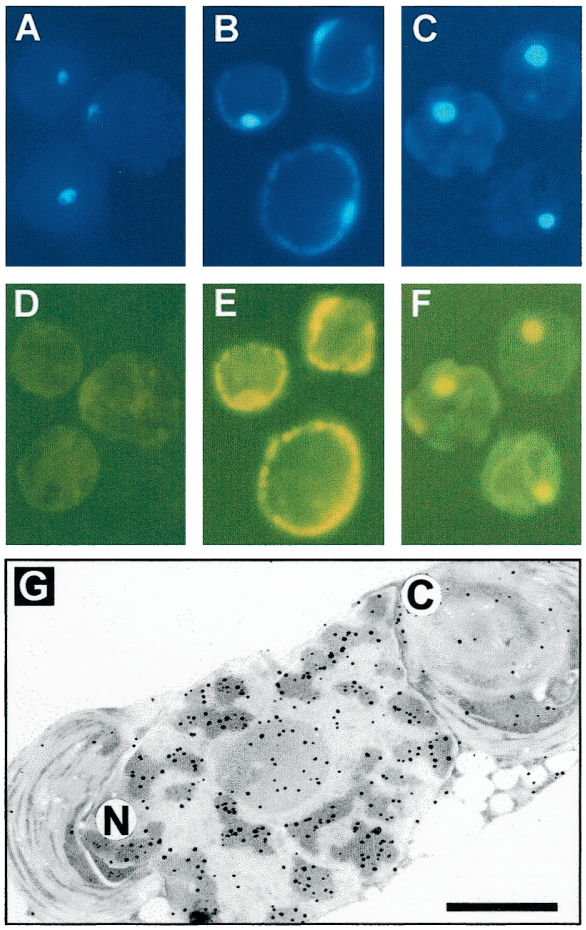


Figure 1. Targeting of aequorin to tobacco cell nuclei. Protoplasts of wild-type tobacco, MAQ 2.4, and MAQ 7.11 stained with DAPI are shown in A, B, and C, respectively. The same protoplasts treated with anti-apoaequorin and FITC-labeled secondary antibody are shown in D, E, and F. G shows a MAQ 7.11 protoplast treated with anti-apoaequorin and gold-labeled secondary antibody. Bar = 1 μm. C, Cytoplasm; N, nucleus.

Confirmation of this distribution was obtained using gold-labeled secondary antibody. Figure 1G shows a nucleus and two associated areas of chloroplast/cytoplasm of a MAQ 7.11 protoplast. The intact nucleolus and nuclear envelope are clearly visible. Staining with gold-labeled secondary antibody revealed a gold particle distribution that was much more highly concentrated over the nuclear regions than the neighboring cytoplasm and strongly localized in dense chromatin. We quantified the gold particle distribution on a large number of sections and observed that 86% was localized in nuclei. Of the remainder, 9% was found in the chloroplasts and 5% in the cytoplasm. The distribution of nucleoplasmin/aequorin between the nu-

cleus and other cytoplasmic compartments was similar to that reported for the distribution of nucleoplasmin in HeLa cells, 90% to 92% nuclear localization (Greber and Gerace, 1995), with a slightly higher proportion outside the nucleus.

Isolation of Wind- and Cold-Shock-Induced and Non-Induced Tobacco Calmodulin Genes

In all plants examined so far, calmodulin is represented by multigene families, and the individual calmodulin members exhibit both tissue-specific and developmental-stage-specific expression (Ling et al., 1991; Takezawa et al., 1995). As the length and the sequence of 3′-UTRs of calmodulin isoforms were reported to be different (Takezawa et al., 1995), 3′-RACE was carried out in tobacco to identify differentially expressed calmodulin genes. Using this technique, several potential calmodulin transcripts were identified. One of these putative calmodulin transcripts, designated *NpCaM-1*, appeared to be induced by wind and cold shock, while another, *NpCaM-2*, was not (data not shown). These two cDNAs were cloned and sequenced. In Figure 2, the partial sequence of two calmodulin isoforms is shown starting from the first Ca^{2+}-binding site of calmodulin. The partial sequences of *NpCaM-1* and *NpCaM-2* are different in nucleotide sequence; however, they encode polypeptides with the same amino acid sequence. The 3′-UTRs were subcloned and used as DNA hybridization probes to study the expression kinetics of *NpCaM-1* and *NpCaM-2* using northern-blot analysis. As shown in Figure 3, this type of analysis indicated that *NpCaM-1* mRNA accumulates after wind and cold shock signaling, whereas *NpCaM-2* does not.

Wind- and Cold-Shock-Induced Ca^{2+} Changes in MAQ 2.4 and MAQ 7.11 and *NpCaM-1* mRNA Accumulation

Changes in $[Ca^{2+}]_{cyt}$ in tobacco seedlings in response to wind and cold shock have been previously reported (Knight et al., 1991, 1992). By constructing plants in which the distribution of aequorin is clearly different from cytoplasmic aequorin, we were able to examine the spatial organization of the Ca^{2+} signal in response to wind and cold shock. Prior to $[Ca^{2+}]_i$ measurements, coelenterazine was placed between the cotyledons to allow the reconstitution of aequorin. Wind and cold shock stimulation were achieved respectively by injecting air instantly or ice-cold water gently from above into the sampling housing of the luminometer. Conversions of emitted luminescence at each time point to free Ca^{2+} levels were performed as described in "Materials and Methods."

Figure 4 shows the effects of wind and cold shock signaling in MAQ 2.4 and MAQ 7.11. Because individual seedlings varied slightly in their absolute response, we have indicated only the SES of the peak values. For wind response the mean peak Ca^{2+} increase in the MAQ 2.4 and MAQ 7.11 were, respectively, 1.08 μM ($n = 7$) and 0.79 μM ($n = 8$) (Fig. 4A) and 1.25 μM ($n = 7$) and 0.55 μM ($n = 8$) for the cold shock response (Fig. 4B).

The kinetics of the signals in the nucleus and cytoplasm differ in response to both stimuli. For wind stimulation, the

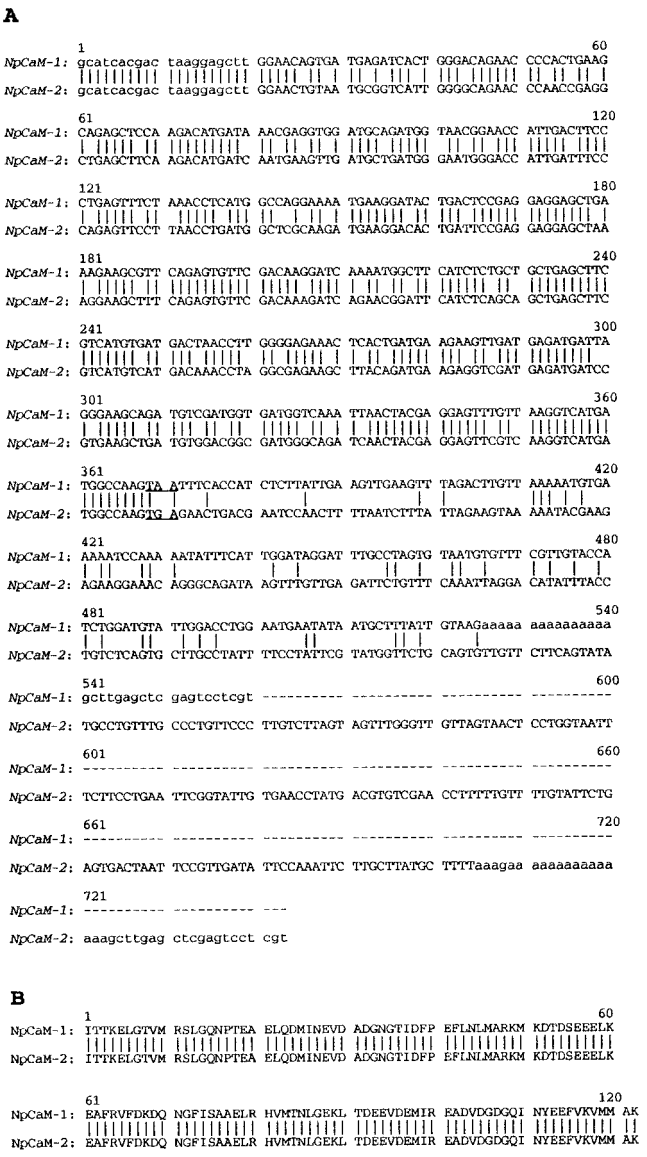


Figure 2. Partial cDNA sequence of *NpCaM-1* and *NpCaM-2* showing nucleotide and predicted amino acid identities. A, Nucleotide sequence; B, amino acid sequence. Primers used for 3′-RACE and subsequent PCR are indicated in lowercase; stop codons are underlined. Homology is indicated with bars.

average rise time (the time required to reach the peak) for MAQ 2.4 (cytoplasm) was 0.31 ± 0.04 and 0.60 ± 0.04 s for MAQ 7.11. For cold shock the average rise times for MAQ 2.4 and MAQ 7.11 were, respectively, 4.8 ± 0.3 and 9.0 ± 1.1 s. The MAQ 7.11 signals always peaked later than those in the cytoplasm, and the peak value was always lower. In more recent unpublished studies of ours using heat shock, signal-induced elevations of MAQ 2.4 and MAQ 7.11 were separated by minutes (M. Gong, A.H. van der Luit, and A.J. Trewavas, unpublished observations). This response, a much later and lower peak value found in MAQ 7.11 (compared with cytoplasmic MAQ 2.4), was similar to that recorded for $[Ca^{2+}]_{nuc}$ in mammalian cells. The average length of the Ca^{2+} transient was similar in both compart-

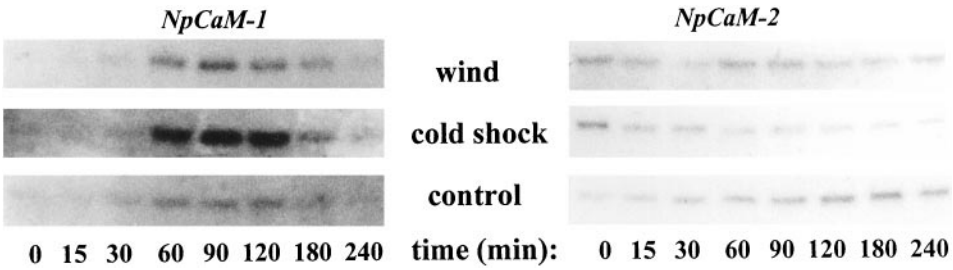


Figure 3. Expression kinetics of *NpCaM-1* and *NpCaM-2* determined by northern-blot analysis after stimulation by a single wind signal or a single cold shock. The 3′-UTRs of *NpCaM-1* and *NpCaM-2* were used as DNA hybridization probes to study the expression kinetics of *NpCaM-1* and *NpCaM-2*. Water of room temperature was used as a control.

ments for wind stimulation, but was about 6 s longer in cold-shocked MAQ 7.11 compared with the cytoplasm.

In separate experiments, tobacco seedlings were given wind signals (one treatment of 5 mL of air) or cold shock signals (one treatment of 1 mL of ice-cold water) similar to those used for Figure 4, A and B. RNA was extracted and the levels of *NpCaM-1* and *NpCaM-2* mRNAs estimated from northern blots. These data ($n = 3$) are shown in Figure 4C. The total increase of *NpCaM-1* mRNA after wind stimulation was about 5-fold after 60 to 90 min, whereas after cold shock it was about 10-fold after 90 to 120 min. *NpCaM-2* exhibited only a slight increase throughout the experimental period.

Use of Inhibitors on MAQ 2.4 and MAQ 7.11 Emphasize That Spatially Separate Ca^{2+} Pathways Can Regulate Calmodulin Gene Expression

To try to deduce which Ca^{2+} compartment is used to regulate *NpCaM-1* RNA concentrations, we treated seedlings with several inhibitors that modify $[Ca^{2+}]_i$ kinetics. To establish suitable concentrations for use, we titrated the concentrations of these inhibitors to obtain an inhibition of about 50% or less in the Ca^{2+} signal. We then quantified the inhibitor-induced alterations in the $[Ca^{2+}]_i$ kinetics and the alterations, if any, in *NpCaM-1* and *NpCaM-2* accumulation.

MAQ 2.4 and MAQ 7.11 seedlings treated with thapsigargin, ruthenium red, or BAPTA-acetoxymethyl ester (AM) were subjected to wind signals (Fig. 5). There was a clear correlation between the behavior of the $[Ca^{2+}]_i$ signals in the MAQ 7.11 compartment and *NpCaM-1* RNA accumulation. Thapsigargin increased the MAQ 7.11 Ca^{2+} signal and subsequent calmodulin RNA accumulation, ruthenium red had no effect on the Ca^{2+} signal in MAQ 7.11 or the subsequent accumulation of calmodulin RNA, whereas BAPTA-AM decreased both. Ruthenium red did decrease the MAQ 2.4 signal but with no effect on *NpCaM-1* accumulation. BAPTA-AM led to a slight decrease in the mean Ca^{2+} peak height in the MAQ 2.4 seedlings, but the difference was not significant, falling within the SE of the experiment. Treatment with the inhibitors alone had no detectable effect on either mRNA levels or cytosolic or nuclear Ca^{2+} (data not shown).

MAQ 2.4 and MAQ 7.11 seedlings were treated with lanthanum and gadolinium chlorides and subjected to cold shock (Fig. 6). With both inhibitors there was a substantial

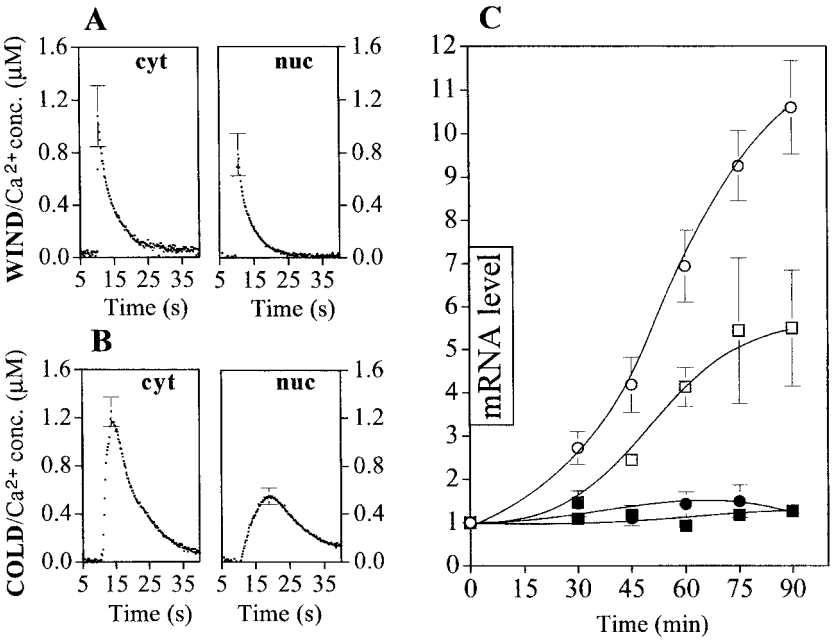
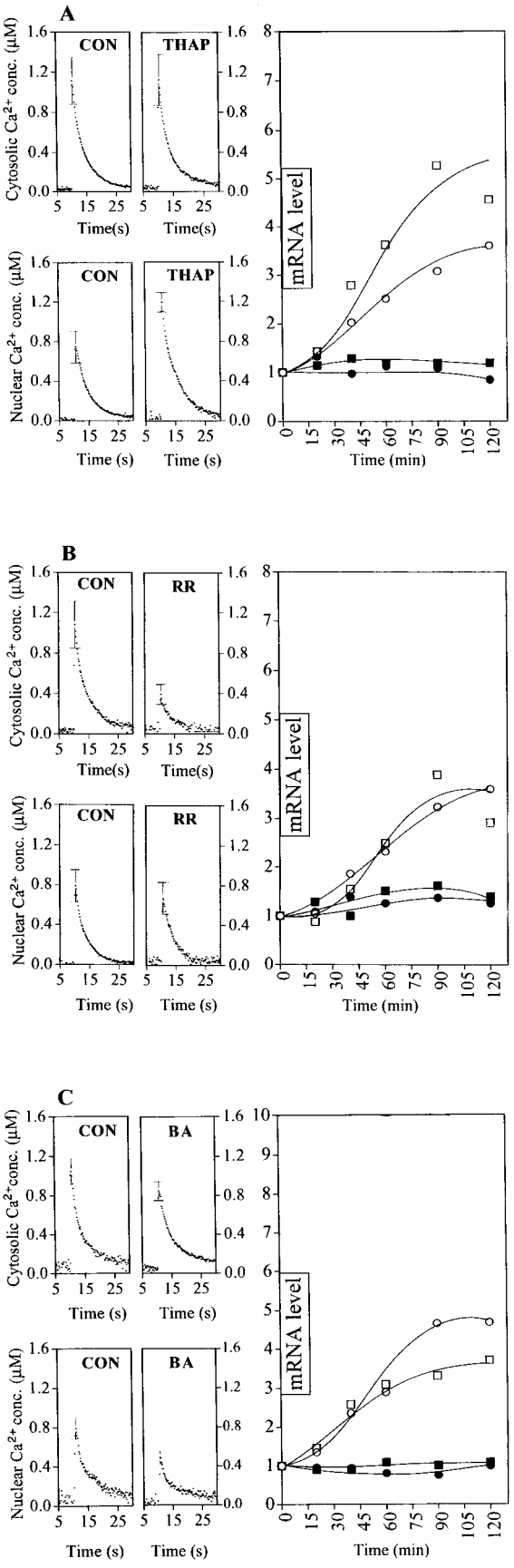


Figure 4. Wind- and cold-shock-induced changes in the cytosolic and nuclear free Ca^{2+} concentrations and the expression levels of *NpCaM-1* and *NpCaM-2*. A, Ca^{2+} changes in cytoplasm (cyt) and nucleoplasm (nuc) after stimulation with 5 mL of air at $t = 10$ s. B, Ca^{2+} changes in cytoplasm (cyt) and nucleoplasm (nuc) after stimulation with 1 mL of ice-cold water at $t = 10$ s. C, Wind- (□ and ■) and cold-shock (○ and ●)-induced changes in mRNA levels of *NpCaM-1* and *NpCaM-2* are indicated and are averages of three experiments. Data are shown as hybridization relative to non-induced mRNA levels (given a value of 1) and is plotted against time in minutes. ○, *NpCaM-1*; ●, *NpCaM-2*; □, *NpCaM-1*; ■, *NpCaM-2*.



decrease in the MAQ 2.4 signal, which was associated with a severe inhibition of subsequent *NpCaM-1* accumulation. The different behavior of the MAQ 7.11 seedlings, in which a slight increase in Ca^{2+} response was observed when the lanthanides were present, emphasizes a correlation between cold-shock-induced $[Ca^{2+}]_i$ kinetics in MAQ 2.4 and *NpCaM-1* expression. Treatment with the inhibitors alone had no detectable effect on mRNA levels or on cytosolic or nuclear Ca^{2+} (data not shown).

DISCUSSION

MAQ 7.11 Reports Changes in Nuclear Ca^{2+}

We transformed tobacco seedlings with a nucleoplasmin aequorin construct to investigate further the apparent compartmentalization of wind and cold shock Ca^{2+} signals. For a variety of reasons, we believe that MAQ 7.11 seedlings report $[Ca^{2+}]_{nuc}$ in response to wind and cold shock stimulation.

Immunolocalization techniques indicated that 86% of the nucleoplasmin/aequorin fusion protein was found located in the nucleus of MAQ 7.11 cells. To be nuclear targeted, this oocyte polypeptide must be recognized by the nuclear import machinery of plants. Increasing evidence suggests that the mechanism of nuclear protein translocation is highly conserved among higher eukaryotes. About 9% of the aequorin in MAQ 7.11 was associated with chloroplasts. We have previously targeted aequorin to chloroplasts in tobacco (designated MAQ 6.3, Johnson et al., 1995). No changes in the chloroplastic Ca^{2+} levels were detected during mechanical and cold shock treatment of these seedlings (A.H. van der Luit, A. Haley, and A.J. Trewavas, unpublished observation). The low level of aequorin in the chloroplast therefore did not contribute to the measurements described here. Another 5% of the nucleoplasmin aequorin was found in the cytoplasm. The nucleoplasmin/aequorin construct is synthesized in the cytoplasm and then partitions to the nucleus. However, this residual cytoplasmic aequorin does not contribute significantly to the luminescence signal of MAQ 7.11. The Ca^{2+} kinetics of the MAQ 7.11 are different from those of MAQ 2.4. Furthermore, there was no evidence of MAQ 7.11 kinetics of two components or two peaks, or even a broadening of the MAQ 7.11 peak, which might have resulted from a contaminating cytoplasmic signal.

There was a clear difference in the kinetics of the Ca^{2+} response to cold shock between MAQ 2.4 and MAQ 7.11.

Figure 5. The effect of Ca^{2+} modulators on wind-induced changes in cytosolic and nuclear Ca^{2+} and *NpCaM-1* and *NpCaM-2* mRNA accumulation. Wind stimulation was applied by 5 mL of air at t = 10 s. The SE for the peak values from eight experiments is indicated for the mean peak. CON, Control; THAP, thapsigargin; RR, ruthenium red; BA, BAPTA-AM. A, The effect of 200 μM thapsigargin. ○, CON *NpCaM-1*; ●, CON *NpCaM-2*; □, THAP *NpCaM-1*; ■, THAP *NpCaM-2*. B, 50 μM Ruthenium red. ○, CON *NpCaM-1*; ●, CON *NpCaM-2*; □, RR *NpCaM-1*; ■, RR *NpCaM-2*.; C, 1 mM BAPTA-AM; solvents were used as control. ○, CON *NpCaM-1*; ●, CON *NpCaM-2*; □, BA *NpCaM-1*; ■, BA *NpCaM-2*.

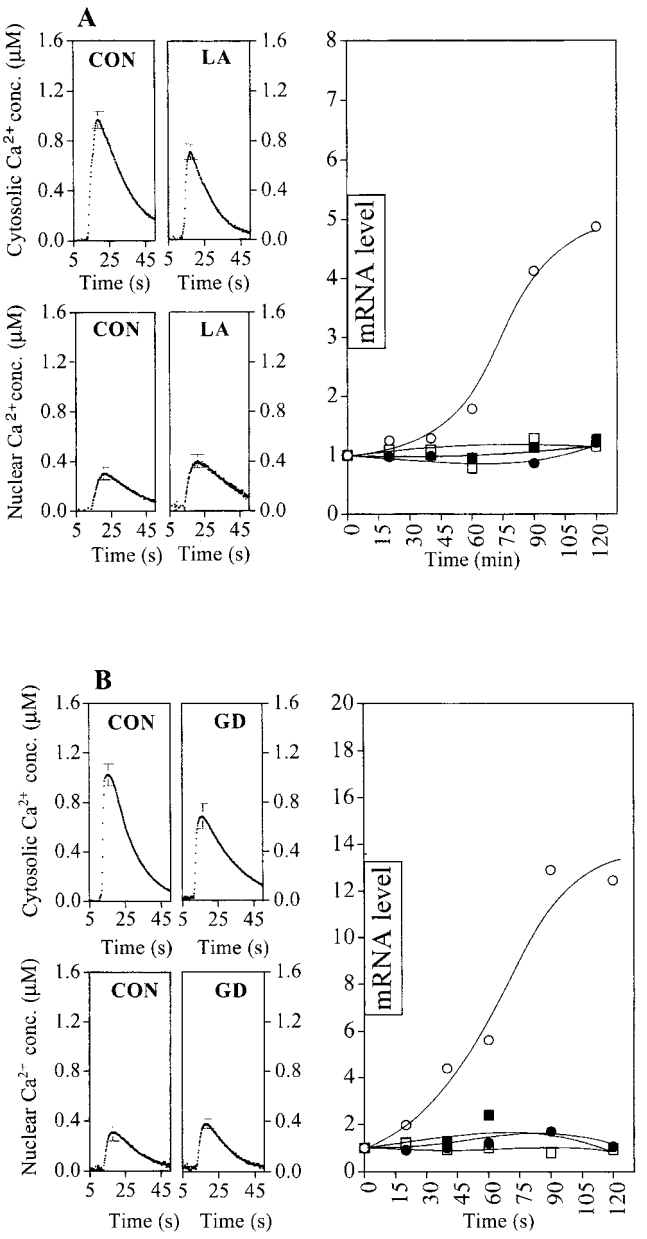


Figure 6. The effect of Ca^{2+} modulators on cold shock-induced changes in cytosolic and nuclear Ca^{2+} and *NpCaM-1* and *NpCaM-2* mRNA accumulation. Cold shock stimulation was applied by a 1-mL injection of ice-cold water at $t = 10$ s. The SE for the peak values from eight experiments is indicated for the mean peak. A, The effect of 10 mM $LaCl_3$ (LA). ○, CON *NpCaM-1*; ●, CON *NpCaM-2*; □, LA *NpCaM-1*; ■, LA *NpCaM-2*; B, 20 mM $GdCl_3$ (GD); $MgCl_2$ concentrations of identical ionic strength were used as a control (CON). ○, CON *NpCaM-1*; ●, CON *NpCaM-2*; □, GD *NpCaM-1*; ■, GD *NpCaM-2*.

This difference in kinetics was not due to fusion to nucleoplasmin, as aequorin in the cytoplasm and nucleoplasm reported identical Ca^{2+} values (Badminton et al., 1998). Wind signals induced $[Ca^{2+}]_{cyt}$ (MAQ 2.4) to peak at 0.3 s, while the MAQ 7.11 peaked later at 0.6 s. The quick response of the nuclear and cytoplasmic signals to wind stimulation probably resulted in part from the speed with which the mechanical signal is perceived. Wind induced a slight movement of the seedling around the hypocotyl/root junction that lasted 0.02 to 0.3 s. In animal cells $[Ca^{2+}]_{nuc}$ usually peaks later than $[Ca^{2+}]_{cyt}$, and the peak height is lower (Badminton et al., 1995, 1996, 1998). With cold shock stimulation, in which seedlings were irrigated with ice-cold water, MAQ 2.4 peaked at 4 to 5 s but MAQ 7.11 peaked at 9 s. Furthermore, MAQ 7.11 Ca^{2+} transients peaked at a substantially lower $[Ca^{2+}]$ than MAQ 2.4 (Fig. 4) in both cases.

Unpublished evidence using MAQ 2.4 and MAQ 7.11 supports the apparent independence of the Ca^{2+} response in the different compartments. Heat shock treatments induce $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ transients, which are separated by minutes (M. Gong, A.H. van der Luit, and A.J. Trewavas, unpublished observations). While we could detect circadian variations in $[Ca^{2+}]_{cyt}$ in MAQ 2.4, we could not detect them in MAQ 7.11 (N.T. Wood, A. Haley, M. Moussaid, A.H. van der Luit, and A.J. Trewavas, unpublished data). There is therefore some autonomy in nuclear Ca^{2+} signaling in plant cells, much as there seems to be in animal cells.

There is an ongoing debate as to the extent to which the nucleus regulates $[Ca^{2+}]_{nuc}$ (Carafoli et al., 1997; Malviya and Rogue, 1998). A common view is that alterations in $[Ca^{2+}]_{cyt}$ are the basic element in Ca^{2+} signaling and that they pass through the nuclear membrane, albeit in an attenuated and later form; in this case the nucleus is not thought to independently regulate $[Ca^{2+}]_{nuc}$. The alternative view regards the nuclear envelope and associated endoplasmic reticulum as an intracellular store of Ca^{2+} able to respond to signals independently of cytoplasmic changes. This latter view does not preclude parallel changes in $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{cyt}$. Meyer et al. (1995) suggested that if Ca^{2+} signals in the cytoplasm and nucleus differ from each other in kinetics by at least 1 s, then the nuclear membrane is a substantial barrier to Ca^{2+} movement from the cytoplasm, greatly increasing the likelihood of separate regulation of nuclear Ca^{2+}. In the case of cold shock at least, the nuclear membrane may act as a significant barrier to Ca^{2+} movement, because there is a 4-s difference between the peak values of MAQ 2.4 and MAQ 7.11.

Distinct Ca^{2+} Signaling Pathways Regulate Calmodulin Gene Expression in Tobacco

There is definite evidence that the flow of Ca^{2+} resulting from activation of different receptors regulates different pathways of gene expression (Bading et al., 1993; Finkbeiner and Greenberg, 1997), presumably through spatial separation of the pathways themselves. Hardingham et al. (1997) microinjected dextran-linked BAPTA into nuclei and concluded that some signals require a pathway through $[Ca^{2+}]_{cyt}$, while others involve $[Ca^{2+}]_{nuc}$. This technology is not yet currently feasible with cells in tobacco seedlings. In the experiments described in this paper for wind signals, some component of the signaling pathways controlling *NpCaM-1* expression could clearly be through $[Ca^{2+}]_{nuc}$. Prior treatment with BAPTA-AM inhibited the nuclear Ca^{2+} signal, leaving the cytosolic Ca^{2+} signal unaffected,

ruthenium red greatly reduced the cytoplasmic signal without influencing that in the nucleus, while treatment with thapsigargin increased the subsequent nuclear signal without influencing the subsequent cytoplasmic signal. Variations in the accumulation of *NpCaM-1* mRNA as a result of inhibitor treatments were correlated with $[Ca^{2+}]_{nuc}$ but not with $[Ca^{2+}]_{cyt}$. Selective inhibition of the cold-shock-induced cytosolic Ca^{2+} signal by lanthanum and gadolinium chlorides, indicative of a cytosolic pathway for regulation of *NpCaM-1* calmodulin gene expression, helps confirm the spatial separation of signaling pathways between wind and cold shock stimuli.

This apparent spatial distribution of signaling pathways may be further complicated by clear evidence that different downstream events are switched on at different stages of the Ca^{2+} transient (Dolmetsch et al., 1997) and by a requirement that cytoplasmic signaling must take place near the plasma membrane. This latter observation of Finkbeiner and Greenberg (1997) might explain why reductions of about 40% in the cold-shock-dependent cytoplasmic Ca^{2+} signal nevertheless completely blocks *NpCaM-1* mRNA accumulation. Based on previously reported effects of neomycin, we suspect that only part of the cold-shock-induced cytosolic signal originates with increased Ca^{2+} flux through the plasma membrane, with the remainder being released from internal stores by $InsP_3$ (Knight et al., 1996). The reduction of 40% might then disguise a quantitatively greater inhibition of Ca^{2+} flux through the plasma membrane by the lanthanides, the cellular region critical perhaps to switching on the cytosolic pathway leading to *NpCaM-1* transcription.

By generating artificial Ca^{2+} transients, Dolmetsch et al. (1997) implicated early events in the rise time, peak value, and duration of the decay back to resting levels as controlling different transduction processes, including changes in gene expression. It is for this reason that we included measurements of rise time, peak Ca^{2+} values, decay times, and resting values where relevant for the data in Figures 4 to 6. However, in tobacco seedlings the kinetics of the Ca^{2+} transient seemed to be directly determined by the nature of the original signal. A wind signal induced a transient lasting some 20 s but reaching a peak within less than 0.5 s. Cold shock induced a transient lasting some 40 s and reaching a peak within 5 to 9 s. Both signals induced *NpCaM-1* mRNA accumulation, although cold shock accumulations were higher than those of wind induction. Even when inhibitors are used, there is little alteration to the overall kinetics except in the peak height. There is a slight lengthening of about 5 s of the transient with thapsigargin; only more detailed studies directly modifying Ca^{2+} transients will determine whether this is a significant change. Certainly at present for the *NpCaM-1* gene, peak height seems to be the more critical factor determining final mRNA accumulation.

At present, two possible ways can be proposed in which $[Ca^{2+}]_{nuc}$ exerts transcriptional regulation. The first may operate through Ca^{2+} or Ca^{2+}-sensitive protein kinases located in the nucleus. As reported many years ago (Trewavas, 1979; Melanson and Trewavas, 1981), plant nuclei contain protein kinase activity and changes in specific phosphorylation of discrete nuclear proteins during cell development or cell division could be detected using two-dimensional electrophoretic separations. Clearly, plant nuclei have the potential for the regulation of transcription through phosphorylation, although whether there are Ca^{2+} or Ca^{2+}/calmodulin-sensitive protein kinases in the plant nucleus remains to be established.

The second possibility is that there is direct interaction of Ca^{2+}/calmodulin with promoters or particular transcription factors. This mechanism is supported by recent work by Corneliussen et al. (1994), who reported binding of calmodulin to the basic helix-loop-helix domains of several mice basic helix-loop-helix transcription factors that inhibit their DNA binding in vitro, and with those of Szymanski et al. (1996), who reported that calmodulin isoforms enhance the binding of TGA3 to the Arabidopsis *CaM-3* promoter.

The mechanism whereby wind signals can apparently selectively modify nuclear Ca^{2+} requires further investigation. Nuclei are surrounded by a basket of microfilaments. Distortion of these microfilamentous structures is thought to be one of the major means by which plant cells sense mechanical signals (Trewavas and Knight, 1994). In addition, Ca^{2+} channels localized to nuclei of amphibian epithelial cells (Prat and Cantiello, 1996) have been shown to be associated with actin filaments. Equivalent channels in tobacco cells might regulate nuclear Ca^{2+} levels in plant cells after wind stimulation.

ACKNOWLEDGMENTS

We would like to thank Dr. M. Badminton for the nucleoplasmin-aequorin construct, Dr. T. Collins for his assistance with the Polyvar epifluorescence microscope, and John Findlay for his assistance with the electron microscope.

Received May 16, 1999; accepted July 20, 1999.

LITERATURE CITED

Allen DG, Blinks JR, Prendergast FG (1977) Aequorin luminescence: relation of light emission to calcium concentration: a calcium-independent component. Science **195:** 996–998

Bachs O, Agell N, Carafoli E (1992) Calcium and calmodulin function in the cell nucleus. Biochim Biophys Acta **1113:** 259–270

Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurones by distinct calcium signaling pathways. Science **260:** 181–186

Badminton MN, Campbell AK, Rembold CM (1996) Differential regulation of nuclear and cytosolic Ca^{2+} in HeLa cells. J Biol Chem **271:** 31210–31214

Badminton MN, Kendall JM, Rembold CM, Campbell AK (1998) Current evidence suggests independent regulation of nuclear calcium. Cell Calcium **23:** 79–86

Badminton MN, Kendall JM, Sala-Newby G, Campbell AK (1995) Nucleoplasmin-targeted aequorin provides evidence for a nuclear calcium barrier. Exp Cell Res **216:** 236–243

Biro RL, Daye S, Serlin BS, Terry ME, Datta N, Sopory SK, Roux SJ (1984) Characterization of oat calmodulin and radioimmunoassay of its subcellular distribution. Plant Physiol **75:** 382–386

Braam J (1992) Regulated expression of the calmodulin-related TCH genes in cultured *Arabidopsis* cells: induction by calcium and heat shock. Proc Natl Acad Sci USA **89:** 3213–3216

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60:** 357–364

Carafoli E, Nicotera P, Santella L (1997) Calcium signaling in the cell nucleus: a symposium report. Cell Calcium **22:** 313–319

Collinge M, Trewavas AJ (1989) The location of calmodulin in the pea plasma membrane. J Biol Chem **264:** 8865–8872

Corneliussen B, Holm M, Waltersson Y, Onions J, Hallberg B, Thornell A, Grundström T (1994) Calcium/calmodulin inhibition of basic helix-loop-helix transcription factor domains. Nature **368:** 760–764

Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. Nature **386:** 855–858

Draper J, Scott R, Armitage P, Walden R (1988) Plant Genetic Transformation and Gene Expression: A Laboratory Manual. Blackwell Scientific Publications, Oxford

Finkbeiner S, Greenberg ME (1997) Spatial features of calcium-regulated gene expression. Bioessays **19:** 657–660

Gannon MN, McEwen BS (1994) Distribution and regulation of calmodulin mRNAs in rat brain. Mol Brain Res **22:** 186–192

Gilchrist JSC, Czubryt MP, Pierce GN (1994) Calcium and calcium-binding proteins in the nucleus. Mol Cell Biochem **135:** 79–88

Gilroy S, Bethke PC, Jones RL (1993) Calcium homeostasis in plants. J Cell Sci **106:** 453–462

Gong M, van der Luit AH, Knight MR, Trewavas AJ (1998) Heat-shock-induced changes of intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. Plant Physiol **116:** 429–437

Greber UF, Gerace L (1995) Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus. J Cell Biol **128:** 5–14

Haley A, Russell AJ, Wood N, Allan AC, Knight MR, Campbell AK, Trewavas AJ (1995) Effects of mechanical signaling on plant cell cytosolic calcium. Proc Natl Acad Sci USA **92:** 4124–4128

Hardingham GE, Chawla S, Johnson CM, Bading H (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. Nature **385:** 260–265

Hepler PK, Wayne RO (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439

Jena PK, Reddy ASN, Poovaiah BW (1989) Molecular cloning and sequencing of a cDNA for plant calmodulin: signal-induced changes in the expression of calmodulin. Proc Natl Acad Sci USA **86:** 3644–3648

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas AJ (1995) Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science **269:** 1863–1865

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol **27:** 137A–138A

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489–503

Knight H, Trewavas AJ, Knight MR (1997) Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. Plant J **12:** 1067–1078

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effect of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524–526

Knight MR, Read ND, Campbell AK, Trewavas AJ (1993) Imaging calcium dynamics in living plants using semi-synthetic recombinant aequorins. J Cell Biol **121:** 83–90

Knight MR, Smith SM, Trewavas AJ (1992) Wind-induced plant motion immediately increases cytosolic calcium. Proc Natl Acad Sci USA **89:** 4967–4971

Kocsis JD, Rand MN, Lankford KL, Waxman SG (1994) Intracellular calcium mobilization and neurite outgrowth in mammalian neurones. J Neurobiol **25:** 252–264

Ling V, Perera I, Zielinski RE (1991) Primary structures of *Arabidopsis* calmodulin isoforms deduced from the sequences of cDNA clones. Plant Physiol **96:** 1196–1202

López-Gómez R, Gómez-Lim MA (1992) A method for extracting intact RNA from fruits rich in polysaccharides using ripe Mango mesocarp. Hortic Sci **27:** 440–442

Malviya AN, Rogue PJ (1998) "Tell me where is calcium bred": clarifying the roles of nuclear calcium. Cell **92:** 17–23

Melanson D, Trewavas AJ (1981) Changes in tissue protein pattern in relation to auxin induction of DNA synthesis. Plant Cell Environ **5:** 53–64

Meyer T, Allbritton NL, Oancea E (1995) Regulation of nuclear calcium concentration. *In* GR Bock, K Ackrill, eds, Calcium Waves, Gradients and Oscillations. Wiley Press, Chichester, UK, pp 252–262

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol **15:** 473–497

Perera IY, Zielinski RE (1992) Structure and expression of the *Arabidopsis* CaM-3 calmodulin gene. Plant Mol Biol **19:** 649–664

Philpott A, Leno GH (1992) Nucleoplasmin remodels sperm chromatin in Xenopus egg extracts. Cell **69:** 759–767

Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res **14:** 5857–5868

Poovaiah BW, Reddy ASN (1993) Calcium and signal transduction in plants. Crit Rev Plant Sci **12:** 185–211

Prat AG, Cantiello HF (1996) Nuclear ion channel activity is regulated by actin filaments. Am J Physiol **270:** C1532–C1543

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shimoda K, Ikeshima H, Matsuo K, Hata J, Maejima K, Takano T (1995) Spatial and temporal regulation of the rat calmodulin gene-III directed by a 877-base promoter and 103-base leader segment in the mature and embryonal central-nervous-system of transgenic mice. Mol Brain Res **31:** 61–70

Shimomura O, Musicki B, Kishi Y, Inouye S (1993) Light-emitting properties of recombinant semi-synthetic aequorins and recombinant fluorescein-conjugated aequorin for measuring cellular calcium. Cell Calcium **14:** 373–378

Solà C, Tusell JM, Serratosa J (1996) Comparative study of the pattern of expression of calmodulin messenger RNAs in the mouse brain. Neuroscience **75:** 245–256

Szymanski DB, Liao B, Zielinski RE (1996) Calmodulin isoforms differentially enhance the binding of cauliflower nuclear proteins and recombinant TGA3 to a region derived from the Arabidopsis Cam-3 promoter. Plant Cell **8:** 1069–1077

Takahashi K, Isobe M, Knight MR, Trewavas AJ, Muto S (1997) Hypoosmotic shock induces increases in cytosolic Ca^{2+} in tobacco suspension-culture cells. Plant Physiol **113:** 587–594

Takezawa D, Liu ZH, An G, Poovaiah BW (1995) Calmodulin gene family in potato: developmental and touch-induced expression of the mRNA encoding a novel isoform. Plant Mol Biol **27:** 693–703

Trewavas AJ (1979) Phosphorylated nuclear proteins in germinating cereal embryos and their relationship to messenger RNA synthesis. *In* LD Laidman, RG Wyn Jones, eds, Recent Advances in the Biochemistry of Cereals. Academic Press, New York, pp 175–208

Trewavas AJ, Knight MR (1994) Mechanical signaling, calcium and plant form. Plant Mol Biol **26:** 1329–1341

Watillon B, Kettmann R, Boxus P, Burny A (1992) Cloning and characterization of an apple (*Malus domestica* L. Borkh.) calmodulin gene. Plant Sci **82:** 201–212

Yang TB, Segal G, Abbo S, Feldman M, Fromm H (1996) Characterization of the calmodulin gene family in wheat: structure, chromosomal location, and evolutionary aspects. Mol Gen Genet **252:** 684–694

Zimprich F, Torok K, Bolsover SR (1995) Nuclear calmodulin responds rapidly to calcium influx at the plasmalemma. Cell Calcium **17:** 233–238