The Regulation of Calmodulin Gene Expression by Nuclear Calcium in Plants

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钙调素

("kai tiao suo" is calmodulin in Chinese; "kai" means calcium)

Declaration

I hereby declare that the work presented is my own and has not been submitted in any form for any degree at this or any other university.

Arnold H. van der Luit.

Preface

During this project I have had the support of many people whom I would like to acknowledge. In 1993, Tony Trewavas gave me the opportunity to do a training period in his laboratory. I had become interested in this field after writing a literature survey on the role of calcium in signal transduction pathways in plants. The reason to come to his laboratory was the brilliant technique that he and Marc Knight had developed for measuring changes in calcium concentrations inside intact living plants using transgenic aequorin. I was, hence delighted that I could start a Ph.D. on this topic in 1994. Both Tony and Marc provided me with a lot of support during the course of the project. Others in the lab gave me additional advice and friendship; Ming Gong came in 1995 for 2 month and together we carried out a lot of heat shock measurements, furthermore, he was a very inspiring student when I taught him molecular biology techniques in 1997; Claudio Olivari made the nucleoplasmin construct during his stay in 1993 and kindly left me the transgenic plants; John Love gave his friendship and advice on various matters that were very important; Mario Roccaro was there for the vital coffee breaks, Paola Perrone was there at 12 o'clock to go up for lunch, Jason gave me advice on the difficult mathematical problems; Richard Smith was a good friend by going with me for weights, beer and kebab lifting. A very special thank goes out to Jan Dijksterhuis, who gave very useful advice while writing this thesis. Finally I would like to thank, Gidi Baum, Tony Collins, John Findley, Ann Haley, Rui Mahló, Martine Moussaid, Ian Oliver, and all the others at the Institute of Cell and Molecular Biology for their help and friendship that made my stay in Edinburgh unforgettable.

Then there are the people who operated from behind the scenes; my parents, who gave me the means to study, my brother and my sister who gave me encouragement during my student time and close friends in Holland; Marcel, the "silence" of Amsterdam, Liesbeth, P.J., Elske, and Martien with his family in San Francisco, who supported me by electronic mail. Finally, I want to mention somebody who was there from the very start, has supported me all the way through and listened to the many calcium stories at weekends, Virginie.

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Abbreviations

A23187	Compound A23187.
ABA	Abscisic acid.
AMPS	Ammonium persulfate.
Ann.	Annealing.
AtCaM	Calmodulin from *Arabidopsis.*
At-IMPα	Importin from *Arabidopsis.*
ATP	Adenosine triphosphate.
ATPase	Adenosine triphosphatase.
BA	Cytokinine.
BAPTA	1,2 bis-(*o*-Aminophenoxy)ethane-N,N,N',N' tetraacetic acid.
BAPTA-AM	1,2 bis-(*o*-Aminophenoxy)ethane-N,N,N',N' tetraacetic acid tetra(acetoxymethyl) ester.
bHLH	Basic helix-loop-helix.
BSA	Bovine serum albumine.
cAMP	Adenosine 3',5'-cyclic monophosphate.
$[Ca^{2+}]_{cyt}$	Cytosolic calcium concentration.
$[Ca^{2+}]_{int}$	Intracellular calcium concentration.
$[Ca^{2+}]_{nuc}$	Nuclear calcium concentration.
Ca^{2+}-CaM	Calcium calmodulin complex or activated calmodulin.
CaM	Calmodulin.
cDNA	Complementary DNA.
CDPK	Calcium-dependent protein kinase.
5' cap	m(7)GpppNNN (with N = any nucleotide, p = phosphate group, m = methyl group).
cas	Cold-acclimation specific.
CaMV	Cauliflower mosaic virus.
Da	Daltons.
DAG	Diacylglycerol.
DAPI	Diamidino-2-phenylindole.
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide.
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleoside triphosphate.
DTT	Dithiothreitol.
EcoRI	EcoRI restriction endonuclease.
EDTA	Ethylenediamine tetraacetic acid.
EGTA	Ethyleneglycol-*bis*(ß-aminoethyl)-N,N,N',N'-tetraacetic acid.

EMBL	European Molecular Biology Library.
et al.	Et alii or, and others.
FITC	Fluorescein isothiocyanate.
g	Gravity ($9.81\ m{\cdot}s^2$).
GA	Gibberellic acid.
GFP	Green fluorescent protein.
GUS	ß-glucuronidase.
HMG	High mobility group.
HS	Heat shock.
HSE	Heat shock element.
HSP	Heat shock protein.
HSTF	Heat shock transcription factor.
IgG	Immunoglobulin G.
IP_3	Inositol 1,4,5-trisphosphate.
IPTG	Isopropyl ß-D-thiogalactoside.
MAPK	Mitogen-activated protein kinase.
MES	2-[N-morpholino]ethanesulfonic acid.
mRNA	Messenger RNA.
Mol.	Molecular.
MOPS	3-[N-morpholino]propanesulfonic acid.
NAA	1-naphthylacetic acid.
NLS	Nuclear localisation signal.
NPC	Nuclear pore complex.
NpCaM	Calmodulin from *Nicotiana plumbaginifolia*.
OD	Optical density.
PBS	Phosphate-buffered saline.
PCaM	Calmodulin from *Solanum tuberosum*.
^{32}P-dCTP	^{32}Phosphate-labelled 2'-deoxycytidine triphosphate.
PCR	Polymerase chain reaction.
pers. commun.	Personal communication.
pfu	*Pyrococcus furiosus*
PIP_2	Phosphatidyl inositol 4,5-bisphosphate.
3' poly (A)	Polyadenylate tail at the 3' end.
PVP-40	Polyvinylpolyprorrolidone.
RACE	Rapid Amplification of cDNA Ends.
RNA	Ribonucleic acid.
rRNA	Ribosomal RNA.
RuBisCo, *rbcS*	Ribulose 1,5'-bisphosphate carboxylase.

SalI	SalI restriction endonuclease.
SDS	Sodium dodecyl sulfate.
SmaI	SmaI restriction endonuclease.
SV40	Simian virus 40.
Taq	*Thermus aquaticus.*
TCH	Touch.
T_Δ	Time signal remains elevated over resting levels.
TEMED	N,N,N',N'-tetramethylethyleneamine.
TGA	Tobacco transcription activator.
T_p	Time signal takes to reach maximal levels.
UTR	Untranslated region.
UV	Ultra-Violet.
v/v	Volume per volume.
wk	Week.
w/v	Weigth per volume.
XbaI	XbaI restriction endonuclease.
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YT	Yeast extract.

Amino acids in one letter code:

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

Nucleotides in one letter code:

A	adenine	G	guanine
C	cytosine	T	thymine
N	adenosine, cytosine, guanine, or thymine	V	adenosine, cytosine, or guanine

Abstract

Plants are constantly subjected to environmental changes, e.g. mechanical forces and temperature fluctuations which they perceive through signal transduction pathways. DNA constructs for Agrobacterium-mediated transformation into *Nicotiana plumbaginifolia* were prepared. The Ca^{2+}-sensitive luminescent protein, aequorin was placed in frame with the nuclear localisation signal (NLS) peptide of the simian virus SV40 Large T-antigen, *Xenopus laevis'* nucleoplasmin, and high mobility group protein, HMG1 from *Pisum sativum*. After analysing individual transformants, nucleoplasmin was shown to successfully target aequorin to nuclei. These plants, together with plants transgenic for cytosolic aequorin were used to study Ca^{2+} dynamics when exposed to the environmental stimuli, wind and cold shock. Wind induced immediate Ca^{2+} transients in the cytoplasm and nucleus, while cold shock induced a Ca^{2+} transient in the cytoplasm that was followed by a delayed Ca^{2+} transient in the nucleus. Wind and cold shock induced calmodulin gene expression that followed distinct kinetics. Rapid Amplification of cDNA Ends (3' RACE) identified the presence of two calmodulin transcripts in tobacco seedlings; *NpCaM-1* and *NpCaM-2* of which only *NpCaM-1* was induced by both stimuli. The transcripts comprise two different nucleotide sequences but encode identical polypeptides. The expression kinetics of *NpCaM-1* was related to wind and cold shock-induced nuclear and cytosolic Ca^{2+} changes by using Ca^{2+} agonists/antagonists. Wind-induced nuclear Ca^{2+} changes correlated closely to the subsequent expression and accumulation of *NpCaM-1* while changes in cytoplasmic Ca^{2+} levels did not. Cold shock, on the other hand, did not reveal this correlation, and a role for intracellular Ca^{2+} levels during subsequent expression of *NpCaM-1* remains therefore speculative. Heat shock induced a prolonged but transient increase in cytoplasmic Ca^{2+} level that was followed by a delayed prolonged increase in nuclear Ca^{2+} level. Seedlings pretreated with Ca^{2+} or EGTA showed respectively enhanced or diminished subsequent thermotolerance, therefore it was suggested that these increases in Ca^{2+} levels were required for the acquisition of heat-induced thermotolerance.

Chapter 1

Introduction

Plants grow in almost every environment in which they perceive signals and on which they respond adequately to survive. Most plants are sessile and need to adapt to any sudden change in the habitat. These environmental fluctuations can be mechanical in nature, including wind and rain, or abrupt changes in temperature. Plants have developed sensory mechanisms to detect these changes and growth mechanisms to respond. Ways to transduce this information into cellular responses are known as signal transduction pathways which can be mediated by calcium and cAMP as second messengers or directly through phosphorylation of proteins. These will alter growth and developmental patterns of the plant and allow it to adapt to the environment. Calcium has been found to be involved in a wide variety of signal transduction pathways, however, the precise function of calcium in specifying a cellular response remains elusive.

1.1   Calcium as Second Messenger in Plants

Plants require a constant supply of calcium (Ca^{2+}) in the range of 1-10 mM corresponding to 3-15 $g \cdot kg^{-1}$ (Clarkson and Hanson, 1980; Larcher, 1995) to maintain growth and development. Calcium uptake by roots leads to millimolar concentration of Ca^{2+} and in most plants calcium is arrayed as the second cation in abundancy and the sixth element, after carbon, hydrogen, oxygen, nitrogen and potassium (Epstein, 1972). Calcium is sequestered in the apoplast and in calcium storage organelles, such as the vacuole and the endoplasmic reticulum. The relationship between the apoplastic and the symplastic calcium pool is poorly understood. The apoplast and several cellular organelles, are thought to sustain Ca^{2+} levels at millimolar concentrations, however, the cytoplasm maintains a Ca^{2+} level four orders of magnitude lower (sub-micromolar, Hepler and Wayne, 1985). The maintenance of this low concentration of cytoplasmic calcium, $[Ca^{2+}]_{cyt}$, leads to a very large electrochemical gradient of Ca^{2+}. A minor change in membrane permeability for Ca^{2+} will induce a very fast and significant increase in $[Ca^{2+}]_{cyt}$. Under normal conditions the $[Ca^{2+}]_{cyt}$ is kept low by the action of Ca^{2+}/H^{+}-antiport systems and Ca^{2+}-ATPases to prevent lethal precipitation of calcium phosphate (Weber, 1976). Additionally, the significant buffer capacity of the cytoplasm mediated by Ca^{2+}-binding proteins, ensure an ion concentration of Ca^{2+} at sub-micromolar levels and results in a remarkable low mobility of free calcium

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(Hodgkin and Keynes, 1957, Rose and Loewenstein, 1976, Allbritton et al., 1992). Characteristics listed above makes calcium a good candidate to act as a signal transducer molecule in higher organisms.

The exploration towards a fully understanding of the mechanisms that underlie the transduction of environmental changes into cellular responses has become one of the most fascinating and progressing aspects of current plant physiology. Thus far our knowledge is fragmented and many questions remain unanswered (Trewavas and Mahló, 1997). It is well established that different environmental factors, also known as the primary signals, induce changes in the level of second messengers. Calcium has been shown to meet all the criteria to act as a second messenger (Jaffe, 1980). First, agonist-induced stimulation should be accompanied by a change in $[Ca^{2+}]_{cyt}$. Second, inhibition of changes in $[Ca^{2+}]_{cyt}$ should inhibit the presumed effect. Third, an artificial increase in $[Ca^{2+}]_{cyt}$ should stimulate the event in the absence of the normal agonist. Because of its cytotoxicity one could dispute calcium acting as second messenger. One plausible key to this controversy is the need of proteins to bind the messenger tightly and specifically. Other cations and monovalent anions appear unsuitable for this purpose since they normally form only loose complexes with proteins, due to their large ionic radii and low charge (Williams, 1976).

The large electrochemical force on Ca^{2+} allows rapid fluctuations in $[Ca^{2+}]_{cyt}$ upon a change in membrane permeability and its low mobility allows significant local fluctuations. In addition, a suitable ionic radius and charge for specific binding to proteins, could have led to the evolutionary development of calcium as second messenger.

1.2   Calcium and Signal Transduction

Extensive information from animal systems indicates that the primary signal perceived on the surface of an individual cell is transmitted into the metabolic machinery of the cell by at least two major signal transduction pathways. One employs the second messenger cAMP (Rasmussen and Barrett, 1984), whose role in plant cells is not fully established, and another employs a combination of second messengers that include Ca^{2+}, inositol trisphosphate (IP$_3$), and diacylglycerol (DAG) (Berridge and Irvine, 1984; Fig. 1.1). An increase in $[Ca^{2+}]_{cyt}$ occurs either indirectly through hydrolysis of membrane phosphoinositides or directly through activation of Ca^{2+}-channels located at membranes of the plasma or certain organelles resulting in a complex network called signal transduction (Trewavas and Gilroy, 1991).

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Post-translational modification of the amino acids, serine, threonine, and tyrosine by proteins kinases and phosphatases is another major transduction route for many signals. These modifications afford the potential for extensive interaction and "cross-talking" among transduction pathways (Bourne, 1995). To date >70 plant protein kinases that regulate many different aspects of metabolism, growth, and division have been isolated and characterised (Stone and Walker, 1995). One group comprises the MAPK cascade that regulates cell proliferation (Jacobs, 1997) and another class of protein kinases of the CDPK family (Harper et al., 1991) found to be attached to microfilaments and to the plasma membrane. Several protein phosphatases have been detected in plant cells, and phosphatase inhibitors can have striking effects on growth and development, possibly through the inhibition of protein kinase cascades (Stone and Walker, 1995).
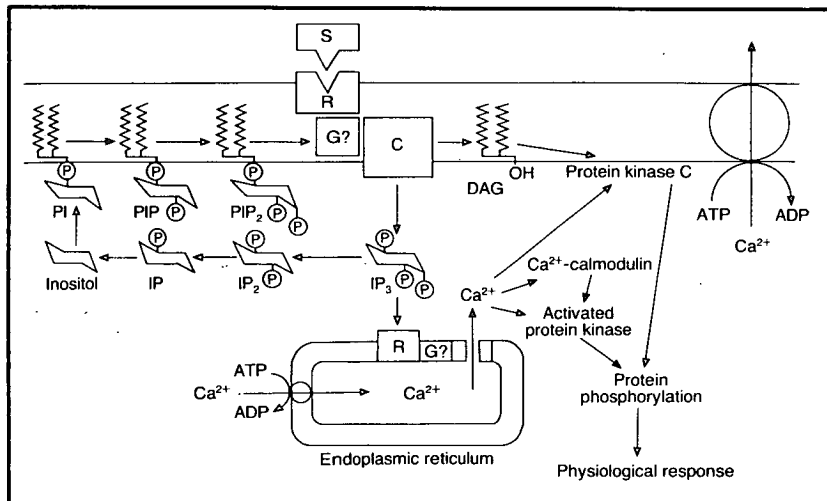
Figure 1.1: A summary of events which takes place when a stimulus (S) is perceived by a receptor (R) at the plasma membrane. The signal is believed to be transduced by a G-protein (G) to phospholipase C (C). Phospholipase C hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). DAG activates protein kinase C which phosphorylates specific proteins. IP$_3$ binds to its receptor in membrane of the endoplasmic reticulum in animals or vacuole in plants to release calcium. Elevated [Ca^{2+}]$_{cyt}$ activate protein kinases either directly or indirectly through calmodulin. Ca^{2+}-ATPase in the plasma membrane and in the membrane of the endoplasmic reticulum lower the Ca^{2+} concentration to steady state levels to prevent cytotoxic events (Taken from Trewavas and Gilroy, 1991).

1.3 Calcium and Plant Development

Environmental and hormonal stimuli affect many aspects of growth and developmental processes in plants. Receptors that perceive these primary signals remain largely unknown and are not discussed here.

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In the last two decades substantial progress has been made towards the understanding of the mechanisms underlying the translation of these primary signals, by means of second messengers, into cellular responses. Changes in $[Ca^{2+}]_{cyt}$ have been observed when cells were exposed to endogenous stimuli, such as plant hormones, as well as to exogenous stimuli, such as light, heat, salt, elicitors, and touch (Table 1.1). Such Ca^{2+} signals have complex temporal arrangements and at least three basic spatio-temporal patterns can be discerned. First, transient and apparently large increases occur in response to cold shock, mechanical stimulation, hypoosmotic shock, abscisic acid (ABA) and elicitors. Second, sustained, apparently modest increases occur in response to gibberellic acid, cytokinins, and light. Third, oscillatory changes with regular or irregular periods occur in response to auxin. This suggests that distinct temporal patterns in the Ca^{2+} signal modulate the response with information encoded in the amplitude, frequency, shape and spatial localisation of the Ca^{2+} signal. Indeed, ratio and confocal imaging have revealed that changes in Ca^{2+} levels are often highly temporal and spatially localised, implying that different parts of the cytoplasm may be regulated differently in response to a stimulus. At present it remains to be established whether these distinct dynamics of Ca^{2+} signals play a part in specifying a cellular response in plants.

Table 1.1: *Summary of stimulus-induced changes in $[Ca^{2+}]_{cyt}$ in plant cells.*
(1) Kauss and Jeblick, 1991; (2) Knight et al., 1991; (3) Shacklock et al., 1990; (4) Miller and Sanders, 1987; (5) Russ et al., 1991; (6) Takahashi et al., 1997; (7) Price et al., 1994; (8) Gilroy et al., 1991; (9) Gehring et al., 1990; (10) Felle, 1988; (11) Bush and Jones, 1987; (12) Gilroy and Jones, 1992; (13) Hahm and Saunders, 1991.

Stimulus	Cell system/type	Ca²⁺-response	origin	Reference
Elicitor	*Catharanthus roseus*	Transient increase	Extracellular	(1)
	Tobacco seedlings			(2)
Cold	Tobacco seedlings	Transient increase	Extracellular	(2)
Mechanical	Tobacco seedlings	Transient increase	Intracellular	(2)
Red light	Wheat protoplasts	Transient increase	Intracellular	(3)
White light	*Nitellopsis*	Sustained decrease		(4)
UV-A	*Mougeotia scalaris*	Sustained increase	Intracellular	(5)
Hypoosmotic	Tobacco cultured cells	Biphasic increase	Extracellular	(6)
Oxidative	Tobacco seedlings	Transient increase	Intracellular	(7)
ABA	*Commelina* Guard cells	Transient increase	Extracellular	(8)
	Corn coleoptiles and roots	Transient increase	N.D.	(9)
	Parsley hypocotyls and roots	Transient increase	N.D.	(9)
Auxin	Corn coleoptiles and roots	Sustained increase	N.D.	(9)
	Parsley hypocotyls and roots	Sustained increase	N.D.	(9)
	Maize epidermal cells	Oscillations	N.D.	(10)
GA	Aleurone cells	Sustained increase	Extracellular	(11,12)
BA	*Funaria*	Sustained increase	Extracellular	(13)

N.D. = Not determined

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A wide variety of cellular activities are induced by fluctuation in $[Ca^{2+}]_{cyt}$. They include cell division, cell growth, phytochrome response, hormone response (Guilfoyle, 1986, Theologis, 1986), somatic embryogenesis (Timmers and Schel, 1992), gravitropism, osmoregulation, fruit ripening, photosynthesis, and plant diseases (Marmé and Dieter, 1983, Hepler and Wayne, 1985, Poovaiah and Reddy, 1987; Saunders, 1990). Ca^{2+} was shown to act as a second messenger to meet the criteria (Jaffe, 1980). Primary stimuli were shown to evoke temporal and spatial fluctuations in $[Ca^{2+}]_{cyt}$ (Table 1.1; Thorn et al., 1993, Cheek, 1989, Bush, 1993) and in the case of stomatal closure, artificial manipulation of $[Ca^{2+}]_{cyt}$, by means of Ca^{2+} release from injected caged probes (Gilroy et al., 1990) results in the predicted cellular response.

1.4   Calcium and Calcium Channels

Energised Ca^{2+} transport maintains low $[Ca^{2+}]_{cyt}$ against a gradient of four orders of magnitude. Ca^{2+} re-enters the cytoplasm by diffusion through protein pores known as channels, which may be specialised for Ca^{2+} or non-selective for ions. For Ca^{2+} to act as messenger, the action of Ca^{2+}-channels must be tightly regulated. Opening of these channels may be controlled by a variety of factors including, membrane potential, ligand binding, covalent modification (causing sensitisation and desensitisation) or stretch activation (Tsien and Tsien, 1990). These factors are integrated in the complex of signal transduction which controls the release of calcium making it suitable to amplify and translate primary signals into cellular responses.

Evidence for the localisation of Ca^{2+}-channels at the membranes of plasma and organelles suggests that both extracellular and internal calcium pools have a role in signal transduction pathways (Johannes et al., 1991; Schroeder and Thuleau, 1991). Each membrane will contain more than one class of Ca^{2+}-channels, each having its own specific opening requirements. Pharmacological properties have provided a mean to distinguish between action of specific Ca^{2+}-channels. Ca^{2+}-channels identified at the plasma membrane of *Charophyte* algae, were shown to be inhibited by the 1,4-dihydropyridine, nifedipine, but to be insensitive to phenylalkylamines (Shina and Tazama, 1987). Although distribution of the 1,4-dihydropyridine-sensitive Ca^{2+}-channel in higher plants is unknown, both 1,4-dihydropyridine-derived Ca^{2+}-channel agonists and antagonists have been reported to interfere with cytokinin-induced budding in the moss *Funaria* (Conrad and Hepler, 1981) in which membrane-associated Ca^{2+} increase was induced (Saunders and Hepler, 1981). A second class of plasma membrane Ca^{2+}-channels shown to be inhibited by phenylalkalines (e.g.

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verapamil) but not by 1,4-dihydropyridine were identified in carrot root protoplasts (Graziana et al., 1988).

A search for Ca^{2+}-channels located at membranes of organelles using patch-clamp technology, showed the presence of a different class of gating mechanisms. Alexandre et al. (1990) identified a Ca^{2+}-channel located at the tonoplast of red beet whose aperture was regulated by IP_3. IP_3 shown to induce elevations of $[Ca^{2+}]_{cyt}$ in animal cells (Berridge and Irvine, 1989), was shown to participate in signal transduction pathways in plants (Gilroy et al., 1990). Besides occupying 80-90% of cells' volume, the vacuole has an important role as a storage organelle. The identification an IP_3-gated Ca^{2+}-channel and the discovery of a IP_3-insensitive voltage-dependent Ca^{2+}-channels (Johannes et al., 1992) located at the tonoplast proves the importance of Ca^{2+} pool of the vacuole.

Stretch-activated ion-channels were first recognised in endothelial cells which were permeable to Ca^{2+}. This channel possibly acts as a mechanosenser to link hemodynamic stresses to endothelial secretion of factors that affect the vascular tone. Stretch-activated Ca^{2+}-permeable channels may also be important in smooth muscle (Kirber et al., 1988), skeletal muscle (Franco and Lansman, 1990), and osteoclasts (Wiltink et al., 1995). The presence of mechanosensitive Ca^{2+}-channels in plants was shown using patch-clamp techniques on epidermal cells of sweet red onion (*Allium cepa*; Pickard and Ding, 1993, Ding and Pickard, 1993, Pickard, 1994). It was concluded that *in vivo* these channels are mechanosensory and mechanoregulatory. Opening time of the channels was shown to depend on cytosolic magnesium concentration, $[Mg^{2+}]_{cyt}$, transmembrane voltage, temperature and was inhibited by lanthanum and gadolinium (Ding and Pickard, 1993). Mechanosensitive Ca^{2+}-channels could serve to transduce shear forces generated in the integrated wall-membrane-cytoskeleton system during turgor changes and cell expansion as well as transducing the stresses induced by gravity, touch and flexure.

The indications for Ca^{2+}-channels at the nuclear envelope in mammalian cells (Mazzanti et al., 1990; Bustamante et al., 1995), explained earlier reports on membrane potentials across the nuclear envelope (Loewenstein and Kanno, 1962; 1963). Recently, Ca^{2+}-channels localised to the nucleus of amphibian epithelial cells were shown to be associated with actin filaments (Prat and Cantiello, 1996). Like in mammalian cells, plant 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). Microfilament-like structures that interact with stretch-activated Ca^{2+}-channels in the endoplasmic

reticulum and at the nuclear envelope could affect calcium levels in the cytoplasm, in regions close to the nucleus and in the nucleoplasm.

1.5 Regulation of Nuclear Calcium

In mammalian cells numerous studies have emphasised the importance of intracellular Ca^{2+} levels in nuclear based events such as the induction and regulation of gene transcription (Morgan and Curran, 1986; Prywes and Roeder, 1986), cell cycle (Hesketh et al., 1985; Poenie et al, 1985; Steinhardt and Alderton, 1988), chromosomal movement (Zhang et al, 1990), DNA synthesis and repair (Wang et al, 1996) and phosphorylation of nuclear proteins (Himpens et al., 1994a; Kawczynski and Dhindsa, 1996). In nuclei of some plant species, protein kinase activity was identified and changes in specific phosphorylation of discrete nuclear proteins during development or cell division were detected (Trewavas, 1979; Melanson and Trewavas, 1981). Plant nuclei have the potential for regulation of transcription through phosphorylation although whether there are Ca^{2+}-regulated protein kinases in the plant nucleus remains to be established.

In animal systems, Ca^{2+}-dependent nuclear enzymes have been identified, for instance, endonucleases (Jones et al., 1989, McMahon et al., 1985, Muel et al., 1986) that are associated with the initiation of mitogenesis and a Ca^{2+}-ATPase have been identified at the nuclear envelope (Burgoyne et al., 1989, Nicotera et al., 1989) where its precise localisation was reported to be at the outer nuclear membrane (Gerasimenko et al., 1995; 1996; Humbert et al., 1996). Additionally, an increase in the nuclear calcium concentration upon several stimuli (Shankar et al., 1993, Sorensen et al., 1993, Katagari et al., 1993), strongly argue for a regulatory role for Ca^{2+} in the nucleus. The second messenger, IP_3 has been suggested to play a role in the regulation of nuclear Ca^{2+} levels (Mak and Foskett, 1994; Hennager et al., 1995). The IP_3-receptor has been co-localised to the outer nuclear membrane (Mak and Foskett, 1997) using patch-clamp studies, whereas others suggest the present of such receptors at the inner nuclear membrane (Gerasimenko et al., 1996; Humbert et al., 1996).

At present, nuclear Ca^{2+} levels have not been measured in plants and in animals mechanisms by which $[Ca^{2+}]_{nuc}$ is regulated remains highly controversial. It is thought that the nuclear membrane provides a Ca^{2+} barrier based on several studies where concentration differences as well as temporal differences have been observed (Birch et al., 1992; Shankar et al., 1993; Himpens et al., 1994b; Kocsis et al., 1994; Badminton et al., 1995), however this theory has been disputed by the fact that molecules with molecular weights under 10 kDa can diffuse freely through the nuclear pores that

perforate the nuclear membranes. Therefore, it has been argued that the nuclear envelope that encloses the nucleus is permeable to ions like Ca^{2+} (Brini et al., 1993; Almohanna et al., 1994). Increases in cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$ could change the phosphorylation status of transcription factors located in the cytoplasm and direct these to the nucleus (Whiteside and Goodbourn, 1993; Pruschy et al., 1994; Greber and Gerace, 1995; Sweitzer and Hanover, 1996). Increases in nucleoplasmic free Ca^{2+} concentrations, $([Ca^{2+}]_{nuc})$ could activate nuclear localised protein kinases (Meek and Street, 1992; Gilchrist et al., 1994), change the phosphorylation status of nuclear localised transcription factors either directly or indirectly through calcium-binding proteins present in the nucleus (Bachs and Carafoli, 1987), and therefore, rapidly control gene expression and other nuclear events. This mechanism is supported by recent work in mice by Corneliussen et al. (1994). An *in vitro* study revealed that the ubiquitous Ca^{2+}-binding protein, calmodulin or CaM (see below) was shown to regulate the transcription factors of the basic helix-loop-helix (bHLH) group by directly inhibiting their DNA binding. In plants, Szymanski et al. (1996) reported that calmodulin isoforms differentially enhanced the binding of cauliflower nuclear proteins and recombinant TGA3 to a region derived from the *Arabidopsis CaM-3* promoter.

1.6   Calmodulin

In plant cells one of the primary sensors of changes in $[Ca^{2+}]_{cyt}$ and mediator of subsequent cellular response is calmodulin or CaM. Calmodulin is a highly conserved protein amongst eukaryotes and is considered to be multifunctional because of its ability to interact with and regulate the activity of numerous proteins (Klee et al., 1980). Over 30 enzymes and structural proteins have been identified that bind calmodulin with high affinity (Means, 1988). This regulatory protein is involved in a wide variety of Ca^{2+}-dependent pathways. Calmodulin has a binding affinity for Ca^{2+} of 10^{-6} M and binding induces a conformational change and makes it to act as molecular switch when the concentration of free Ca^{2+} rises transiently from a resting value of approximately 10^{-7} to 10^{-5} M. Twenty-four amino acids of the total of 148 facilitate the binding of Ca^{2+} to calmodulin (Fig. 1.2) in three-dimensional structures, so-called EF-hands or calmodulin-folds (Babu et al., 1985). A superfamily of proteins utilises this configuration to bind Ca^{2+} but calmodulin is the only known member that serves such a plethora of functions (Weinstein and Mehler, 1994). Upon sequestering Ca^{2+}, calmodulin reveals two hydrophobic patches that bind to specific domains of calmodulin-binding proteins (Török and Whitaker, 1994). The primary structures of these calmodulin-binding domains show very little homology (O'Neil and DeGrado,

1990), although all share the propensity to form a basic amphiphilic α-helix (James et al., 1995). In animals, the Ca^{2+}-calmodulin complex has been shown to regulate the intracellular concentration of second messengers including Ca^{2+}, cAMP and IP_3 metabolism (James et al., 1995). The importance of calmodulin was also shown after the examination of several temperature-sensitive yeast mutants (Ohya and Botstein, 1994) and by the fact that disruption of the single gene in yeast and other fungi is lethal (Rasmussen and Means, 1987; 1989).
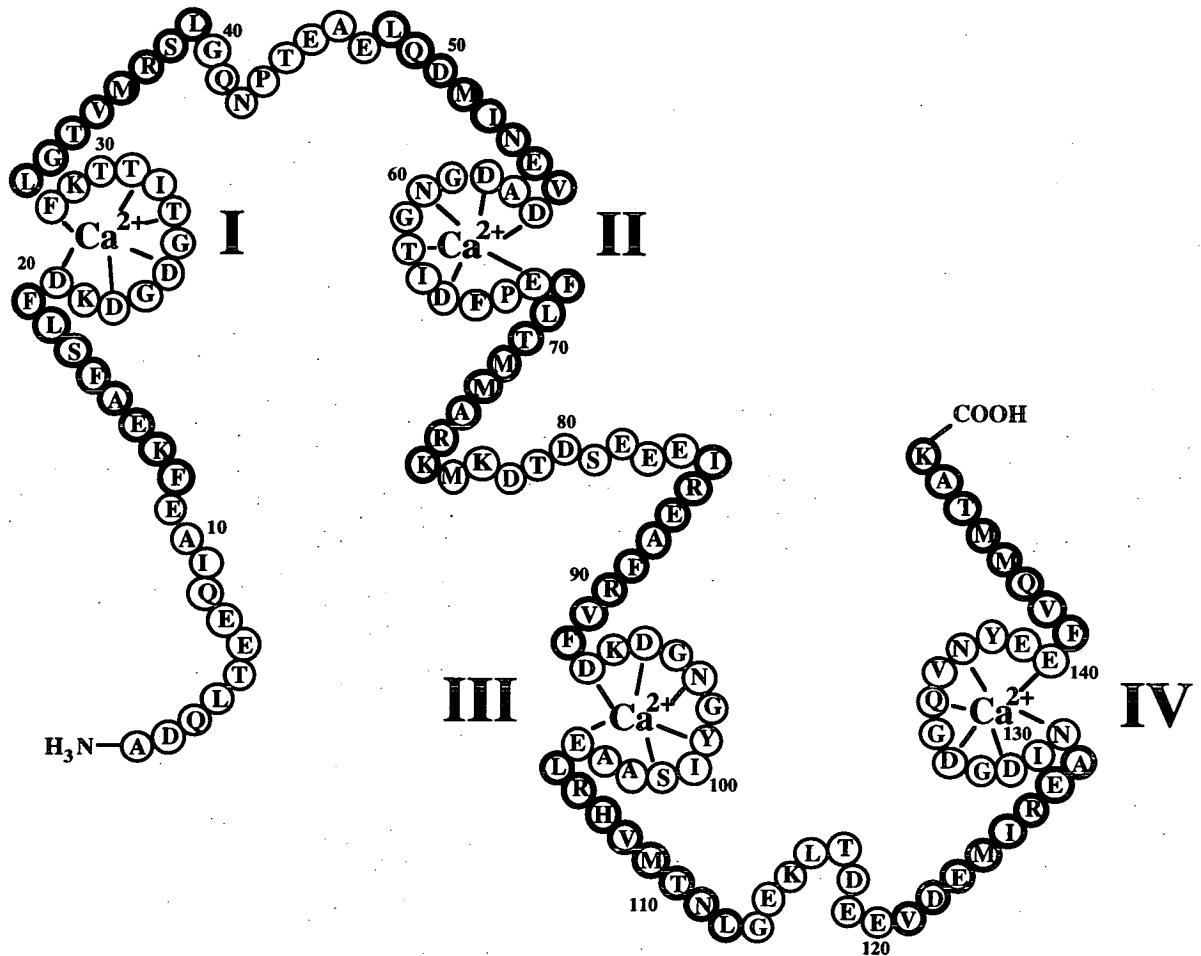
Figure 1.2: Schematic representation of vertebrate calmodulin with the four Ca^{2+}-binding loops flanked by stretches of α-helices indicated as circles in bold (Taken from Klee and Vanaman, 1982).

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While many protein kinases activated by calmodulin have been described, a novel type has been recognised that is activated directly by Ca^{2+}. The kinase, so-called CDPK, contains both a catalytic domain and a Ca^{2+}-binding regulatory domain similar to calmodulin (Roberts and Harmon, 1992).

Recently, other Ca^{2+}-binding proteins have been identified in plants. A cDNA encoding a 21.7 kDa polypeptide (CaBP-22) shares 66% amino acid sequence identity with *Arabidopsis* calmodulin and exhibits similar calcium-binding characteristics (Ling and Zielinski, 1993). Two touch-induced genes (*TCH-3* and *TCH-4*) encoding calmodulin-related proteins upon mechanical stimulation (Braam and Davis, 1990) and their expression kinetics, localisation and characterisation have been reported recently (Sistrunk et al., 1994; Xu et al., 1995, 1996; Polisensky and Braam, 1996; Khan et al., 1997).

1.7   Calmodulin Gene Expression

The presence of calmodulin in plants was first reported in the late 70s. Calmodulin protein has since been purified and cDNA and genomic clones characterised from a number of plant species, including *Arabidopsis* (Ling et al., 1991; Perera and Zielinski, 1992), barley (Ling and Zielinski, 1989), potato (Takezawa et al., 1995), and wheat (Yang et al., 1996) (Table 1.2). In all higher eukaryote species genes encoding different calmodulin isoforms are under control of different promoters and exhibit distinct temporal and spatial expression (Ling et al., 1991; Shimoda et al., 1995). Upon translation, some of these isoforms have been shown to regulate the activity of downstream target proteins with different kinetics (Lee et al., 1995; 1997; Liao et al., 1996; Harding et al., 1997).

Many studies have emphasised that calmodulin (CaM) genes are highly responsive to environmental and chemical signals (Table 1.2). Auxin induces calmodulin gene expression in strawberry fruit (Jena et al., 1989). In apple, the expression of calmodulin is induced by wounding (Watillon et al., 1992). In *Arabidopsis thaliana*, there appear to be several calmodulin genes (*AtCaM-1, -2*, and *-3*) which are differentially regulated (Ling et al., 1991, Perera and Zielinski, 1992). In roots, mRNA corresponding to AtCaM-1 was expressed, whereas those encoding AtCaM-2 and AtCaM-3 were not, suggesting that different regulatory elements exist (Perera and Zielinski, 1992). Recently, eight clones of potato calmodulin were isolated and characterised (Takezawa et al., 1995). Transgenic potato plants carrying the *PCaM-1* promoter fused to the ß-glucuronidase (GUS) reporter gene showed developmentally regulated and touch-induced GUS expression. It was investigated

whether the GUS mRNA expression driven by the introduced *PCaM-1* promoter and the endogenous *PCaM-1* were positively correlated. The expression of GUS mRNA was higher in the roots and slightly lower in developing tubers, which could be due to different stabilities of the two transcripts in roots and the developing tuber. This argues strongly against the use of reporter genes in such expression studies unless proper controls are performed.

Table 1.2: *Summary of stimulus-induced changes in calmodulin gene expression in plant cells*

Stimulus	Cell system	Reference
Heat shock	*Arabidopsis*	Braam, 1992
Mechanical	*Arabidopsis*	Braam and Davis, 1992
	Potato	Takewaza et al., 1995
Light	Merit Corn roots tips	Jena et al., 1989
Wounding	*Arabidopsis*	Braam and Davis, 1992
	Apple	Watillon et al., 1992
Abscisic acid	Aleurone layers	Schuurink et al., 1996
Auxin	Strawberry	Jena et al., 1989
Gibberellic acid	Aleurone layers	Schuurink et al., 1996

1.8   Thigmomorphogenesis of Plants

The effects of mechanical perturbations on plant growth were first reported in 1841 (Knight, 1841) and later described by Darwin (1880). In 1973, M.J. Jaffe examined the effects of touch on the growth and development of plants. Most vascular plants studied were shown to respond by elongating more slowly and increasing in diameter, resulting in short, stiff plants, often only 40 to 60 per cent as tall as controls (Fig. 1.3). Bending stems also causes these responses, and in nature the bending effects of wind influences plant development in a similar manner. Spraying tomato plants with water for 10 seconds once daily reduced their growth in a greenhouse to about 60 per cent of the growth of controls resulting in stockier plants with significantly reduced fruit yields (Wheeler and Salisbury, 1979). Inhibitory effects on flowering with dramatic effects on reproduction of a few species have also been observed (Mitchell and Myers, 1995). These and similar developmental responses to mechanical stress are called thigmomorphogenesis (Jaffe, 1973). In addition, shaking induced premature dormancy in young trees and substantial height and yield reductions in maize (Neel and Harris 1971, 1972a,b). Morphological changes induced by shaking are called seismomorphogensis.

The ability of the plant to sense minor physical strains led to the suggestion that the specification of plant form find its original basis in mechanical interactions and may be as important as responses to light, temperature, or gravity (Trewavas and Knight, 1994). In mammalian systems, such mechanical interactions can be modelled through

the effects of tension and compression on the cytoskeleton of a single cell, providing the cell with positional information for cell differentiation and patterning formation (Wolpert, 1994). The plane of cell division will be set to minimise the tension and compression stress within the whole tissue (Hardy et al., 1995; Wolpert, 1996).

Wind is a well-recognised thigmomorphogenetic stimulus (Grace, 1977). In woody plants the wind-induced rocking of the stem about the roots leads to the induction of stem thickening and lignification (Jones and Mitchell, 1989). The stem becomes more resistant to motion which helps to reduce shoot damage. It is thought that the primary yield differences between glasshouse-grown crops and their equivalents in the field reflect the absence of wind stimulation in the greenhouse as the diversion of carbohydrate to increase stem thickening reduces the food reverses available to the seeds (Trewavas and Knight, 1994).
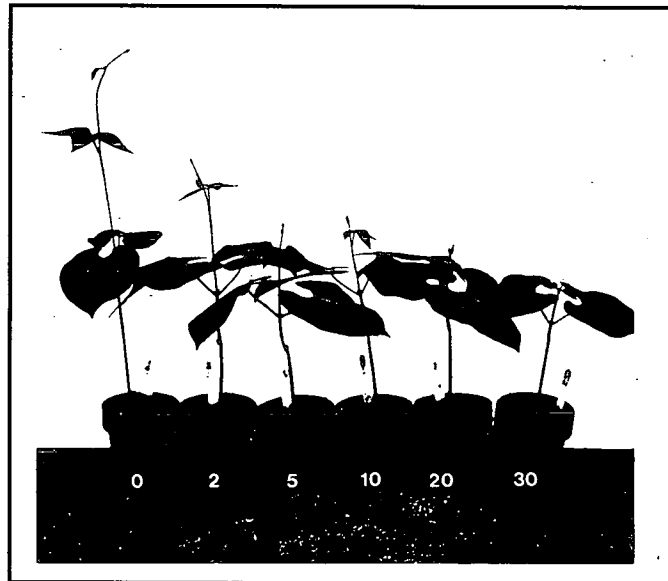
Figure 1.3: The effect of the number of daily rubs of the stem (once up and down, between thumb and forefinger, with moderate pressure) on the growth of young bean plants. From left to right, the number of stimuli were 0, 2, 5, 10, 20, or 30 (Taken from Jaffe, 1976).

1.9   Wind and Touch-Induced Increases in Calcium Levels

Recent studies have shown that thigmomorphogenic phenomena are influenced by calcium-modulating compounds. Jones and Mitchell (1989) demonstrated that a calcium influx caused by the ionophore A23187 added to the zone of maximum hypocotyl elongation of dark-grown soybean seedlings mimicked the effects of thigmic stress. The calcium chelator EGTA and the calmodulin antagonists calmidazolium and chlorpromazine counteracted the growth-inhibitory effects. A symmetrical accumulation of $[Ca^{2+}]_{cyt}$ within the stem growth zone leads to analogous inhibition of straight growth (Kauss, 1987). Physical perturbation of the plasma membrane leads to the uptake of Ca^{2+} into cells resulting in increased $[Ca^{2+}]_{cyt}$ which are thought to induce a cascade of biochemical events leading to seismo- and thigmomorphogenesis (Kauss, 1987). Further evidence for such a Ca^{2+}-mediated signal transduction pathway was obtained from studies on mechanically stressed soybean seedlings, in which EGTA and calmodulin-binding inhibitors prevented rubbing-induced growth reductions (Jones and Mitchell, 1989). One effect of Ca^{2+} could be the promotion of wall-bound peroxidases, as EGTA and chlorpromazine suppressed this activity (Basra et al., 1992). In another study with cultured soybean cells direct physical pressure led to the induction of an oxidative burst (Yahraus et al., 1995). Whether oxidative species have a pronounced role during mehanosensing remains unclear at present. A central role for Ca^{2+} in mechanosensing was confirmed by experiment using transgenic tobacco seedlings expression the light-sensitive luminescent protein, aequorin. Knight et al. (1992) showed that mechanical stimulation of seedlings by touching or wind exposure resulted in an immediate increase in $[Ca^{2+}]_{cyt}$. This transient increases in $[Ca^{2+}]_{cyt}$ only persisted while the seedling was in motion. When motion ceased, $[Ca^{2+}]_{cyt}$ returned to basal levels after 15 seconds (Knight et al., 1992, Fig. 1.4).

1.10   Wind and Touch-Induced Increases in Calmodulin Gene Expression

Mechanical stimulation of *Arabidopsis* seedlings by touch signals increased calmodulin mRNA levels 10-100 fold after ten minutes (Braam and Davis, 1990; Fig. 1.5). The induction of these so-called touch or *TCH* gene expression was suggested to be regulated directly or indirectly by an increased $[Ca^{2+}]_{cyt}$, as an increase in extracellular calcium led to an increase in calmodulin gene expression in cultured *Arabidopsis* cells (Braam, 1992). Many studies have emphasised that calmodulin expression is highly responsive to environmental and chemical signals (see paragraph 1.7; Table 1.2).

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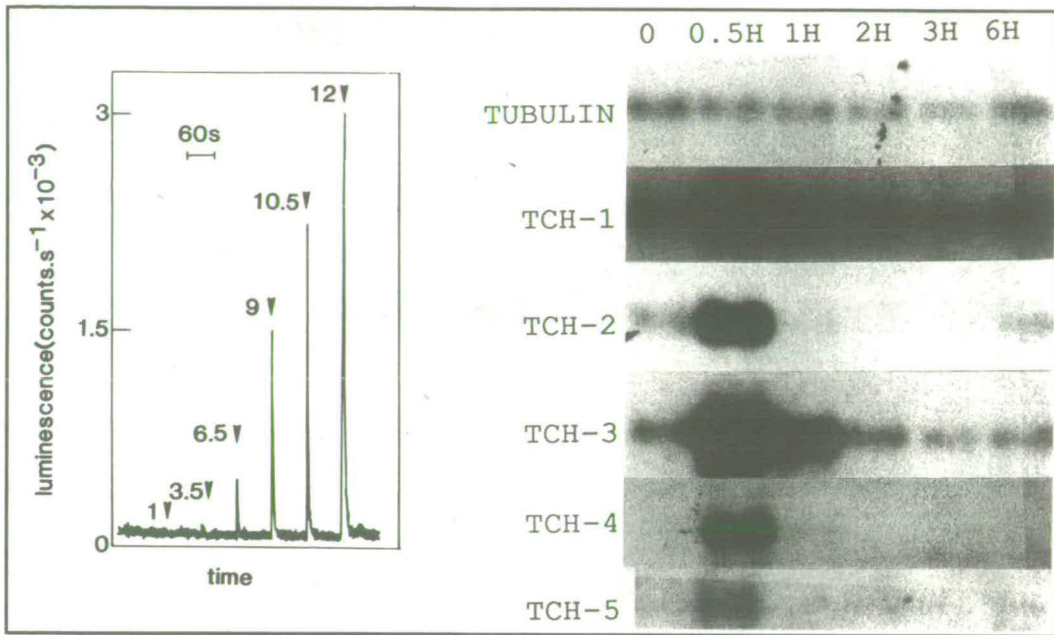
Figure 1.4: Effect of wind stimuli of increasing the force on seedling cytosolic calcium-responsive luminescence. A trace from a chart recorder connected to a chemiluminometer shows the changes in seedling luminescence in response to a wind force of 1, 3.5, 6.5, 9, 10.5, and 12 N, as indicated (Taken from Knight et al., 1992).

Figure 1.5: Northern blot analysis of time course of induction of the touch or *TCH* genes following touch stimulation of *Arabidopsis* plants (Taken from Braam and Davis, 1990).

1.11   Cold Shock Treatment of Plants

Many plants native to warm habitats display abrupt reductions in the rates of physiological processes and exhibiting signs of injury following exposure to temperatures in the range of 0 to 15°C. Many crop species including corn, rice, tomato, cucumber, soybeans, and cotton fall into this category of chilling-sensitive plants. Several crops of temperate origin, including potatoes, asparagus, and apples, also exhibit chilling sensitivity, however at lower temperatures (0-5°C) (Bramlage, 1982). Chilling injury depends not only on the species and tissue type, but also on the extent of exposure to low temperature. Other environmental variables contribute to the extent of injury manifest as a consequence of chilling. High humidity partially ameliorates the effects of wilting that occurs as a consequence of increased stomatal water loss from the leaves and diminished hydraulic conductivity of the roots at low temperatures. Exposure to chilling temperatures in the light is more injurious than in darkness (Garber, 1977; Powles, 1984). The most prominent chill-induced metabolic and structural effects include the cessation of protoplasmic streaming (Woods et al., 1984), altered rates of respiration (Lyons, 1973), impaired photosynthesis (Peeler and Naylor, 1988), alterations in protein synthesis (Cooper and Ort, 1988) and changes in

membrane permeability (Minorsky, 1985). The diversity of responses to chilling injury and the many types of biochemical-metabolic dysfunctions suggests that either (1) many cellular-molecular sites are sensitive to low temperature in chilling-sensitive plants or (2) one primary site or lesion initiates a cascade of events, leading to cell injury and/or death.

Mounting evidence points to the involvement of a genetic component associated with the ability to cold-acclimate and the induction of freezing tolerance. The most well-established indication is the inheritance pattern of hardiness in progeny of parents with different tolerance levels (Gaudet, 1994; Close, 1996) but it also includes (1) accumulation of soluble proteins, (2) change in protein electrophoretic pattern, (3) alterations in isoenzyme composition, (4) appearance of new proteins, (5) accumulation of rRNA and soluble RNAs, (6) changes in RNA base composition, (7) altered mRNA content, and (8) induction of freezing tolerance by ABA.

1.12   Cold Shock-Induced Increases in Calcium Levels

For a plant to acclimate to cold, it must perceive low temperature signals and transduce them into a biochemical response. Several lines of evidence suggest that Ca^{2+} is involved in signal transduction in response to cold shock. Mechanosensitive Ca^{2+}-channels are modulated by a decrease in temperature and have been suggested to function as temperature sensors during cold acclimation (Ding and Pickard, 1993). Cytosolic Ca^{2+} levels were shown to increase transiently as a result of cold shock (Knight et al., 1991) and this was partially inhibited by gadolinium, a putative inhibitor of stretch-activated Ca^{2+}-channels. Similarly, protoplasts of alfalfa were shown to take up extracellular Ca^{2+} after exposure to cold shock and that the influx was inhibited by Ca^{2+}-channel blockers and EGTA (Monroy and Dhindsa, 1995). Furthermore, treatment of alfalfa cell suspension cultures with Ca^{2+}-channel blockers and EGTA resulted in an inhibition of cold acclimation (Monroy et al., 1993). Surprisingly, the duration and the magnitude of the influx is not different between chilling-sensitive plants, like tobacco and chilling-resistant plants, like *Arabidopsis* (Knight et al., 1996) suggesting that Ca^{2+} signalling perhaps does not play a role in the differences observed between the physiology of these two species in response to low temperatures. However, tobacco plants were able to recover their ability to respond fully to cold shock 30 min after stimulation, whereas *Arabidopsis* plants were not. The longer recovery time of *Arabidopsis* plants may form part of a "cold memory", that might enable cold-resistant plants to cold acclimate. Cold-sensitive plants are less able to

retain information on previous cold treatment which might prevent them from cold acclimation.

1.13   Cold Shock-Induced Gene Expression

Freezing tolerance in plants develops in response to declining temperatures before the onset of winter. During this period of cold acclimation, the exposure to low but non-freezing temperatures, novel mRNAs accumulate and new proteins are synthesised (Guy, 1990). There have been several studies, using a variety of plant species, that have demonstrated altered gene expression during cold acclimation (Mohapatra et al., 1989; Cattivelli and Bartels, 1990; Kurkela and Franck, 1990). At low temperatures enzymatic processes including protein synthesis are significantly reduced, therefore increased protein-synthetic capacity is needed to compensate the reduced rate of synthesis. When cold-tolerant winter wheat was subjected to cold acclimation, both rRNA and total RNA were increased as compared to less-hardy spring-wheat (Sarhan and d'Aoust, 1975). Increases in rRNA can probably be explained on the basis of more ribosomes being associated in polysomes in cold-acclimated tissues. In addition the base composition of the total RNA was altered during cold acclimation (Sarhan and d'Aoust, 1975).

Evidence from cell culture systems of several plant species suggests that ABA could play an important role in the induction of freezing tolerance and control the expression of genes responsible for increased hardiness (Robertson et al., 1987). It has been demonstrated that ABA alone can provoke greater freezing tolerance in cell cultures. As for cold shock, ABA also influences the expression of a number of genes in a temporal fashion, thus it could play a direct role in the induction of freezing tolerance (Robertson et al., 1987).

A single cold shock treatment was sufficient to cause elevated levels of *kin1* mRNA after 1 hr in *Arabidopsis* (Knight et al., 1996). An influx of Ca^{2+} was required to induce full *kin1* response as EGTA, lanthanum and external Ca^{2+} affected the expression (Knight et al., 1996). Cold shock induces changes in gene expression and the necessity of a Ca^{2+} influx, was further observed in alfalfa, where the expression of *cas* (cold-acclimation genes) was affected by Ca^{2+} levels (Monroy and Dhindsa, 1995). In alfalfa, the requirement for elevated levels of intracellular Ca^{2+} for the induction of cold shock-induced *cas* gene expression appears to be temporary, since if EGTA was added to lower intracellular Ca^{2+} levels after the on-set of cold acclimation the expression of the cold-induced *cas* genes was not affected. When intracellular Ca^{2+} levels were artificially increased with a Ca^{2+} ionophore, A23187, a

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transient expression was obtained without any cold shock treatment, indicating that the influx of extracellular Ca^{2+} can induce the expression transietly but is not to sustain it (Monroy and Dhindsa, 1995).

1.14   Heat Shock Treatment of Plants

The heat shock response is induced by exposing an organism to supraoptimal temperatures (generally 5-10°C above normal growth temperature) for a period of 15 minutes to a few hours. Heat shock induces the synthesis of a set of proteins that were either not present or present only at low levels in unstressed cells, the so-called heat shock proteins (HSPs). As it is now understood, the heat shock response involves the perception of heat via changes in different parameters including key molecules, the internal environment and the membrane of the cell. These alterations communicates the stimulus that affects a change in transcription activity; genes for HSPs are actively transcribed while those for normal cellular proteins become quiescent. In animal cells, it was shown that the *hsp* mRNAs are subsequently translated at relatively high rate and preferentially above other transcripts (Lindquist, 1986). The heat-induced HSPs are thought to play some role in alleviating the deleterious result of the heat stress. Consequently, cells that have synthesised HSPs are able to withstand subsequent, otherwise damaging heat exposures-a state described as thermotolerance.

The conditions under which *hsp* expression is elicited provide important clues about the perception and regulation of the heat shock response. It is fairly evident that the mechanism for perception is not analogous to hormone receptor mechanisms where there is the presence of an inductive molecule. Because heat shock involves changes in the physical parameter of temperature, it is much more likely that changes in the physiology or biochemistry of heat-shocked cells initiates the heat shock event. These include (1) changes in the oxidative/reductive environment of the cell, (2) changes in ion levels inside the cell, (3) the presence of heat-denatured proteins.

Numerous investigators have noted that many other stress conditions elicit the synthesis of at least a subset of the HSPs (Sachs and Ho, 1986). Incubation of cell in the presence of arsenite, arsenate, mercury ions, cadmium, hydrogen peroxide, and sulfhydryl reagents (Brodl, 1990) mimics, at least in part, the heat shock response, often establishing thermotolerance. These stresses are, in common, oxidative in nature. In support to this observed change in the oxidative/reductive environment of the cell, increase levels of glutathione levels were observed during continued heat shock in maize roots (Nieto-Sotelo and Ho, 1986). This increase provides a mechanism for returning the cell's environment to a less oxidised state (Brodl, 1990).

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Membranes are highly sensitive to alterations in temperature, and it is likely that some of the earliest effects of heat shock impact membrane properties. It has been long established that exposure to high temperatures affects photosynthesis, probably through alterations of the permeability properties of partitioning membranes (Berry and Björkman, 1980). Heat alters thylakoid permeability to protons, thereby disturbing the proton gradient that drives photophosphorylation and to other charge-compensating ions reducing the photophosphorylation during heat exposure. Heat appears to have an impact on the partitioning of ions across membranes in general. The influx of K^+ and the efflux of H^+ is arrested, likely resulting in changes in the cell's ionic balance and potentially affecting other biochemical changes. In addition, heat may alter the binding efficiencies of membrane pumps in a manner analogous to the mode by which heat alters the K_m of enzymes.

The presence of heat-denatured proteins appear to induce HSP synthesis in many systems (Ananthan et al., 1986). Conversy, agents that are putatively capable of protecting proteins from thermal damage, such as glycerol or D_2O, block the induction of HSPs by high temperature (Edington et al., 1989). Such data give support to the hypothesis of Munro and Pelham (1985) that the level of heat-denaturated protein in the cell is responsible for the perception of heat shock. They proposed that heat shock result in a large increase in denaturated proteins, which are then tagged by ubiquitin for degradation and form the signal for the heat shock response.

1.15   Heat Shock-Induced Increases in Calcium Levels

Several authors have suggested that Ca^{2+}-mediated second messenger systems are involved in heat shock responses of animal cells (Lamarche et al., 1985; Calderwood et al., 1988; Landry et al., 1988; Mosser et al., 1990), although other results indicated that Ca^{2+} was not strictly required for HSP synthesis *per se* (Drummond et al., 1986, 1988). In plant cells, Klein and Ferguson (1987) observed that uptake of Ca^{2+} by suspension-cultured pear cells or protoplasts was significantly enhanced during heat stress. Wu et al. (1992) also indicated that pretreatment of hypocotyl segments and etiolated seedlings of *Brassica napus* with the Ca^{2+} ionophore A23187 or EGTA to modify Ca^{2+} homeostasis resulted in changes of the synthesis of HSPs. Furthermore, using the fluorescent dye Indo-1, Biyaseheva et al. (1993) reported that heat shock induced a fourfold increase of $[Ca^{2+}]_{cyt}$ in pea mesophyll protoplasts but further dynamic changes of $[Ca^{2+}]_{cyt}$ during heat shock could not be detected because of limitations in the technique.

The effects of Ca^{2+} on the induction of the heat shock response make it quite attractive to propose that early perception of the heat shock event is communicated in the cell via some Ca^{2+}-dependent protein kinase (Landry et al, 1988; Burke and Orzech, 1988). The fact that calmodulin antagonists can alter many of the cell's responses to heat shock supports this hypothesis (Landry et al., 1988; Gong et al., 1997a,b).

1.16   Heat Shock-Induced Gene Expression

Much work has been done to uncover the molecular mechanism by which *hsp* gene transcription is initiated. In the 5' regulatory region of all *hsp* genes, there is a 14-base sequence (CTNGAANNTTCNAG) which is conserved among different organisms (Pelham, 1982). This so-called heat shock element or HSE seems to have a crucial role for the high temperature induction of HSPs; deleting of the element prevented expression in *Drosophila* (Dudler and Travers, 1984). Proteins that bind HSE, called HSTFs have been isolated and characterised in yeast and *Drosophila* (Weiderrecht et al., 1987). Genes encoding HSPs from maize (Rochester et al., 1986) and soybean (Gurley et al., 1986) contain 5' elements confirming the heat shock consensus sequence of animals. The calmodulin gene, *AtCaM-3* might be regarded as a heat shock protein as it contains the HSE sequence and Braam (1992) demonstrated that heat shock induced the expression of calmodulin-related *TCH* genes in cultured *Arabidopsis* cells. In addition, external Ca^{2+} was required for maximal heat shock induction of these *TCH* genes, thus connecting the calcium signalling pathway and heat shock response.

The fate of mRNA remaining from cellular gene transcription under non-heat-shocked conditions varies with the organism that is heat-shocked (Brodl, 1989). Upon heat shock in tomato (Scharf and Nover, 1982) and soybean (Key et al., 1981) non-heat-treated-responsive cellular mRNAs are not translated, yet they remain, stable in the cytoplasm and are reactivated during recovery. In isolated aleurone layers from mature imbibed barley seeds that are heat-shocked, selective cessation of normal cellular protein synthesis is achieved; some continue to be synthesised while others are arrested (Belanger et al., 1986). Exceptions to the rule include the mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in cultured soybean cells. Levels of this mRNA decrease during heat shock, presumably at the rate of non-heat-responsive mRNA turnover in the absence of new transcription (Vierling and Key, 1985). How this translational bias is established is currently not known. Heat shock was demonstrated to disrupt the 5' cap and the 3' poly (A) tail, two regulatory elements that work in concert to establish an efficient level of translation of introduced reporter

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mRNAs (Gallie et al., 1995). It has been found that the 5' untranslated regions of *hsp* mRNA are unusually long, rich in adenine, and have little secondary structure (McGarry and Lindquist, 1985).

1.17   Measuring Calcium Fluctuations

Direct demonstration of the involvement of Ca^{2+} in signal transduction requires the measurement and localisation of fluctuations in intracellular calcium concentrations ($[Ca^{2+}]_{int}$) during stimulation of the fully physiological conditions. The first measurements of cytosolic free Ca^{2+} in living cells were made with Ca^{2+}-sensitive photoprotein, aequorin in giant muscle cell in the late 1960s (Ridgway and Ashley, 1967). During the 1970s two new methods were introduced, bis-azo absorbance dyes (mainly arsenazo III) and Ca^{2+}-selective microelectrodes (Ashley and Campbell, 1979). In 1978, Gilkey and co-workers used aequorin-injected eggs of the fresh water fish, medaka, and observed an explosive rise in free calcium during fertilisation that was followed by a slow return to the resting levels. Imaging intensification techniques revealed a spreading wave of high free calcium up to 100-1,000 times resting levels during fertilisation from the animal pole (were the sperm enters) and that traversed the egg as a shallow band which vanished at the antipode some minutes later. However, reports on successful measuring and quantification of $[Ca^{2+}]_{int}$ remained limited to a very few cell types due to the large restrictions of the applied techniques. Visualisation of $[Ca^{2+}]_{int}$ heterogeneities and gradients at higher magnification in much smaller cells then fish eggs were made possible by the introduction of fluorescent indicators (Williams et al., 1985, Tsien, 1989). However, Ca^{2+} gradients that were maintained over longer periods of time remained difficult to demonstrate due to artefacts such as photometric inaccuracy, improper substraction of background fluorescence, and dye compartmentalisation into organelles that contained high Ca^{2+} ion concentrations. In addition, effects of the cytoplasmic micro-environment on the dye could simulate such gradients (Williams et al., 1985). In recent years, continuous work has been performed to study Ca^{2+} temporal and spatial dynamics in detail by means of fluorescent dyes, and in numerous cases techniques have been improved (Cheek, 1989, Shacklock et al., 1992, Jaffe, 1993, Kasai et al., 1993, Thorn et al., 1993, Malhó et al., 1994).

1.18   Aequorin

The characteristic greenish bioluminescence of the jellyfish *Aequorea victoria*, clearly visible as a ring of bright light (Harvey, 1952), is due to the action of two closely associated proteins: aequorin, a small Ca^{2+}-binding protein (21.4 kDa, fluorescence L_{max} = 470 nm), and a green fluorescent protein (GFP, 27 kDa, fluorescence L_{max} = 508 nm). The latter contains a chromophore that is the ultimate emitter in the bioluminescence reaction in the jellyfish (Shimomura, 1979). Aequorin is made up of a complex of apoaequorin (apoprotein), coelenterazine, which is a hydrophobic luminophore that is permeable to cells (an imidazole compound with a molecular weigth of 423 Da) and molecular oxygen. When aequorin is mixed *in vitro* with Ca^{2+} a bluish light is observed due to an intramolecular reaction in which the coelenterazine substrate is oxidised to coelenteramide (figure 1.6 and 1.7). The products are light (L_{max} = 470 nm), CO_2, coelenteramide, apoaequorin. When the reaction is carried out in the presence of GFP, greenish luminescence is produced (L_{max} = 508 nm) as a result of energy transfer between excited aequorin and GFP (Morise et al., 1974).

$$\textbf{coelenterazine} + \textbf{O}_2 + \textbf{Ca}^{2+} \xrightarrow{\textbf{Apoaequorin}} h\nu_{\lambda_{max}} + \textbf{coelenteramide} + \textbf{CO}_2$$

470 nm

GFP

$$h\nu_{\lambda_{max}}$$

508 nm
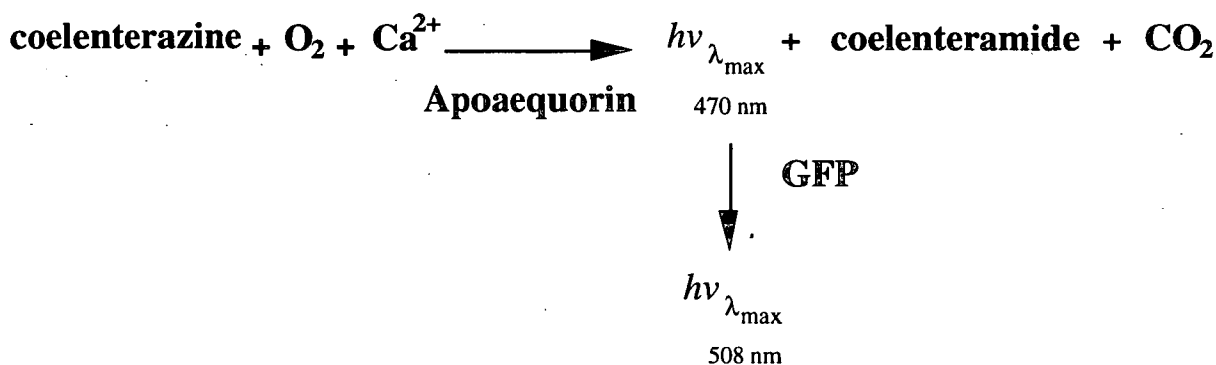
Figure 1.6: A simplified model of the luminescent reactions of the blue and green fluorescent proteins.
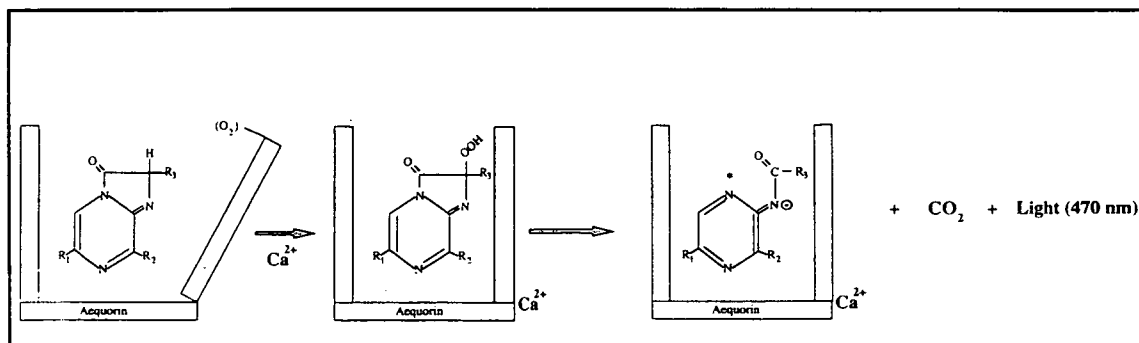
Figure 1.7: Aequorin is regarded as an oxygenase. The presence of Ca^{2+} induces a conformational change of aequorin. The result is the production of blue light due to the generation of an excited state of the protein-coelenteramide complex.

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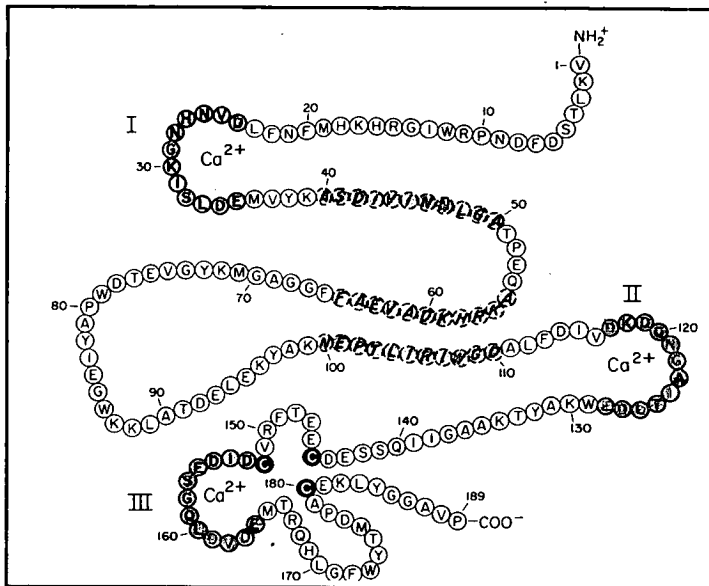
Figure 1.8: The primary structure of apoaequorin and the Ca^{2+}-triggered bio-luminescence reaction of aequorin. The sequence is arranged to show the Ca^{2+}-binding sites (heavily stippled, in dark grey), and hydrophobic regions (broken circle, lightly stippled, in light grey) (taken from Tsuji et al., 1986).

1.19   Aequorin as Tool to Measure Calcium Levels

Microinjected aequorin has been widely used for measuring $[Ca^{2+}]_{int}$ in animal cells (Blinks et al, 1978; Campbell, 1983), but in plants this technique has been limited to photomultiplier measurements of large cells (Williamson and Ashley, 1982, Callaham and Hepler, 1991). Recently, a totally new approach was developed to measure $[Ca^{2+}]_{int}$ using aequorin. *Nicotiana plumbaginifolia* was transformed to express apoaequorin (Knight et al., 1991, 1992) and addition of coelenterazine to the plants, reconstituted active aequorin. After the binding of three Ca^{2+} ions to aequorin, the luminophore is discharged and emits blue light (Fig. 1.8). The light emission is in direct proportion to the concentration of free Ca^{2+} (Campbell, 1983). This technique shows numerous advantages over the use of Ca^{2+}-sensitive fluorescent dyes (Read et al., 1992). Aequorin is not cytotoxic and does not affect plant growth and development and is a highly negatively charged protein which has not been reported to compartmentalise within organelles or leak out of cells. Since aequorin does not require irradiation, potential cell damage is avoided. The protein has a very low affinity for free Ca^{2+} thereby avoiding $[Ca^{2+}]_{int}$ buffering problems and enabling accurate measurements over a wide range of Ca^{2+} concentrations (50 nM - 100 mM) (Trewavas et al., 1992). Some of the fluorescent dyes, like quin-2 have high affinity for Ca^{2+} and will artificially lower cytosolic Ca^{2+} levels under various conditions thereby affecting the assessment of the calcium signal (Tscharner et al., 1986).

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A very important aspect in measurement and imaging Ca^{2+} levels in living cells has been the development of non-disruptive ways of introducing Ca^{2+}-sensitive indicators in cells. The fluorescent dyes are highly hydrophobic and do not cross the plasma membrane easily. Introducing dyes into cells by microinjecting or electroporating, however, may lead to perturbation or even partial distortion of the cell, often affecting resting steady state levels of Ca^{2+} (Rand et al., 1994). Introducing fluorescent dyes by ester loading or pH loading may lead to cytotoxic effects. Furthermore, problems arise with dye sequestration into organelles or leakage of dye out of cells (Read et al., 1992). Most fluorescent dyes require irradiation before imaging that can result in dye photobleaching and potentially damaging the cells.

Advantages of fluorescent dyes over using aequorin are the brighter signals emitted by fluorescent dyes. Each molecule of an indicator like Fura-2 can emit on average over 10,000 photons prior to extinction, whereas each molecule of aequorin has only about an 0.15 chance of emitting even a single photon (Shimomura and Johnson, 1979). Fluorescent dyes, on the other hand, require *in vitro* calibration and, therefore, the ionic composition and physical characteristics (e.g. viscosity) of the dye-ion calibration solution must be modified accordingly to mimic the plant cytosol. The use of apoaequorin reconstituted with ε-coelenterazine and measuring the ratio of the bimodal spectrum of luminescence emission at two wavelengths, 421 and 477 nm, allows a simple method for quantification of $[Ca^{2+}]_{int}$ *in vivo* (Knight et al., 1993).

1.20   Regulation of Calmodulin Gene Expression by Intracellular Calcium Levels

An increase in intracellular calcium levels, $[Ca^{2+}]_{int}$ and calmodulin gene expression upon mechanical stimulation has led to a simple but intriguing hypothesis. Calmodulin mRNA levels are regulated by $[Ca^{2+}]_{int}$ or $[Ca^{2+}-CaM]_{int}$ as illustrated in figure 1.9. Mechanical stimulation may release Ca^{2+} from internal stores (Knight et al., 1992). Ca^{2+} either binds to calmodulin present in the cytoplasm and the Ca^{2+}-activated calmodulin translocates to the nucleus, or an increase in nuclear Ca^{2+} levels activates calmodulin present in the nucleus. The first possibility is supported by studies with fluorescein-labelled calmodulin microinjected into tissue cultured cells of PtK1, where translocation of calmodulin to the nuclei was observed (Pruschy et al., 1994). The second possibility is supported by data from rat osteoclasts where a calcium signal was localised to the nucleus region following stimulation of the integrin receptor, that links the cell cytoskeleton to the extracellular matrix in order to sense mechanical stimulation (Shankar et al., 1993). This result, together with the existence of a signal transduction pathway within the nucleus, including nuclear metabolism of phosphatidylinositol

bisphophate and IP$_3$ receptors (Gerasimenko et al., 1996; Humbert et al., 1996), the presence of calmodulin and calmodulin-binding proteins in the nucleus (Bachs and Carafoli, 1987, Bachs et al., 1992, Gilchrist et al., 1994) and protein kinases in the nucleus (Meek and Street, 1992, Brusa et al., 1994) support this assumption. Therefore, calmodulin gene expression could be increased either directly by nuclear calcium or through Ca^{2+}-calmodulin dependent protein kinases and/or transacting factors within the nucleus. A role of Ca^{2+}/calmodulin in regulating gene expression has been suggested previously in mice (Corneliussen et al., 1994). Binding of Ca^{2+}-loaded calmodulin to the basic helix-loop-helix (bHLH) domains of several bHLH transcription factor proteins directly inhibit their DNA binding *in vitro*. Alternatively, calmodulin mRNA constitutively present in the cytoplasm could be stabilised by a process mediated through an increased [Ca^{2+}]$_{cyt}$. Many of these data remains to be established in plant cells although plant nuclei were shown to contain protein kinase activity (Brusa et al., 1994) and changes in specific phosphorylation of discrete nuclear proteins during development or cell division were detected using two dimensional separations (Trewavas, 1979; Melanson and Trewavas, 1981). Moreover, calmodulin has been detected in purified plant nuclei using a variety of quantitative techniques (Collinge and Trewavas, unpublished). Enhanced levels of cellular calmodulin may enable the cell to respond to repetitive episodes of wind stimulation, overcome cytotoxic effects by lowering the concentration of free Ca^{2+} and is believed to be involved in tertiary responses such as lignification (Kaiser et al., 1994) and ethylene biosynthesis (Jaffe and Forbes, 1993).
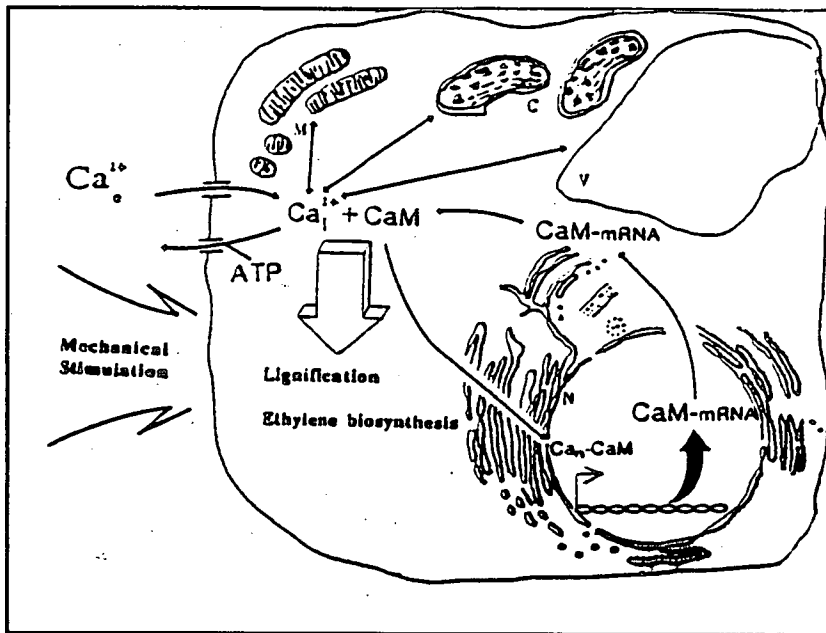
Figure 1.9: A model that illustrates possible way to regulate of calmodulin gene expression by mechanical stimulation-induced increases in $[Ca^{2+}]_{cyt}$. Abbreviations: CaM, calmodulin; N, nucleus; V, vacuole; C, chloroplasts and M, mitochondria.

In the present work the model of figure 1.9 was tested. The Ca^{2+}-sensitive luminescent protein, aequorin was fused to nuclear localisation signals to target aequorin to nuclei of tobacco plants (Chapter 3). Using rapid amplification of cDNA ends, RACE PCR, two partial calmodulin cDNAs, designated *NpCaM-1* and *NpCaM-2* were isolated that were actively expressed in tobacco seedlings. The accumulation of *NpCaM-1* transcript was induced after wind and cold shock stimulation, whereas that of *NpCaM-2* remained unaffected under these conditions (Chapter 4). The changes in spatio-temporal Ca^{2+} dynamics were related to level of calmodulin gene expression by using modulators of Ca^{2+}-channel action. Changes in cytoplasmic and nuclear Ca^{2+} levels were measured and compared to induced levels of expression of *NpCaM-1* by the environmental stimuli, wind (Chapter 5) and cold shock (Chapter 6). In chapter 7 the relationship is described between heat shock-induced cytosolic calcium levels and heat shock-induced thermotolerance in tobacco seedlings. Chapter 2 describes all used materials and methods and chapter 8 holds the final discussion.

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Chapter 2

Materials and Methods

2.1   Materials

<u>Bacterial strains</u>

Agrobacterium tumefaciens LBA4404.

Escherichia coli JM101.

Escherichia coli XL1-blue.

Escherichia coli Stratagene Epicurian Supercompetent cells (Stratagene, Cambridge, UK).

<u>Buffers, broths and gels</u>

TAE buffer	20 mM Tris-acetate (pH 8.0), 1 mM EDTA.
DNA loading buffer	3% (w/v) Ficoll™400, 0.001% (w/v) bromophenol blue and xylene cyanole dye.
RNA running buffer	20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA.
RNA loading buffer	RNA running buffer, 3% (w/v) Ficoll™400, 0.025% (w/v) bromophenol blue and xylene cyanole dye, 50 $\mu g \cdot mL^{-1}$ ethidium bromide, 0.5% (v/v) formamide, 6.6% (v/v) formaldehyde.
PCR buffer	67 mM Tris·HCl (pH 8.8), 6.7 mM $MgCl_2$, 1.7 $mg \cdot mL^{-1}$ bovine serum albumin, 16.6 mM $(NH_4)_2SO_4$.
RTC buffer	50 mM Tris·HCl (pH 8.15 at 41°C), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT and 0.5 mM of each dNTP.
Denhardt's solution	0.02% (w/v) bovine serum albumin, 0.02% (v/v) Ficoll™400, 0.02% (v/v) PVP-40.
TfbI buffer	30 mM potassium acetate, 100 mM RbCl, 15 mM $CaCl_2$, 50 mM $MnCl_2$ (pH 5.8 with 0.2 M acetic acid) and 15% (v/v) glycerol, sterilised by filter.
TfbII buffer	10 mM MOPS, 100 mM $CaCl_2$, 10 mM RbCl, pH 6.5 with KOH and 15% (v/v) glycerol, sterilised by filter.
Acrylamide gel (10%)	10% (v/v) acrylamide, 0.27% (v/v) N,N,-methylenebisacrylamide, 750 mM Tris·HCl (pH 8.9), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (AMPS), 0.04% (v/v) N,N,N',N',-tetramethyl-ethylenediamine (TEMED).

Stacking gel	62.5 mM Tris·HCl (pH 6.8), 5% (v/v) acrylamide, 0.13% (v/v) N,N,-methylenebisacrylamide, 0.1% (w/v) SDS, 0.1% (w/v) AMPS, 0.04% (v/v) TEMED.
Protein running buffer	50 mM Tris·HCl (pH 8.3), 200 mM glycine, 0.1% (w/v) SDS.
SDS loading buffer	62.5 mM Tris·HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) ß-mercaptoethanol, 0.01% (w/v) bromophenol blue.
Transfer buffer	30 mM Tris·HCl (pH 8.3), 192 mM glycine, 10% (v/v) methanol.
Coomassie brilliant blue stain	0.025% (w/v) Coomassie brilliant blue R., 40% (v/v) methanol, 7% (v/v) ethanoic acid.
Destain solution	7% (v/v) ethanoic acid, 4% (v/v) methanol, 1% (v/v) glycerol.
TBS-T buffer	10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.4% (v/v) Tween 20.
Aequorin reconstitution buffer	50 mM Tris·HCl (pH 7.4), 10 mM EGTA, 500 mM NaCl, 5 mM ß-mercaptoethanol and 0.1% (w/v) BSA.
20X SSC	3 M NaCl, 0.3 M sodium citrate.
RNA lysis buffer	2% (w/v) SDS, 1% (v/v) ß-mercaptoethanol, 50 mM EDTA, and 150 mM Tris with pH adjusted to 7.5 with 1 M boric acid.
TE buffer	10 mM Tris·HCl (pH 7.6) and 1 mM EDTA.
Luria-Bertani broth	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 85.5 mM NaCl.
2X YT broth	1.6% (w/v) bacto-tryptone, 1% yeast extract, 85.5 mM NaCl with pH adjusted to 7.4 with NaOH.

Chemical

All chemicals were purchased from Sigma (Dorset, UK) unless otherwise stated.

Plants

wild type; *Nicotiana plumbaginifolia*

MAQ 2.4; Aequorin transgenic *Nicotiana plumbaginifolia* (Knight et al., 1991).

MAQ 6.3a; Aequorin transgenic *Nicotiana plumbaginifolia* expressing chloroplast targeted aequorin (Johnson et al., 1995).

MAQ 7.11; Aequorin transgenic *Nicotiana plumbaginifolia* described here.

Plasmids

pMAQ 2.4; pMAQ 6.3a; pBIN19; pBI101 (Jefferson et al., 1987); pCASAQ.SP, a pDH51 derivative provided by Dr. Marc R. Knight (University of Oxford, Oxford, UK); pCASMAQ.SP, a pDH51 derivative provided by Dr. Marc R. Knight; pUC19

derivative, carrying the cDNA of HMG1, a kind gift from Dr. John Gray (University of Cambridge, Cambridge, UK); pSV7d, an expressing vector carrying the cDNA of nucleoplasmin, a kind gift from Dr. Mike N. Badminton (University of Wales, Cardiff, UK); pNiCaM, pBluescript SK derivative, carrying the partial cDNA clone of tobacco calmodulin, a kind gift from Dr. Anne Taylor (University of Aberdeen, UK).

Primers

MKA, MKB, Xhmg, Shmg, E086, Q_T, Q_o, Q_i, M13 forward and reverse primer for sequence analysis.

Restriction and Modifying Enzymes

All restriction and modifying enzymes were purchased from Biogene (Bedfordshire, UK); Biolabs (Hertfordshire, UK); Boehringer (Mannheim, D); Promega (Southampton, UK); Stratagene (Cambridge, UK).

2.2   Seeds Sterilisation, Germination and Plant Growth Conditions

All plant material used throughout this project was germinated from F_3 generation seeds unless otherwise stated. Seeds were surface sterilised by a 15 min incubation in 10% (w/v) NaOCl followed by five consecutive washes in sterile water. Sterilised seeds were incubated in 1 mM gibberellic acid at 4°C overnight in the dark to break dormancy and synchronise growth. Seeds were germinated on germination medium containing half-strength MS salts (Murashige and Skoog, 1962) and 0.8% (w/v) bacto-agar (Difco Laboratories, Detroit, USA) and cultured for 7-10 days at 25°C with 16 hr photoperiod. Transgenic seeds were germinated as described above with the addition of 200 μg·mL^{-1} kanamycin. Under these conditions over 99% of the seeds germinated successfully. Seedlings were either used for further experimentation or for the production of seed or mature tissue.

2.3   Preparation of Tobacco Protoplasts

Six expanded leaves (6 cm in length) from Nicotiana plumbaginifolia were surface sterilised with 70% (v/v) ethanol for 30 s followed by 10% (v/v) NaOCl for 15 min. Leaves were washed 6 times in sterilised distilled water. Leaves were cut to remove the mid rib and major veins, and sliced into "ribbons" of 2 mm width. Cut leaves were incubated overnight in filter-sterilised 400 mM mannitol, 1% (w/v) Cellulase and 0.2% (w/v) Macerozyme (pH 5.7). Macerozyme and Cellulase were purchased from Yakult

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Honsha (Tokyo, Japan). Protoplasts were filtered 2 times through a sterile 100 μm mesh filter by gravity flow. Protoplasts were washed 3 times in 400 mM mannitol in 10 mM MES (pH 5.7) and centrifuged 5 min at 100g to replace enzyme solution.

2.4   Standard Molecular Biology Techniques

<u>Restriction analysis</u>

Restriction digests were performed in 5-10 units per μg DNA in a 10-20 μL volume, and digested for 1-2 hr in buffer supplied with the enzyme according to manufactures' instructions. For multiple restriction digests "universal" buffers according to manufacture guidelines were used. After digestion, size-fractionation of digested DNA was performed on 0.5-3% (w/v) agarose gels prepared in TAE buffer containing 0.5 μg·mL^{-1} ethidium bromide. Gels were run of 1 hr at 10 V·cm^{-1} afterwhich a photograph was taken under UV illumination. For fragment isolation, size-fractionated DNA bands were either transferred onto a DEAE-membrane (Schleicher and Schuell, Dassel, D) or isolated with Gene Clean (pB101, Vista, CA, USA) according to manufactures' instructions.

<u>Hybridisation probe</u>

In order to prepare of probes for hybridisation purposes, DNA fragment was labelled with ^{32}P-dCTP (Amersham, Buckinghamshire, UK) by random priming labelling with mixed hexadeoxyribonucleotides (Pharmacia Biotech, Uppsala, S) according to manufactures' instructions. After overnight incorporation the fragments were purified by means of a G-25 Sephadex column (Pharmacia Biotech, Uppsala, S) and denaturated before hybridisation.

<u>PCR</u>

For amplification of DNA fragments to produce chimaeric nuclear targeted aequorin fusion proteins a PCR reaction was carried out in PCR buffer containing 2.5 units Taq polymerase (Biogene, Bedfordshire, UK) using the following conditions: one cycle at a sequence of 5 min 95°C, 1 min 55/60/65°C, and 2 min 72°C followed by 30 cycles at a sequence of 25 sec 95°C, 1 min 55/60/65°C, and 2 min 72°C on a Hybaid Omnigene temperature Cycler (Hybaid Ltd., Middlesex, UK). Fragments were subcloned into pCASAQ.SP and pCASMAQ.SP.

Ligations

For ligations DNA fragments were inserted into cloning vectors using a 3 to 1 insert to vector ratio at 4°C using T4 DNA ligase. When subcloned in pCR-Scripts Amp SK(+) Cloning Kit (Stratagene, Cambridge, UK) manufactures' instructions were followed.

Bacterial transformation

Transformation of competent XL1.blue, JM101 *E. coli* cells was carried out by growing the cells in Luria-Bertani broth to an OD_{550} of 0.3-0.4. Cells were cooled for 5 min on ice, centrifuged at 2,600g, 4°C for 5 min and the pellet was resuspended in 0.4 of the original volume TfbI. Cells were kept on ice for 5 min and centrifuged at 2,600g, 4°C for 5 min and resuspended in 0.04 of the original volume TfbII. Cells were left on ice for 5 min and aliquoted in pre-chilled eppendorfs and snap-frozen in liquid nitrogen and stored at -70°C. The cells were defrozen on ice and left for 10 min. Transformation was performed by adding 10-100 ng DNA and incubated on ice for 15-45 min. Cells were heat-shocked at 42°C for 90 s and left on ice for 10-30 min. To recover cells and to allow expression of the introduced DNA, cells were incubated 45 min at 37°C. Cells were plated out on Luria-Bertani agar (Luria-Bertani broth with 1.5% (w/v) bacto-agar) plates containing 10 mg·L^{-1} tetracycline, 20 mg·mL^{-1} 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal), 238 mg·mL^{-1} isopropyl ß-D-thiogalactosidase (IPTG) and an antibioticum selection that was encoded by the introduced plasmid DNA to allow selection of transformed cells. Each transformation yielded between 10 and 10^3 colonies depending on the quantity and purity of DNA used. When *Escherichia coli* Stratagene Epicurian Supercompetent cells (Stratagene, Cambridge, UK) were used, conditions were applied as instructed by the manufactures' guidelines.

For transformation in *Agrobacterium tumefaciens* LBA4404 cells were grown in 2X YT broth containing 100 µg·mL^{-1} rifampicin and 400 µg·mL^{-1} streptomycin at 28°C to an OD_{650} of 0.6. Cells were cooled for 5 min on ice, centrifuged at 2,600g, 4°C for 5 min and the pellet was once washed and then resuspended in 20 mM CaCl$_2$. Cells were aliquoted in pre-chilled eppendorfs and snap-frozen in liquid nitrogen and stored at -70°C. The cells were defrozen on ice and left for 10 min. Transformation was performed by adding 100 ng-1 µg DNA to an aliquot of cells and incubated on ice for 15-45 min. Cells were heat-shocked at 37°C for 5 min before adding 2X YT broth. To recover cells and to allow expression of the introduced DNA, cells were incubated 3 hr at 28°C without shaking. Cells were centrifuged at 2,600g, 20°C for 3 min and spread onto 2X YT broth containing 1.5% (w/v) bacto-agar, 100 µg·mL^{-1} rifampicin,

400 μg·mL^{-1} streptomycin and an antibioticum selection encoded by the introduced plasmid DNA to allow selection of transformed cells.

2.5   Total RNA Extraction and Northern Blot Analysis

For RNA extraction all materials were incubated overnight at 120°C and all solutions were treated with diethyl pyrocarbonate (DEPC) according to Sambrook et al. (1988). Total RNA was extracted from seedlings according to López-Gómez and Gómez-Lim (1992). In short, 1-2 wk-old seedlings were snap-frozen in liquid nitrogen, ground to a greenish pulp using pestle and mortar, transferred into a pre-chilled eppendorf and stored at temperatures below -70°C. On average 100 mg frozen tissue was homogenised in 1 mL RNA lysis buffer using a 1 mL hand-homogeniser at room temperature. The homogenate was quickly vortexed with 20% final concentration of absolute ethanol and 500 mM final concentration of potassium acetate. Vortexing was continued for 1 min to promote dissociation of polysaccharides from RNA, followed by chloroform-isoamyl alcohol (24:1) extraction and centrifugation (11,600g for 10 minutes). The recovered colourless aqueous phase was extracted with phenol-chloroform-isoamyl alcohol (50:49:1) and with chloroform-isoamyl alcohol (24:1) to extract protein and DNA and re-extract phenol. The aqueous phase was carefully removed, and the RNA was precipitated with LiCl (3 M final concentration) at -20°C overnight. RNA was collected by centrifugation (11,600g for 30 minutes at 4°C). The RNA was dissolved in water, potassium acetate was added to a 0.3 M final concentration, and the RNA precipitated with two of the original volumes of absolute ethanol. After incubation in ethanol for 1 hr at -70°C, the RNA was pelleted by centrifugation (11,600g for 10 min), washed twice with 75% (v/v) ethanol, and dissolved in water. The RNA was quantified spectrophotometrically and yielded approximately 100 μg per 100 mg plant material.

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) in RNA running buffer. Gels were run of 1 hr at 10 V·cm^{-1} afterwhich a photograph was taken under UV illumination. To insure that equal amount of RNA was loaded, the picture of the ethidium bromide-stained gel was scanned and quantified with Imagequant™ (Molecular Dynamics, 's-Hertogenbosch, NL). The 3' untranslated regions or UTR of *NpCaM-1* and *NpCaM-2* were used as DNA hybridisation probes, and hybridised in 4X SSC, 1% (w/v) SDS, 200 mM Tris·HCl (pH 7.6), 10% (w/v) dextran sulfate, 100 μg·mL^{-1} herring sperm, 2X Denhardt's solution, at 65°C overnight and washed 20 min in 2X SSC at 65°C followed by a brief wash in 2X SSC, 1% (w/v) SDS at room

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temperature. Filters were either exposed to Hyperfilm™-MP (Amersham, Buckinghamshire, UK) or a phosphor-plate and imaged with a phosphor-imager (Molecular Dynamics, 's-Hertogenbosch, NL). Intensities of hybridising bands were quantified using Imagequant™ (Molecular Dynamics, 's-Hertogenbosch, NL).

2.6 Amplification of the 3' Untranslated Region by Rapid Amplification of cDNA Ends (RACE)

For amplification of cDNA ends by rapid amplification of cDNA ends, or RACE, cDNA was synthesised from 5 µg total RNA in a RTC buffer, 10 units of RNasin (Promega Biotech, Southampton, UK), 100 ng·µL^{-1} of dT$_{17}$-adapter primer, Q$_T$ (5' CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTT$_{17}$VN 3', with V = G, C, A and N = G, C, T, A), 10 units of SuperScript™ II RNase H$^-$ Reverse transcriptase (Life Technologies, Paisley, UK) in a total volume of 20 µL. The mixture was incubated at 5 min at room temperature, 1 hr at 42°C, 10 min at 50°C and 15 min at 70°C. Then RNA was removed with 0.2 units of RNase H (Life Technologies, Paisley, UK) and the whole reaction diluted with 1 mL TE buffer to produce the cDNA pool for amplification. For amplification a PCR cocktail was prepared (5 µL 10X PCR buffer and 42 µL dH$_2$O, 1 µL adapter primer, Q$_i$ (ACGAGGACTCGAGCTCAAGC, 25 pmol·µL^{-1}), 1 µL of a calmodulin-specific primer, E086 (GCATCACGAC TAAGGAGCTT, 25 pmol·µL^{-1}) and 1 µL cDNA pool. The cDNA was denatured 5 min at 95°C and cooled to 72°C. Then 2.5 units of Taq polymerase, 1.25 units of *pfu* polymerase (Stratagene, Cambridge, UK) and 30 µL mineral oil were added. Primers were annealed and extended at 52 or 56°C for 5 min and 72°C for 40 min to ensure correct replication, respectively followed by a 35 cycles of a sequence of 95°C for 40 sec, 52 or 56°C for 1 min, and 72°C for 3 min followed by a 15 min incubation at 72°C to complete the reaction. *NpCaM-1* and *NpCaM-2* were cloned using a pCR-Script Amp SK(+) Cloning Kit (Stratagene, Cambridge, UK) and used for sequence analysis.

2.7 Sequence Analysis

Sequence analysis was carried out on both the DNA strands *in quadruple*. For each strand sequencing reactions were performed using a ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Cheshire, UK) and sequenced using an automatic sequencer (Perkin-Elmer Corporation, Cheshire, UK).

2.8 Protein Extraction and Western Blot Analysis

For total protein extraction leaf material was snap-frozen in liquid nitrogen and homogenised in Aequorin reconstitution buffer. The homogenate was centrifuged 3 min at 11,600g using a MSE Micro Centaur table centrifuge (Scotlab, Coatbridge, UK) to remove the cell debris before transferring the supernatant into another eppendorf. Determination of the total protein content of the extracts was performed as described by Bradford (1976) using a Biorad protein assay kit (BioRad Life Sciences, Hertfordshire, UK). Ten μL total protein extract for each homogenate was added to 1 mL dye reagent and the optical density measured at 595 nm against a blank containing 10 μL Aequorin reconstitution buffer. Standards were prepared using bovine serum albumin (BSA).

Samples of 3 μg total protein were diluted with 2 μL 2X SDS Loading buffer and incubated at 100°C for 3 min to denature proteins. Samples were chilled on ice, centrifuged at 11,600g, 2 min and loaded onto the 10%-polyacrylamide gel together with 1.5 μL rainbow coloured protein molecular weight markers (Amersham Life Science, Buckinghamshire, UK). Protein gels were run vertically in running buffer at 5 V·cm^{-1} in Biogel mini electrophoresis units (Biotech Instruments Ltd., Bedfordshire, UK). Proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) in Transfer buffer at 120 V, 480 mA for 1 hr using a TE series Transphor Electrophoresis unit (Hoefer Scientific Instruments, San Francisco, USA). For immunodetection, non-specific binding sites on the nitrocellulose membranes were first blocked by overnight incubating in 5% (w/v) Marval non-fat milk powder (Chivers and Sons Ltd., Dublin, IRL) in TBS-T buffer at 4°C. Membranes were briefly washed (1 time 1 hr, 2 times 10 min) in TBS-T before being immersed in a 1:1000 dilution of the primary polyclonal (mouse anti-aequorin antiserum), and incubated at room temperature on a 3D rocking platform STR9 (Stuart Scientific, Dorset, UK) with gentle shaking at 25 rpm for 1 hr. The membranes were washed as before and incubated a further one hr in TBS-T to remove any unbound primary antibody. Membranes were incubated in a 1:5000 dilution of horse radish peroxidase-labelled secondary antibody (sheep anti-mouse antisera; Amersham Life Sciences, Buckinghamshire, UK) for 1 hr at room temperature and excess unbound antibody removed by washing as described before. Detection was performed using an ECL detection kit (Amersham Life Sciences, Buckinghamshire, UK) according to the manufactures' instructions and the membranes exposed to preflashed ECL Hyperfilm™ (Amersham Life Sciences, Buckinghamshire, UK).

2.9   Genetic Transformation of Nicotiana plumbaginifolia

Single colonies of *Agrobacterium tumefaciens* LBA4404 carrying the pBIN19 derivatives containing DNA fragments that upon translation specified a fusion product of nuclear targeted aequorin were inoculated in 5 mL Luria-Bertani broth (Sambrook et al., 1989).   The cultures contained 100 μg·mL^{-1} rifampicin, 500 μg·mL^{-1} streptomycin, and 100 μg·mL^{-1} kanamycin and incubated at 30°C for 48 hr. Expanded leaves from *Nicotiana plumbaginifolia* were surface sterilised with 70 % (v/v) ethanol for 30 s followed by 10 % (w/v) NaOCl for 10 min. Leaves were washed 6 consecutive times in sterile distilled water. Leaf discs were cut and placed in 30 mL shooting medium containing 0.47% (w/v) MS salts (Murashige and Skoog, 1962), 10 mg·mL^{-1} sucrose, 2 mg·mL^{-1} kinetin, and 0.2 mg·mL^{-1} 1-naphthylacetic acid (NAA, adjusted with 1 N KOH to 5.8). One mL of an *Agrobacterium* culture was added and left for 10 min to allow successful infection. Discs were then briefly dried on filter paper and transferred onto plates of shooting medium (0.47% (w/v) MS salts (Murashige and Skoog, 1962), 10 mg·mL^{-1} sucrose, 2 mg·mL^{-1} kinetin, and 0.2 mg·mL^{-1} 1-naphthylacetic acid (NAA, adjusted with 1 N KOH to 5.8, 0.8% (w/v) Difco bacto-agar) containing 500 μg·mL^{-1} carbenicillin (disodium salt), 200 μg·mL^{-1} kanamycin (acid sulphate) and cultured at 25°C, 16 hr photoperiod. Shootlets developed after 3 to 6 weeks on average from inoculation. Developed shootlets were transferred onto plates with expanding medium (0.235% (w/v) MS salts, 5 mg·mL^{-1} sucrose, 250 μg·mL^{-1} carbenicillin, 200 μg·mL^{-1} kanamycin (adjusted with 1 N KOH to 5.8) and 0.8% (w/v) Difco bacto-agar. After 2 weeks, those that continued to expand were transferred into 50 mL sterile capped tubes, containing rooting medium (0.235% (w/v) MS salts, 100 μg·mL^{-1} carbenicillin, 100 μg·mL^{-1} kanamycin (adjusted with 1 N KOH to 5.8) and 0.8% (w/v) Difco bacto-agar). Shootlet began to root after 2 weeks on rooting media. Rooting was continued until the plantlets had developed an extensive root system (between 1 and 2 weeks). These were potted into M3 compost (Levington Horticulture Ltd., Ipswich, UK) and kept covered with Saran Rab (Genetic Research Instrumentation, Essex, UK). The plantlets were grown in the conditions as described above. The Saran Rab was gradually pierced to allow the transformants to become accustomed to their new environment. After approximately 5 weeks under greenhouse conditions (25°C, 16 hr photoperiod) the seeds from each plant were collected and used for further analysis.

2.10   In vivo and In vitro Reconstitution of Aequorin

Seedlings germinated and were grown for 7 days under conditions described before. At this stage the average height of the seedlings, including roots, was 8 mm and no true leaves had yet appeared. Aequorin was reconstituted by floating seedlings on water containing 2 µM coelenterazine for at least 4 hr in the dark according to Knight et al., 1991 or by the application of a droplet of 3 µL of 2 µM coelenterazine onto the cotelydons. When calcium agonists/antagonists were used, seedlings were submerged and incubated for 6 hr. Following this treatment the liquid was drained and a droplet of 3 µL of 2 µM coelenterazine with the relevant inhibitor was placed between the cotyledons and left for at least another 4 hr in the dark after which time the liquid was removed and calcium measurement was carried out.   Seedlings treated with coelenterazine at a concentration of 2 µM or in the presence of the calcium modulators showed no signs of toxicity when compared with untreated seedlings (Knight et al., 1991 and data not shown). In order to estimate the amount of aequorin reconstituted *in vivo*, single 7 days old transgenic seedlings were homogenised in 400 µL Aequorin reconstitution buffer. Reconstituted aequorin was discharged by the addition of an equal volume of 100 mM $CaCl_2$ and the total amount of luminescence produced over 10 s was integrated.   Luminescence measurements were made using a digital chemiluminometer with an EMI photomultiplier model 9757AM at 1 kV with a discriminator (Campbell et al., 1985) or EMI photomultiplier model 9829A with an EMI FACT50 cooling system (Badminton et al., 1995). For *in vitro* reconstitution, tissue or lysed protoplasts were first homogenised in Aequorin reconstitution buffer and incubated with coelenterazine for at least 4 hr, and discharged as described above.

2.11   Mechanical, Cold Shock and Heat Shock Stimulation of Aequorin Transgenic Tobacco Seedlings

Wind stimulation was simulated by instantly injecting 5 mL air with a syringe into the sample housing of the luminometer. Cold shock was simulated by injection of 1 mL ice-cold water with a syringe into the sample housing at low velocity to prevent any mechanical disturbance. Heat shock was simulated by placing cuvettes containing transgenic seedlings in a waterbath of a set temperature. The cuvettes were removed at specific times and the luminescence of the seedlings was integrated numerically over 15 s in the chemiluminometer. A thermocouple was placed in the cuvette and actual temperature in the cuvette during heat treatment was recorded. After the luminescence

measurement, cuvettes were placed back into the waterbath to continue heat shock treatment and allow subsequent luminescence measurements.

For wind and cold shock stimulation aequorin was *in vivo* reconstituted as described before and the luminescent light was calibrated into Ca^{2+} concentration by a method based on the calibration curve of Allen and co-workers (1977). The relationship between light and the calcium concentration is non-linear. At the maximum slope of the response, a change in the calcium concentration of 10 fold result in a 300 fold increase in light production. To determine the Ca^{2+} concentrations *in vivo* equation 1 was used. L represents the amount of light per second emitted, L_{max} as total amount of light present in the entire sample over the course of the experiment. The $[Ca^{2+}]$ represents the calculated Ca^{2+} concentration, K_R is the dissociation constant for the first Ca^{2+} ion and K_{TR} for the second Ca^{2+} ion to bind to aequorin. For *cp*-coelenterazine, the values were $26 \cdot 10^6$ M^{-1} for K_R and 57 M^{-1} for K_{TR} and for native coelenterazine $2 \cdot 10^6$ M^{-1} for K_R and 55 M^{-1} for K_{TR}.

$$\text{Equation 1:} \quad L/L_{max} = ((1 + K_R \cdot [Ca^{2+}]) / (1 + K_{TR} + K_R \cdot [Ca^{2+}]))^3$$

For calibration of the heat shock measurements of Ca^{2+} levels, the seedlings were homogenised in Aequorin reconstitution buffer and discharged with an excess of Ca^{2+} in the luminometer. Each group of MAQ 2.4 seedlings produced on average about 120,000 counts $\pm$ 16,000 with a discharge rate of 8,063 $\pm$ 1077 counts·s^{-1} when loaded with coelenterazine via the cotyledons whereas MAQ 7.11 produced on average 25,000 counts $\pm$ 2,000 with a discharge rate of 1,778 $\pm$ 90 counts·s^{-1}. The ratio of the discharge rate, L/L_{max} can be used as a value for measure the actual pCa value (Allen et al., 1977). From the luminescence data, cytosolic and nuclear pCa values were calculated using equation 2 (Knight et al., 1996) with L/L_{max} represents the differences in the rates of discharge between the intracellular Ca^{2+} increase and the total discharge by excess of Ca^{2+} at the end of the experiment.

$$\text{Equation 2:} \quad pCa = 0.332588 \cdot -(\log L/L_{max}) + 5.5593$$

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2.12   Subcellular Fractionation

Protoplasts were obtained by digesting the cell walls of 6 young leaves in 1% (w/v) Cellulase, 0.2% (w/v) Macerozyme and 0.4 M mannitol, on a 3D rocking platform STR9 (Stuart Scientific, Dorset, UK) with gentle shaking at 7 rpm overnight in darkness. Protoplasts were washed in 50 mM MOPS (pH 7.8), 0.15% (w/v) BSA, 0.3 M mannitol, 1.0 mM $MgCl_2$ and 2.5 mM DTT and lysed by passing through a 25 μm nylon filter. Staining with the DNA dye, diamidino-2-phenylindole (DAPI), showed >90% intact nuclei, which were pelleted by centrifugation at 100g. The crude nuclear fractions were examined for aequorin activity.

2.13   Immunolocalisation of Aequorin using FITC-Labelled Antibodies

Protoplasts were washed and collected in 0.5% (w/v) MES (pH 5.8), 80 mM $CaCl_2$, 0.3 M mannitol and fixed for 15 min on poly-L-lysine treated slides using 4% (w/v) paraformaldehyde. Cells were permeabilised 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, 0.3 M mannitol. Specimens were blocked for 5 min with 1% (w/v) BSA followed by a 1.5 hr incubation at 37°C with mouse antiserum raised against aequorin (1:1000) and with secondary FITC-labelled goat anti-mouse IgG solution (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 and photographed with a Polyvar epifluorescence microscope using Kodak Ektachrome T (64) film.

2.14   Immunolocalisation of Apoaequorin using Immuno-Gold-Labelled Antibodies

Fresh protoplasts were prepared as described before. Protoplasts were washed twice in 0.5% (w/v) MES (pH 5.8), 80 mM $CaCl_2$, 0.3 M mannitol and pelleted by a 200g centrifugation for 5 min. The protoplasts were fixed for 15 min using 1/4 strength Karnovsky's fixative (Karnovsky, 1965) in 0.3 M Mannitol, 10 mM MES, 80 mM $CaCl_2$ and 1 unit per 10 mL Protease Inhibitor (pH 5.7), washed twice to replace the fixation solution and stored overnight at 4°C. The fixed tissue was dehydrated by consecutively 10 min incubations in 30, 50, 70, and 90% (v/v) ethanol at room temperature, and for 20 min in three changes in dehydrated absolute ethanol followed by propylene oxide (twice for 15 min). The embedding of fixed and dehydrated tissue was carried out Agar 100 resin (Agar Scientific, Essex, UK). Thin sections (80-90 nm) placed on gold were incubated in 1% (w/v) BSA in PBS for 5 min at room

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temperature. Sections were incubated with mouse antiserum raised against aequorin (1:200) for 2 hr at room temperature or for 18-24 hr at about 4°C and kept in a moist chamber. The antisera or immunosorbent-purified antibodies were diluted in 1% (w/v) BSA in PBS (pH 7.4). The grids were placed on drops of a 20 fold dilution of a 1 nM gold-labelled goat-anti-mouse IgG solution from British BioCell International (Cardiff, UK) for 1 hr at room temperature in a moist chamber. The sections were stained with 5% (v/v) aqueous uranyl acetate (5-7 min), 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 (Sigma, Dorset, UK) before examination using an electron microscope type JEOL 100S.

2.15   Effect of External Calcium and EGTA on Thermotolerance of Tobacco Seedlings

Sterilised tobacco (*Nicotiana plumbaginifolia*, wild type) seeds were sown in plastic petridishes with three compartments, each containing 8 mL half-strength MS medium and 0.8% (w/v) agar, and germinated at 25°C with a 16 hr photoperiod for 2 weeks. For Ca^{2+} or EGTA treatment, 5 mL of sterilised distilled water (control), 10 mM $CaCl_2$ or EGTA (the pH of EGTA solution was adjusted to 6.9) were added to one of three compartments in a same petridish and kept overnight at 25°C. At the end of the incubation, the solutions or distilled water were drained and the seedlings were transferred from 25°C to 38°C or 40°C in an incubator for 2 hr for heat shock treatment, then returned to 25°C for a 4 hr recovery period, and transferred again to 48°C for 4 hr or 50°C for 2 hr and 20 min (140 min) for heat treatment. The control seedlings without initial heat shock treatments were transferred directly from 25°C to 48 or 50°C. After the heat treatments, the seedlings were cultured again at 25°C, with a 16 hr photoperiod for 8 days. In preliminary experiments, 8 days of recovery at 25°C for heat-treated seedlings was sufficient to easily discriminate between seedlings which were alive and those which were dead. Dead seedlings lacking chlorophyll and turgor and with supine often-dry hypocotyls could easily be recognised whilst viable seedlings retained turgor, green leaves and continued to grow.

Chapter 3

Nuclear Targeting of Apoaequorin

The cell is a dynamic entity, molecular messages converge upon cells and evoke intracellular changes. A cascade of events is induced in the cytoplasm and often directed towards the nucleus to affect nuclear processes. An understanding of how these events are transduced into the nuclei remain elusive, due to a lack of knowledge about nuclear transport and the conveyance between cytoplasm and nucleoplasm.

The nucleus is isolated from the cytoplasm by a double membrane, the nuclear envelope, that forms the interface between the nucleoplasm and cytoplasm (Fabre and Hurt, 1994, Powers and Forbes, 1994). Electron microscopy showed that the lumen of the nuclear envelope and the lumen of the endoplasmic reticulum are connected. The outer membrane is continuous with the rough endoplasmic reticulum, and is similar to it with respect to composition and the ability to bind ribosomes. The inner nuclear membrane, although connected to the outer membrane at the nuclear pore complexes, differs from the rough endoplasmic reticulum, and contains its own set of proteins (Goldberg and Allen, 1995). The nuclear envelope is perforated by nuclear pore complexes or NPCs that serve as channels for molecular exchange between the nucleus and cytoplasm. The structure and function of the NPCs appear to be conserved in all eukaryotes studied so far indicating its central role in cellular functioning. The NPC is about 120 nm in diameter, composed of at least 30 distinct protein components in varying stoichiometries and has an approximate mass of 124 MDa (Reichelt et al., 1990). The NPC properties appear to be regulated in response to mitogenic signals (Feldherr and Akin, 1991), probably mediated by the phosphorylation of NPC components (Macaulay et al., 1995) or by changes in the Ca^{2+} concentration within the ER and nuclear envelope lumen (Greber and Gerace, 1995). The NPC acts as primary arbiter of which signals enter the nucleus and which do not (Forbes, 1992). Large molecules are excluded from the nucleus, while small proteins (<20-40 kDa) quickly equilibrate between nucleoplasm and cytoplasm. It has been established that larger nuclear proteins must contain a nuclear localisation signal, NLS in order to be specifically imported through the nuclear pore complex. Gold particles upto 250 Å in diameter coated with NLS-bearing nuclear proteins, have been shown to be transported into the nucleus through the nuclear pore complex (Feldherr et al., 1984). NLSs are recognised and bound by a signal recognition particle or cytosolic receptor that docks the protein to the NPC. NLSs can be grouped in three classes; SV40 large T-antigen-like and bipartite classes of NLS have been found in animals, fungi, and plants, and a

Mat α2-like NLS class that has been found exclusively in plants and fungi (Hicks et al., 1993; 1995). Experimentally, protein import into the nucleus is divided into two distinct steps, docking and translocation. Docking is NLS-dependent and occurs when nuclear proteins bind at the cytoplasmic side of the NPC in an energy-independent fashion. Translocation is an energy-dependent process involving ATP-hydrolysis (Newmeyer et al., 1986; Richardson et al., 1988). *In vitro* import systems using permeabilised vertebrate cells suggested that some of the factors that are necessary for import are soluble (Moore and Blobel, 1993). Four factors were identified that mediate the import *in vitro*; importin α, importin ß, Ran/TC4, and p10 (Hicks and Raikhel, 1995; Görlich and Mattaj, 1996). Nuclear import occurs when a heterodimer of importins α and ß binds to an NLS-containing protein in the cytoplasm via the NLS-binding region of importin α (Radu et al., 1995). Importin ß mediates the docking of the trimeric complex to the cytoplasmic side of the NPC (Radu et al., 1995). Translocation of the trimeric complex through the NPC requires free GTP (Görlich et al., 1996), p10 (Moore and Blobel, 1994) and a small GTPase, Ran (Görlich et al., 1996). After translocation, dissociation of importin α with the NLS-containing protein may lead to the release of importin α into the cytoplasm, where it can participate in another cycle of import. In plants, NLS-binding sites at the NPC and nuclear envelope have recently been characterised (Hicks and Raikhel, 1993; Hicks et al., 1995; Hicks and Raikhel, 1995). *At-IMPα* was cloned from *Arabidopsis thaliana* (Hicks et al., 1996) that is 45-56% identical to other importin α sequences found in vertebrates, fungi and insects and functions as an NLS receptor in plants by recognising all three classes of import signals (Smith et al., 1997).

Three modes of protein transport across nuclear envelopes have been identified: diffusion, facilitated transport and active transport. Proteins larger than 60 kDa accumulate in the nucleus either through active transport if they contain a NLS, or by facilitated transport if they contain sequences allowing them to bind inside the nucleus (Hunt, 1989, Dingwall and Laskey, 1991, Paine, 1993). The NLSs from nuclear targeting proteins are not removed proteolytically upon translocation and thereby retains the ability to re-enter the nucleus after cell division. Targeting signals for chloroplasmic (Keegstra, 1989), mitochondrial (Hartl and Neupert, 1990), and vacuolar (Chrispeels and Raikhel, 1992) proteins are removed upon translocation. A comparison between numerous NLSs has shown no clear consensus in the amino acid sequence (BOX 3.I; Dingwall and Laskey, 1991). The sequences appear to consist in short stretches of positively charged amino acids (lysine and arginine). Although the amino acid sequences of NLS vary, they seem to have the same nuclear transport pathway (Burke, 1990, Silver, 1991). Microinjection of a transcriptional activator

protein from *Saccharomyces cerevisiae*, Mcm1 into *Xenopus laevis* frog oocytes resulted in the import of Mcm1 into the oocyte nucleus indicating that nuclear protein transport is functionally conserved between yeast and higher eukaryotes (Wagner and Hall, 1993).

BOX 3.I: A summary of some characterised nuclear localisation signals or NLSs from yeast to higher eukaryotes. Amino acids important in nuclear targeting are in bold. SV40-NLS, the NLS from simian virus 40 large T-antigen (Kalderon et al., 1984); nucleoplasmin, nuclear abundant structural protein from *Xenopus laevis* frog oocytes (Robbins et al., 1991); Mcm1, transcriptional activator protein from *Saccharamyces cerevisiae* (Passmore et al., 1988); GCN4, transcriptional activator protein from *Saccharomyces cerevisiae* (Hinnebusch, 1984); Opaque2, transcriptional activator protein from *Zea mays* (Varagona et al., 1992); TGA-1A, transcriptional activator protein from tobacco (Katagiri et al., 1989); TAF-1, transcriptional activator protein from tobacco (Oeda et al., 1991); C-FOS, transcriptional activator protein from human (VanStraaten et al., 1983).

SV40 (large T-antigen)	PP**KKKRK**V
Nucleoplasmin (*Xenopus laevis*)	**KRPAATKKAGQAKKKKL**
MCM1 (*Saccharomyces cerevisiae*)	**KERRKIEIKFIENKTRRH**
GCN4 (*Saccharomyces cerevisiae*)	AL**KRARNTEAARRSRARKL**
Opaque2 (*Zea mays*)	V**RKRKESNRESARRSRYRK**
TGA-1A (tobacco)	V**LRRLAQNREAARKSRLRK**
TAF-1 (tobacco)	RE**KRKQSNRESARRSRLRK**
C-FOS (human)	R**IRRERNKMAAAKCRNRRR**

One of the earliest recognised NLS as import signal is the NLS of the simian virus 40 (SV40) large T-antigen (BOX 3.I) and comprises a short stretch of basic amino acids, PKKKRKV. The signal has been shown to be sufficient to target several proteins to the mammalian and plant nuclei (Kalderon et al., 1984; Restrepo et al., 1990, Varagona et al., 1991, Hicks et al., 1995). Van der Krol and Chua (1991) constructed fusion genes encoding chimaeric proteins in which various NLSs were placed in frame to the N-terminus of the ß-glucuronidase (GUS) reporter protein. Plants transgenic for these constructs revealed that the 7 amino acids containing NLS of the SV40 was sufficient to facilitate nuclear import of the GUS protein. Rihs and Peters (1989) suggested that 15 amino acids immediately preceding the NLS increase the efficiency of the existing NLS. It is unknown what enhances the effectively of import but phosphorylation sites and the importance of the hydropathic character of the sequences flanking the NLS have been suggested (Roberts et al., 1987). Placement of the SV40 T-antigen NLS in the hydrophobic domain of pyruvate kinase creates a non-nuclear protein, indicating that the NLS must be accessible to interact with components of the import machinery. In addition, nuclear localisation signals may be masked by subunit interaction or binding with other proteins.

The major nuclear protein of *Xenopus* oocytes and embryos, nucleoplasmin contains another type of nuclear targeting sequence and several studies on its function including chimaeric protein fusions have been performed (Robbins et al., 1991;

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Badminton et al., 1995). The protein is a pentamer of 29 kDa subunits and can bind to the four histones that build up nucleosomes (H2A, H2B, H3, and H4) and it is believed to catalyses the disassembly and re-assembly of nucleosomes *in vivo* (Philpott and Leno, 1992). Furthermore, it stimulates the binding of transcription factors to nucleosomes (Chen et al., 1994). The carboxy-terminal 'tail' region of nucleoplasmin contains two sequences of basic amino acids which are strikingly similar to the SV40 nuclear targeting sequence that are separated by a spacer (BOX 3.I), but neither of these stretches can target pyruvate kinase to the nucleus independently (Robbins et al., 1991). The motif is known to be bipartite and has also been recognised in DNA binding domains of nuclear proteins (BOX 3.I).

The extent of translocation of proteins towards the nucleus might be influenced by phosphorylation (Hunt, 1989, Whiteside and Goodbourn, 1993) and mediated by calcium and calmodulin (Luo et al., 1996). Phosphorylation of specific sites is observed during cell-cycle dependent control of nuclear entry of oncogene products and transcription factors. Nucleoplasmin is phosphorylated during accumulation in the nucleus (Vancurova et al., 1995). The kinetics of the cytoplasm-nucleus transport is affected by the degree of phosphorylation of nucleoplasmin by casein kinase II, a protein that has also been identified in plants (Brusa et al., 1994).

Paine (1993) designated the uptake of nucleoplasmin into nuclei facilitated transport for binding to nuclear compounds, like DNA, may allow the entry of protein down its chemical gradient (Paine, 1993, Vancurova et al., 1993). The uptake of nuclear proteins into nuclei by facilitated transport might account for the uptake of a wide variety of nuclear transcription factors. Another kind of eukaryotic protein known to interact with DNA, has recently been recognised, is known as the high mobility group or HMG (Goodwin et al., 1973). HMG proteins consist in a large number of basic and acidic amino acid residues (Fig. 3.3). HMG proteins have also been identified in plants and their function have been related to HMG proteins in animals (Grasser et al., 1993; Grasser, 1995). Because of their relative abundance, sequence conservation between species and apparent lack of sequence specificity in binding to DNA, they are believed to perform general functions in chromatin, such as structural roles or possibly as general transcription factors (Bustin, 1990, Weir et al., 1993). The HMG proteins consist of HMG domains that have been recognised in a series of transcription factors, sex-determining proteins and other proteins associated with DNA. Studies on HMG and its analogues revealed that they have a major structural role in the bending or looping DNA: such contortions are increasingly found to be required for achieving the correct conformation for transcription and various classes of DNA rearrangements. HMG1 proteins are DNA-binding proteins, with

affinities to sites that are known as AT-hooks (Reeves and Nissen, 1990), and bind in the minor groove of AT tracts of double-stranded DNA (Solomon et al., 1986; Geierstanger et al., 1994). These motifs are found in TATA boxes, homeodomain binding sites, serum response elements, matrix attachment regions, and others. The significant binding within the nucleus of this protein might direct the DNA-binding protein into the nucleus.

In order to investigate the role of nuclear calcium during cell signalling, chimaeric DNA constructs were synthesised which upon translation specified fusion products consisting of the calcium-sensitive bioluminescent protein, apoaequorin and nuclear localisation signals. The NLSs of simian virus 40 large T-antigen (SV40), high mobility group 1 (HMG1) from *Pisum sativum* and nucleoplasmin from *Xenopus laevis* were used. Upon genetic transformation of *Nicotiana plumbaginifolia* the transgenic plant with the highest expression was selected using *in vitro* reconstitution and western blot analysis and precise localisation of the construct was determined by subcellular fractionation and immunolocalisation. These plants together with plants expressing cytosolic aequorin (MAQ 2.4; Knight et al., 1991) were used to study changes in the spatio-temporal calcium response upon exposure to wind and cold shock.

N.B. Preparation of the plasmid containing a construct that encodes the nucleoplasmin apoaequorin fusion protein and transformation into *Nicotiana plumbaginifolia* were carried out by Dr. Claudio Olivari.

3.1   Construction of Nuclear Targeted Apoaequorin Chimaeric cDNAs

To synthesis chimaeric DNA constructs which upon translation specified fusion products consisting of the apoaequorin and nuclear localisation signals for nuclear targeting of aequorin, 5 different constructs were made; two contained SV40 fused to ß-glucuronidase (GUS), two contained HMG1 and one contained nucleoplasmin. The nuclear localisation signals were placed in frame with the cDNA of apoaequorin in the plasmids, pCASAQ.SP and pCASMTAQ.SP that were provided by Dr. Marc R. Knight (University of Oxford, Oxford, UK). These plasmids derive from pHD51 (Pietrzak et al., 1986) that contains the cauliflower mosaic virus (CaMV) 35S transcription promoter and terminator, and the wild-type cDNA of apoaequorin and the mutated derivative respectively (Kendall et al., 1992). The mutated derivative pCASMTAQ has asparagine replaced with alanine at position 119 to produce an active photoprotein with a Ca^{2+} affinity reduced by a factor 20 compared to recombinant aequorin (Kendall et al., 1992). The NLS of the large T-antigen of SV40 was fused to the cDNA that encodes the ß-glucuronidase (GUS) reporter protein in order to facilitate cellular localisation of the fusion protein in plants after transformation. In order to prepare the cDNA of simian virus large T-antigen SV40 linked to GUS coding region from pBI101 (Jefferson et al., 1986) and the cDNA of the high mobility group 1 (HMG1) from *Pisum sativum*, PCR amplification was performed as described in chapter 2 and using primers as indicated in BOX 3.II. The cDNA of HMG1 from *Pisum sativum* is present in a derivative of pUC19 and was kindly provided by Dr. John Gray (University of Cambridge, Cambridge, UK). The PCR fragments were subcloned in frame with the cDNA encoding apoaequorin in pCASAQ.SP and pCASMTAQ.SP as *SalI-XbaI* fragment to produce pCAH and pCAL for SV40-GUS and pCHAH and pCHAL for HMG1, respectively (Fig. 3.1). In a fifth construct, the cDNA of nucleoplasmin from *Xenopus laevis* was placed in frame with the cDNA of apoaequorin by PCR and cloned into pCASAQ.SP (Pietrzak et al., 1986) as a *SmaI-SalI* fragment (Fig. 3.5). The cDNA encoding nucleoplasmin in pSV7d was kindly provided by Dr. M. Badminton (University of Cardiff).

All constructs were subcloned as EcoRI fragments into pBIN19 (Fig. 3.1; Bevan, 1984) to give rise to pBAH, pBHAH, pBAL, pBAH and pBON, respectively (not shown), and transformed into *Nicotiana plumbaginifolia* using *Agrobacterium tumefaciens* LBA4404 by leaf disc transformation according to Draper et al. (1988) as described in Chapter 2. The constructs were analysed using digestion analysis according to Sambrook et al. (1989) as described in chapter 2. In figures 3.2 and 3.3 the sequences of the cDNA encoding GUS from *Escherichia coli* in pBI101 and HMG1 from *Pisum sativum* are shown respectively. In figure 3.4 and 3.5 the sequence encoding nucleoplasmin from *Xenopus laevis* and apoaequorin from *Aequorea victoria* are shown (Prasher et al., 1987).

BOX 3.II: Primers shown in 5'—>3' orientation that were used for the cloning strategy. Restriction site are shown in italics; overhanging sequence as plain text; restriction site in italics; sequence annealing to template in bold. MKA and MKB were kindly provided by Dr. Marc R. Knight.

```
MKA:
GGTCTAGACA ATG GCT CCC AAG AAG AAG AGA AAG GTA CCG GGT GGT CAG TCC CTT ATG
    XbaI     M   A   P   K   K   K   R   K   V   P   G   G   Q   S   L   M
             |————————————————————— SV40 —————————————————————|


MKB:
CCGTCGACCCCA TTG TTT GCC TCC CTG CTG
    SalI      Q   K   G   G   Q   Q


Xhmg:
ATGCTCTAGAGCA CATC ATG AAA GGA GGA AAG TCC
     XbaI            M   K   G   G   K   S


Shmg:
AACGACGTCGACGCGT CTC GTC ATC ATC CTC CTC CTC
     SalI          E   D   D   D   E   E   E
```

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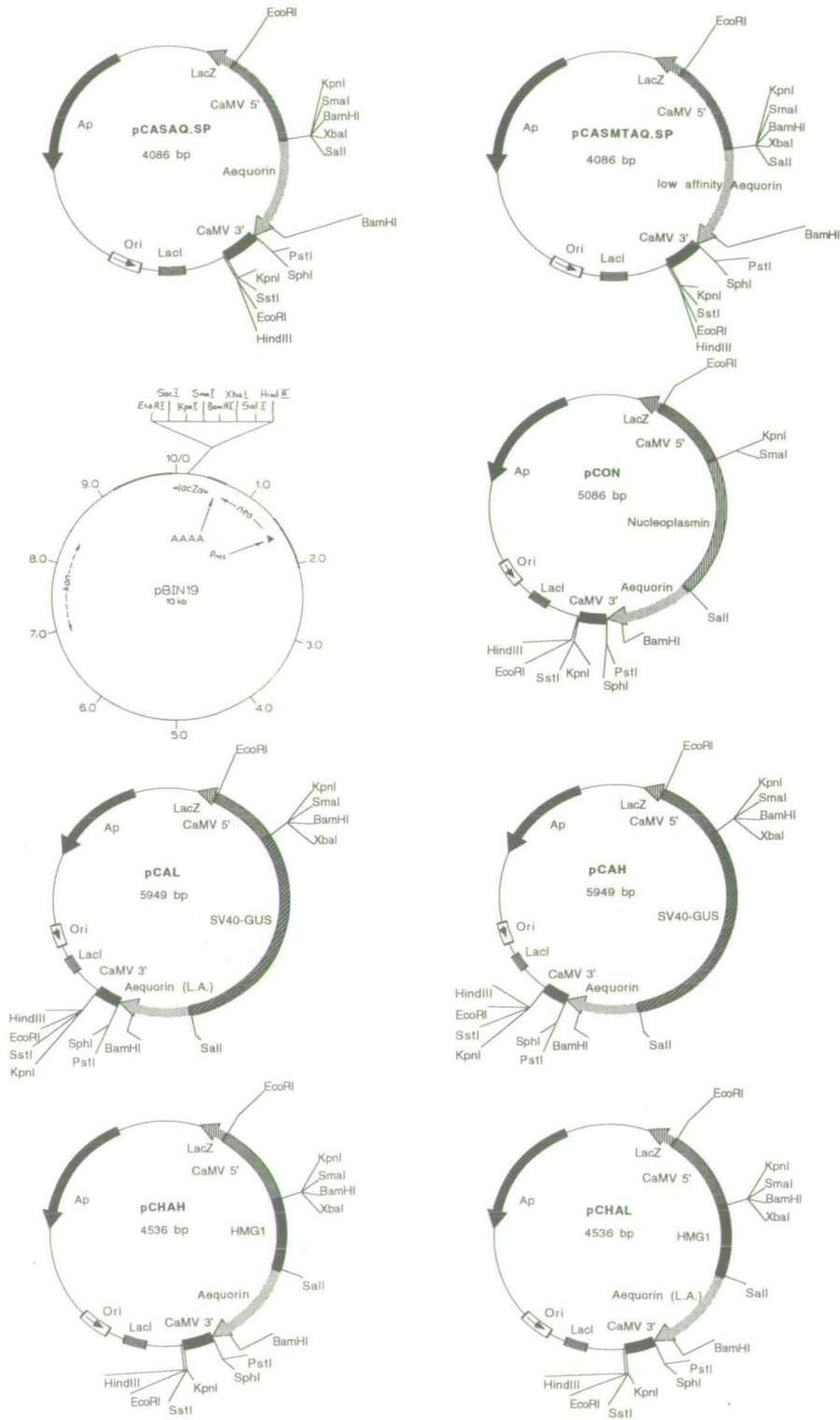
Figure 3.1: Plasmids used for constructing nuclear targeting of apoaequorin constructs. Plasmids derived from pDH51. The plasmids: **pCASAQ.SP** carries the cDNA of apoaequorin directed by CaMV 35S promoter; **pCASMTAQ.SP** carries the cDNA of the mutated cDNA of apoaequorin; **pBIN19** is the binary vector used to transform *Agrobacterium tumefaciens*; **pCON** carries the cDNA of nucleoplasmin in frame with the cDNA of aequorin; **pCAL** carries the cDNA of SV40-GUS in frame with the mutated cDNA of aequorin; **pCAH**; carries the cDNA of SV40-GUS in frame with aequorin; **pCHAH** carries the cDNA of HMG1 from pea in frame with aequorin; **pCHAL** carries the cDNA of HGM1 in frame with the mutated cDNA of aequorin.

```
      CTCTAGAGGATCCCCGGGTAGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCC
  1   ---------+---------+---------+---------+---------+---------+  60
      GAGATCTCCTAGGGGCCCATCCAGTCAGGGAATACAATGCAGGACATCTTTGGGGTTGGG
                                          M  L  R  P  V  E  T  P  T  R

      GTGAAATCAAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAA
 61   ---------+---------+---------+---------+---------+---------+  120
      CACTTTAGTTTTTTTGAGCTGCCGGACACCCGTAAGTCAGACCTAGCGCTTTTGACACCTT
       E  I  K  K  L  D  G  L  W  A  F  S  L  D  R  E  N  C  G  I

      TTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCA
121   ---------+---------+---------+---------+---------+---------+  180
      AACTAGTCGCAACCACCCTTTCGCGCAATGTTCTTTCGGCCCGTTAACGACACGGTCCGT
       D  Q  R  W  W  E  S  A  L  Q  E  S  R  A  I  A  V  P  G  S

      GTTTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATC
181   ---------+---------+---------+---------+---------+---------+  240
      CAAAATTGCTAGTCAAGCGGCTACGTCTATAAGCATTAATACGCCCGTTGCAGACCATAG
       F  N  D  Q  F  A  D  A  D  I  R  N  Y  A  G  N  V  W  Y  Q

      AGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATG
241   ---------+---------+---------+---------+---------+---------+  300
      TCGCGCTTCAGAAATATGGCTTTCCAACCCGTCCGGTCGCATAGCACGACGCAAAGCTAC
       R  E  V  F  I  P  K  G  W  A  G  Q  R  I  V  L  R  F  D  A

      CGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCAGGGCG
301   ---------+---------+---------+---------+---------+---------+  360
      GCCAGTGAGTAATGCCGTTTCACACCCAGTTATTAGTCCTTCACTACCTCGTAGTCCCGC
       V  T  H  Y  G  K  V  W  V  N  N  Q  E  V  M  E  H  Q  G  G

      GCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAGTGTACGTA
361   ---------+---------+---------+---------+---------+---------+  420
      CGATATGCGGTAAACTTCGGCTACAGTGCGGCATACAATAACGGCCCTTTTCACATGCAT
       Y  T  P  F  E  A  D  V  T  P  Y  V  I  A  G  K  S  V  R  I

      TCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCCGCCGGGAATGGTGATTA
421   ---------+---------+---------+---------+---------+---------+  480
      AGTGGCAAACACACTTGTTGCTTGACTTGACCGTCTGATAGGGCGGCCCTTACCACTAAT
       T  V  C  V  N  N  E  L  N  W  Q  T  I  P  P  G  M  V  I  T

      CCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGAA
481   ---------+---------+---------+---------+---------+---------+  540
      GGCTGCTTTTGCCGTTCTTTTTCGTCAGAATGAAGGTACTAAAGAAATTGATACGGCCTT
       D  E  N  G  K  K  K  Q  S  Y  F  H  D  F  F  N  Y  A  G  I

      TCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTGGACGATATCACCGTGG
541   ---------+---------+---------+---------+---------+---------+  600
      AGGTAGCGTCGCATTACGAGATGTGGTGCGGCTTGTGGACCCACCTGCTATAGTGGCACC
       H  R  S  V  M  L  Y  T  T  P  N  T  W  V  D  D  I  T  V  V

      TGACGCATGTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATG
601   ---------+---------+---------+---------+---------+---------+  660
      ACTGCGTACAGCGCGTTCTGACATTGGTGCGCAGACAACTGACCGTCCACCACCGGTTAC
       T  H  V  A  Q  D  C  N  H  A  S  V  D  W  Q  V  V  A  N  G

      GTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAGGCA
661   ---------+---------+---------+---------+---------+---------+  720
      CACTACAGTCGCAACTTGACGCACTACGCCTAGTTGTCCACCAACGTTGACCTGTTCCGT
       D  V  S  V  E  L  R  D  A  D  Q  Q  V  V  A  T  G  Q  G  T

      CTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCT
721   ---------+---------+---------+---------+---------+---------+  800
      GATCGCCCTGAAACGTTCACCACTTAGGCGTGGAGACCGTTGGCCCACTTCCAATAGAGA
       S  G  T  L  Q  V  V  N  P  H  L  W  Q  P  G  E  G  Y  L  Y

      ATGAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGTGATATCTACCCGCTTCGCGTCG
801   ---------+---------+---------+---------+---------+---------+  860
      TACTTGACACGCAGTGTCGGTTTTCGGTCTGTCTCACACTATAGATGGGCGAAGCGCAGC
       E  L  C  V  T  A  K  S  Q  T  E  C  D  I  Y  P  L  R  V  G

      GCATCCGGTCAGTGGCAGTGAAGGGCCAACAGTTCCTGATTAACCACAAACCGTTCTACT
861   ---------+---------+---------+---------+---------+---------+  920
      CGTAGGCCAGTCACCGTCACTTCCCGGTTGTCAAGGACTAATTGGTGTTTGGCAAGATGA
       I  R  S  V  A  V  K  G  Q  Q  F  L  I  N  H  K  P  F  Y  F

      TTACTGGCTTTGGTCGTCATGAAGATGCGGACTTACGTGGCAAAGGATTCGATAACGTGC
921   ---------+---------+---------+---------+---------+---------+  980
      AATGACCGAAACCAGCAGTACTTCTACGCCTGAATGCACCGTTTCCTAAGCTATTGCACG
       T  G  F  G  R  H  E  D  A  D  L  R  G  K  G  F  D  N  V  L
```

47

```
      TGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGC
 981  --------+---------+---------+---------+---------+---------+  1040
      ACTACCACGTGCTGGTGCGTAATTACCTGACCTAACCCCGGTTGAGGATGGCATGGAGCG
       M  V  H  D  H  A  L  M  D  W  I  G  A  N  S  Y  R  T  S  H

      ATTACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTGGTGATTG
1041  --------+---------+---------+---------+---------+---------+  1100
      TAATGGGAATGCGACTTCTCTACGAGCTGACCCGTCTACTTGTACCGTAGCACCACTAAC
       Y  P  Y  A  E  E  M  L  D  W  A  D  E  H  G  I  V  V  I  D

      ATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTTTCGAAGCGGGCAACA
1101  --------+---------+---------+---------+---------+---------+  1160
      TACTTTGACGACGACAGCCGAAATTGGAGAGAAATCCGTAACCAAAGCTTCGCCCGTTGT
       E  T  A  A  V  G  F  N  L  S  L  G  I  G  F  E  A  G  N  K

      AGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGCACTTAC
1161  --------+---------+---------+---------+---------+---------+  1120
      TCGGCTTTCTTGACATGTCGCTTCTCCGTCAGTTGCCCCTTTGAGTCGTTCGCGTGAATG
       P  K  E  L  Y  S  E  E  A  V  N  G  E  T  Q  Q  A  H  L  Q

      AGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGCGTGGTGATGTGGAGTA
1121  --------+---------+---------+---------+---------+---------+  1180
      TCCGCTAATTTCTCGACTATCGCGCACTGTTTTTGGTGGGTTCGCACCACTACACCTCAT
       A  I  K  E  L  I  A  R  D  K  N  H  P  S  V  V  M  W  S  I

      TTGCCAACGAACCGGATACCCGTCCGCAAGTGCACGGGAATATTTCGCCACTGGCGGAAG
1181  --------+---------+---------+---------+---------+---------+  1140
      AACGGTTGCTTGGCCTATGGGCAGGCGTTCACGTGCCCTTATAAAGCGGTGACCGCCTTC
       A  N  E  P  D  T  R  P  Q  V  H  G  N  I  S  P  L  A  E  A

      CAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACG
1141  --------+---------+---------+---------+---------+---------+  1200
      GTTGCGCATTTGAGCTGGGCTGCGCAGGCTAGTGGACGCAGTTACATTACAAGACGCTGC
       T  R  K  L  D  P  T  R  P  I  T  C  V  N  V  M  F  C  D  A

      CTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAACCGTTATTACGGAT
1201  --------+---------+---------+---------+---------+---------+  1260
      GAGTGTGGCTATGGTAGTCGCTAGAGAAACTACACGACACGGACTTGGCAATAATGCCTA
       H  T  D  T  I  S  D  L  F  D  V  L  C  L  N  R  Y  Y  G  W

      GGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGG
1261  --------+---------+---------+---------+---------+---------+  1220
      CCATACAGGTTTCGCCGCTAAACCTTTGCCGTCTCTTCCATGACCTTTTTCTTGAAGACC
       Y  V  Q  S  G  D  L  E  T  A  E  K  V  L  E  K  E  L  L  A

      CCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGTTAG
1221  --------+---------+---------+---------+---------+---------+  1280
      GGACCGTCCTCTTTGACGTAGTCGGCTAATAGTAGTGGCTTATGCCGCACCTATGCAATC
       W  Q  E  K  L  H  Q  P  I  I  I  T  E  Y  G  V  D  T  L  A

      CCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGG
1281  --------+---------+---------+---------+---------+---------+  1240
      GGCCCGACGTGAGTTACATGTGGCTGTACACCTCACTTCTCATAGTCACACGTACCGACC
       G  L  H  S  M  Y  T  D  M  W  S  E  E  Y  Q  C  A  W  L  D

      ATATGTATCACCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATT
1241  --------+---------+---------+---------+---------+---------+  1300
      TATACATAGTGGCGCAGAAACTAGCGCAGTCGCGGCAGCAGCCACTTGTCCATACCTTAA
       M  Y  H  R  V  F  D  R  V  S  A  V  V  G  E  Q  V  W  N  F

      TCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATCT
1301  --------+---------+---------+---------+---------+---------+  1360
      AGCGGCTAAAACGCTGGAGCGTTCCGTATAACGCGCAACCGCCATTGTTCTTTCCCTAGA
       A  D  F  A  T  S  Q  G  I  L  R  V  G  G  N  K  K  G  I  F

      TCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTTCTGCTGCAAAAACGCTGGACTGGCA
1361  --------+---------+---------+---------+---------+---------+  1420
      AGTGAGCGCTGGCGTTTGGCTTCAGCCGCCGAAAAGACGACGTTTTTGCGACCTGACCGT
       T  R  D  R  K  P  K  S  A  A  F  L  L  Q  K  R  W  T  G  M

      TGAACTTCGGTGAAAAACCGCAGCAGGGAGGCAAACAATGAATCAACAACTCTCCTGGCG
1421  --------+---------+---------+---------+---------+---------+  1480
      ACTTGAAGCCACTTTTTGGCGTCGTCCCTCCGTTTGTTACTTAGTTGTTGAGAGGACCGC
       N  F  G  E  K  P  Q  Q  G  G  K  Q  *
```

Figure 3.2: Sequence of the cDNA of ß-glucuronidase from *Escherichia coli*. In EMBL database as cv1012t.em_sy (Jefferson et al., 1986).

```
    CTTCACCGTTCGGTTTCATCATGAAAGGAGGAAAGTCCAAGGGTGAATCGAAGAAAGCTG
1   --------+---------+---------+---------+---------+---------+  60
    GAAGTGGCAAGCCAAAGTAGTACTTTCCTCCTTTCAGGTTCCCACTTAGCTTCTTTCGAC
                      M  K  G  G  K  S  K  G  E  S  K  K  A  E

    AAACGAAGCTTGCGGTGAATAAGAAGGGTGCTCCCGCCACAAAGGGCGGAAAGAAACCAG
61  --------+---------+---------+---------+---------+---------+  120
    TTTGCTTCGAACGCCACTTATTCTTCCCACGAGGGCGGTGTTTCCCGCCTTTCTTTGGTC
    T  K  L  A  V  N  K  K  G  A  P  A  T  K  G  G  K  K  P  A

    CCAAGGGAAAGGAACCAAAGGACCCTAACAAGCCGAAGAGACCTCCAAGTGCTTTCTTCG
121 --------+---------+---------+---------+---------+---------+  180
    GGTTCCCTTTCCTTGGTTTCCTGGGATTGTTCGGCTTCTCTGGAGGTTCACGAAAGAAGC
    K  G  K  E  P  K  D  P  N  K  P  K  R  P  P  S  A  F  F  V

    TTTTCATGGAGGACTTTAGGAAGCAGTTCAAAAAAGGAAACGCTGACAACAAAGCTGTGT
181 --------+---------+---------+---------+---------+---------+  240
    AAAAGTACCTCCTGAAATCCTTCGTCAAGTTTTTTCCTTTGCGACTGTTGTTTCGACACA
    F  M  E  D  F  R  K  Q  F  K  K  G  N  A  D  N  K  A  V  S

    CTGCAGTGGGCAAAGCTGCTGGGGCAAAATGGAAGTCAATGACTGAAGCTGAGAAAGCAC
241 --------+---------+---------+---------+---------+---------+  300
    GACGTCACCCGTTTCGACGACCCCGTTTTACCTTCAGTTACTGACTTCGACTCTTTCGTG
    A  V  G  K  A  A  G  A  K  W  K  S  M  T  E  A  E  K  A  P

    CATATGCTGCTAAGGCAGAGAAAAGGAAGGCTGAATATGAGAAAAGCATGAAGTCCTATA
301 --------+---------+---------+---------+---------+---------+  360
    GTATACGACGATTCCGTCTCTTTTCCTTCCGACTTATACTCTTTTCGTACTTCAGGATAT
    Y  A  A  K  A  E  K  R  K  A  E  Y  E  K  S  M  K  S  Y  N

    ACAAGAAACAAGCTGAAGGTCCAGCTGCTGTTGAAGAAGAAGAAGAATCCGAGAAATCAG
361 --------+---------+---------+---------+---------+---------+  420
    TGTTCTTTGTTCGACTTCCAGGTCGACGACAACTTCTTCTTCTTAGGCTCTTTAGTC
    K  K  Q  A  E  G  P  A  A  V  E  E  E  E  E  S  E  K  S  E

    AATCTGAGGTGCATGATGAGAATGATGATGAGGAAGAAAGTGAGGAGGAGGATGATGACG
421 --------+---------+---------+---------+---------+---------+  480
    TTAGACTCCACGTACTACTCTTACTACTACTCCTTCTTTCACTCCTCCTCCTACTACTGC
    S  E  V  H  D  E  N  D  D  E  E  E  S  E  E  E  D  D  D  E

    AGTAGAAAATTGGATGATGATAACATTTATGTAGGATGCTATGCTGATGATTTGATCATC
481 --------+---------+---------+---------+---------+---------+  540
    TCATCTTTTAACCTACTACTATTGTAAATACATCCTACGATACGACTACTAAACTAGTAG
    *

    TTATGTGGATGATTGTCATTTTATGATAAAAATGTCTTGCTACCCAAATATTTTGCTGCT
541 --------+---------+---------+---------+---------+---------+  600
    AATACACCTACTAACAGTAAAATACTATTTTTACAGAACGATGGGTTTATAAAACGACGA

    CTTGGCTAATGTTTTGTCTGTTCAAAACGTGAATAGTTAAAAATGAGGGAGCAAACTTTT
601 --------+---------+---------+---------+---------+---------+  660
    GAACCGATTACAAAACAGACAAGTTTTGCACTTATCAATTTTTACTCCCTCGTTTGAAAA

    ATTTGTACTTTAAGGCCAACTCTTTAGTTAAGAGATGTTTATCTTTTGTAGAGCTTTTAG
661 --------+---------+---------+---------+---------+---------+  720
    TAAACATGAAATTCCGGTTGAGAAATCAATTCTCTACAAATAGAAAACATCTCGAAAATC

    ATTTTCATGATGGAATCTCTTGATGTTTGTATTAACATAATTTCCTTATTAGCTTTTCAT
721 --------+---------+---------+---------+---------+---------+  780
    TAAAAGTACTACCTTAGAGAACTACAAACATAATTGTATTAAAGGAATAATCGAAAAGTA

    ATCCTTTGTGCTGTATCAAAAAAAAAA
781 --------+---------+------  807
    TAGGAAACACGACATAGTTTTTTTTTT
```

Figure 3.3: The cDNA sequence of HMG1 from *Pisum sativum*. In EMBL database as x76774.em_pl (Webster et al., 1993), kindly provided by Dr. John Gray (University of Cambridge).

49

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      CTTTTAATACCGGATGTGCATGTGCCTGAAAGGAGCTGTTAGTTAGTGGAGTGCAATTGT
  1   ---------+---------+---------+---------+---------+---------+  60
      GAAAATTATGGCCTACACGTACACGGACTTTCCTCGACAATCAATCACCTCACGTTAACA


      TCCTCCGCCTGCAGAGTGTGCCGAAGCCAGTTACTTTGGGTTATCTACGTGACATGGCCT
 61   ---------+---------+---------+---------+---------+---------+  120
      AGGAGGCGGACGTCTCACACGGCTTCGGTCAATGAAACCCAATAGATGCACTGTACCGGA
                                                              M  A  S


      CTACAGTGAGCAACACCAGCAAACTTGAGAAACCTGTGTCCCTTATATGGGGGTGTGAAC
121   ---------+---------+---------+---------+---------+---------+  180
      GATGTCACTCGTTGTGGTCGTTTGAACTCTTTGGACACAGGGAATATACCCCCACACTTG
       T  V  S  N  T  S  K  L  E  K  P  V  S  L  I  W  G  C  E  L


      TGAATGAGCAAGACAAGACGTTTGAGTTTAAGGTAGAAGATGATGAGGAAAAATGTGAGC
181   ---------+---------+---------+---------+---------+---------+  240
      ACTTACTCGTTCTGTTCTGCAAACTCAAATTCCATCTTCTACTACTCCTTTTTACACTCG
       N  E  Q  D  K  T  F  E  F  K  V  E  D  D  E  E  K  C  E  H


      ATCAGTTGGCGTTGCGCACGGTGTGTCTGGGGGACAAGGCAAAGGATGAGTTCAACATTG
241   ---------+---------+---------+---------+---------+---------+  300
      TAGTCAACCGCAACGCGTGCCACACAGACCCCCTGTTCCGTTTCCTACTCAAGTTGTAAC
       Q  L  A  L  R  T  V  C  L  G  D  K  A  K  D  E  F  N  I  V


      TAGAAATCGTTACACAAGAGGAGGGAGCGGAAAAATCTGTTCCAATTGCCACTCTAAAGC
301   ---------+---------+---------+---------+---------+---------+  360
      ATCTTTAGCAATGTGTTCTCCTCCCTCGCCTTTTTAGACAAGGTTAACGGTGAGATTTCG
       E  I  V  T  Q  E  E  G  A  E  K  S  V  P  I  A  T  L  K  P


      CTTCTATTCTACCCATGGCAACTATGGTGGGCATTGAGCTGACTCCTCCAGTTACTTTCC
361   ---------+---------+---------+---------+---------+---------+  420
      GAAGATAAGATGGGTACCGTTGATACCACCCGTAACTCGACTGAGGAGGTCAATGAAAGG
       S  I  L  P  M  A  T  M  V  G  I  E  L  T  P  P  V  T  F  R


      GGTTAAAAGCTGGTTCCGGCCCACTGTACATCAGTGGTCAACACGTAGCGATGGAGGAAG
421   ---------+---------+---------+---------+---------+---------+  480
      CCAATTTTCGACCAAGGCCGGGTGACATGTAGTCACCAGTTGTGCATCGCTACCTCCTTC
       L  K  A  G  S  G  P  L  Y  I  S  G  Q  H  V  A  M  E  E  D


      ATTACTCATGGGCAGAAGAGGAAGATGAAGGGGAAGCTGAAGGAGAAGAGGAGGAAGAAG
481   ---------+---------+---------+---------+---------+---------+  540
      TAATGAGTACCCGTCTTCTCCTTCTACTTCCCCTTCGACTTCCTCTTCTCCTCCTTCTTC
       Y  S  W  A  E  E  E  D  E  G  E  A  E  G  E  E  E  E  E


      AGGAAGAAGATCAAGAATCTCCACCCAAAGCTGTAAAGAGGCCTGCGGCTACCAAAAAAG
541   ---------+---------+---------+---------+---------+---------+  600
      TCCTTCTTCTAGTTCTTAGAGGTGGGTTTCGACATTTCTCCGGACGCCGATGGTTTTTTC
       E  E  D  Q  E  S  P  P  K  A  V  K  R  P  A  A  T  K  K  A


      CAGGCCAGGCAAAGAAGAAGAAACTTGACAAAGAGGACGAGAGCTCCGAGGAAGACAGTC
601   ---------+---------+---------+---------+---------+---------+  660
      GTCCGGTCCGTTTCTTCTTCTTTGAACTGTTTCTCCTGCTCTCGAGGCTCCTTCTGTCAG
       G  Q  A  K  K  K  K  L  D  K  E  D  E  S  S  E  E  D  S  P


      CAACCAAAAAGGGCAAAGGAGCCGGAAGAGGAAGAAAGCCGGCTGCTAAGAAGTGAACCT
661   ---------+---------+---------+---------+---------+---------+  720
      GTTGGTTTTTCCCGTTTCCTCGGCCTTCTCCTTCTTTCGGCCGACGATTCTTCACTTGGA
       T  K  K  G  K  G  A  G  R  G  R  K  P  A  A  K  K  *


      CTTGGAGGCTGTTCCTTGCCCCTATTCCCTCCTGTGTAATTTTTTTTGTTGTTTAAAATA
721   ---------+---------+---------+---------+---------+---------+  780
      GAACCTCCGACAAGGAACGGGGATAAGGGAGGACACATTAAAAAAAAACAACAAATTTTAT


      AAATATAAATAAGCTTAAAC
781   ---------+---------+  800
      TTTATATTTATTCGAATTTG
```

Figure 3.4: The cDNA sequence of nucleoplasmin from *Xenopus laevis*. In EMBL database as x04766.em_ov (Dingwall et al., 1987).

50

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    CTTTGCACCAAAACACCACATCAAATCTCCAGTTGATAAACTAAATCGTCCCAACGGCAA
  1 ---------+---------+---------+---------+---------+---------+ 60
    GAAACGTGGTTTTGTGGTGTAGTTTAGAGGTCAACTATTTGATTTAGCAGGGTTGCCGTT

    CAGGCCAACATGACCAGCGAACAATACTCAGTCAAGCTTACACCAGACTTCGACAACCCA
 61 ---------+---------+---------+---------+---------+---------+ 120
    GTCCGGTTGTACTGGTCGCTTGTTATGAGTCAGTTCGAATGTGGTCTGAAGCTGTTGGGT
             M  T  S  E  Q  Y  S  V  K  L  T  P  D  F  D  N  P

    AAATGGATTGGACGACACAAGCACATGTTTAATTTTCTTGATGTCAACCACAATGGAAGG
121 ---------+---------+---------+---------+---------+---------+ 180
    TTTACCTAACCTGCTGTGTTCGTGTACAAATTAAAAGAACTACAGTTGGTGTTACCTTCC
    K  W  I  G  R  H  K  H  M  F  N  F  L  D  V  N  H  N  G  R

    ATCTCTCTTGACGAGATGGTCTACAAGGCGTCCGATATTGTTATAAACAATCTTGGAGCA
181 ---------+---------+---------+---------+---------+---------+ 240
    TAGAGAGAACTGCTCTACCAGATGTTCCGCAGGCTATAACAATATTTGTTAGAACCTCGT
    I  S  L  D  E  M  V  Y  K  A  S  D  I  V  I  N  N  L  G  A

    ACACCTGAACAAGCCAAACGTCACAAAGATGCTGTAGAAGCCTTCTTCGGAGGAGCTGGA
241 ---------+---------+---------+---------+---------+---------+ 300
    TGTGGACTTGTTCGGTTTGCAGTGTTTCTACGACATCTTCGGAAGAAGCCTCCTCGACCT
    T  P  E  Q  A  K  R  H  K  D  A  V  E  A  F  F  G  G  A  G

    ATGAAATATGGTGTAGAAACTGAATGGCCTGAATACATCGAAGGATGGAAAAGACTGGCT
301 ---------+---------+---------+---------+---------+---------+ 360
    TACTTTATACCACATCTTTGACTTACCGGACTTATGTAGCTTCCTACCTTTTCTGACCGA
    M  K  Y  G  V  E  T  E  W  P  E  Y  I  E  G  W  K  R  L  A

    TCCGAGGAATTGAAAAGGTATTCAAAAAAACCAAATCACACTTATTCGTTTATGGGGTGAT
361 ---------+---------+---------+---------+---------+---------+ 420
    AGGCTCCTTAACTTTTCCATAAGTTTTTTGGTTTAGTGTGAATAAGCAAATACCCCACTA
    S  E  E  L  K  R  Y  S  K  N  Q  I  T  L  I  R  L  W  G  D

    GCATTGTTCGATATCATTGACAAAGACCAAAATGGAGCTATTTCACTGGATGAATGGAAA
421 ---------+---------+---------+---------+---------+---------+ 480
    CGTAACAAGCTATAGTAACTGTTTCTGGTTTTACCTCGATAAAGTGACCTACTTACCTTT
    A  L  F  D  I  I  D  K  D  Q  N  G  A  I  S  L  D  E  W  K

    GCATACACCAAATCTGATGGCATCATCCAATCGTCAGAAGATTGCGAGGAAACATTCAGA
481 ---------+---------+---------+---------+---------+---------+ 540
    CGTATGTGGTTTAGACTACCGTAGTAGGTTAGCAGTCTTCTAACGCTCCTTTGTAAGTCT
    A  Y  T  K  S  D  G  I  I  Q  S  S  E  D  C  E  E  T  F  R

    GTGTGCGATATTGATGAAAGTGGACAGCTCGATGTTGATGAGATGACAAGACAACATTTA
541 ---------+---------+---------+---------+---------+---------+ 600
    CACACGCTATAACTACTTTCACCTGTCGAGCTACAACTACTCTACTGTTCTGTTGTAAAT
    V  C  D  I  D  E  S  G  Q  L  D  V  D  E  M  T  R  Q  H  L

    GGATTTTGGTACACCATGGATCCTGCTTGCGAAAAGCTCTACGGTGGAGCTGTCCCCTAA
601 ---------+---------+---------+---------+---------+---------+ 660
    CCTAAAACCATGTGGTACCTAGGACGAACGCTTTTCGAGATGCCACCTCGACAGGGGATT
    G  F  W  Y  T  M  D  P  A  C  E  K  L  Y  G  G  A  V  P  *

    GAAACTCTGCGG
661 ---------+-- 672
    CTTTGAGACGCC
```

Figure 3.5: The cDNA sequence of apoaequorin from *Aequorea victoria*. In EMBL database as m16103.em_in (Prasher et al., 1987).

3.2 Selection of Nuclear Targeted Apoaequorin Transgenic Tobacco Plant by In Vitro *Reconstitution and Western Blot Analysis*

In order to select transgenic plants expressing aequorin, *in vitro* reconstitution and western blot analysis on aequorin transgenic plants were performed (Chapter 2). For each of the 5 constructs about 20 independent transformants were obtained. From each of these independent transgenic plants total protein was extracted from young almost fully expanded leaf tissue. For *in vitro* reconstitution of active aequorin, 2 μg of total protein was incubated for 4 hr with native coelenterazine, the luminophore for apoaequorin in Aequorin reconstitution buffer and discharged with an equal volume of 100 mM $CaCl_2$. Light emitted by the samples was measured with an EMI photomultiplier model 9757AM at 1 kV with a discriminator (Campbell, 1983). For western blot analysis 3 μg of total protein was size-fractionated on a 10% acrylamide gel electrophoresis and blotted onto ECL membrane (Amersham, Buckinghamshire, UK). Non-specific sites on the membranes were blocked with skimmed milk from Marval (Dublin, Rep. Ireland) and incubated with mouse anti-apoaequorin (1:1000) according to manusfacture's procedures (Amersham, Buckinghamshire, UK). For each line one individual transformant was selected based on highest *in vitro* reconstitution and strongest appearing band after western blot analysis (Table 3.I). The aequorin transgenic lines, MAQ 2.4 and MAQ 6.3a were used as controls and were provided by Marc R. Knight. For SV40-GUS-apoaequorin transgenic plants, MAQ 8 (pCAL) and MAQ 9 (pCAH) the expression of apoaequorin was very low as it was undetectable in western blot analysis (data not shown). *In vitro* reconstitution revealed an aequorin activity for MAQ 8 and MAQ 9 transgenic lines three magnitudes lower as compared to MAQ 2.4. The amount of light recorded was within the range of background readings of the luminometer that resulted from thermal noise. For the other constructs, western blot analysis revealed a 42 kDa band for HMG1-apoaequorin transgenic plants, MAQ 10 (pCHAH) and MAQ 11 (pCHAL) and a 45 kDa band for nucleoplasmin-apoaequorin transgenic plants (MAQ 7) (data not shown). The two controls revealed bands of 24 kDa for cytosolic apoaequorin (MAQ 2.4) and 24 kDa for chloroplast targeted apoaequorin (MAQ 6.3a, with the sequence of the gene encoding the leader signal peptide of the small subunit of ribulose 1,5'-bisphosphate carboxylase, RuBisCo, *rbcS* from pea that was fused to the apoaequorin coding region) (Johnson et al., 1995) (data not shown). The sizes of the bands were expected based on their molecular weight estimated from the amino acid sequence (Fig. 3.2-5). The amount of light recorded after *in vitro* reconstitution was within the order of magnitude of the other apoaequorin transgenic plants.

The transformant MAQ 7.11, expressing the fusion protein between nucleoplasmin and apoaequorin was selected for further experimentation based on comparative results obtained by *in vitro* reconstitution, western blot analysis, the responses to cold shock and wind stimulation and based on genetical analysis that two T-DNA insertions were predicted (data not shown). The other transgenic lines await further investigation and in particular line MAQ 10.32 promises good results.

Table 3.I: Selection of the aequorin transgenic plant line with the highest expression level of aequorin. MAQ 7.11: apoaequorin fused to nucleoplasmin; MAQ 8.5: low affinity apoaequorin fused to SV40-GUS; MAQ 9.8: apoaequorin fused to SV40-GUS; MAQ 10.32: apoaequorin fused to HMG1; MAQ 11.7: low affinity apoaequorin fused to HMG1. Relative band intensity is shown as well as *in vitro* reconstitution after discharging 2 μg total protein previously reconstituted with 2 μM native coelenterazine for 4 hr in the dark by 100 mM $CaCl_2$. MAQ 2.4* and MAQ 6.3a* are included in the table as reference and were kindly privided by Marc R. Knight. MAQ 7.11‡ was selected and used for further experimentation.

transgenic plant line MAQ	fusion protein with low or high affinity apoaequorin protein	western blot relative band intensity in arbitrary units	*in vitro* reconstitution in counts·s⁻¹
MAQ 2.4*	none high	1	15,000
MAQ 6.3a*	small subunit of RuBisCo high	10	28,000
MAQ 7.11‡	nucleoplasmin high	15	65,000
MAQ 8.5	SV40-GUS low	N.D.	50
MAQ 9.8	SV40-GUS high	N.D.	100
MAQ 10.32	HMG1 high	10	75,000
MAQ 11.7	HMG1 low	2	22,000

N.D.=not detectable

3.3   Nuclear Targeting of Apoaequorin

Subcellular Fractionation

To study whether the fusion proteins were translocated successfully into the nucleus subcellular fractionation was performed (Fig. 3.6). In this method, protoplasts were prepared from young leaves, lysed and nuclei sedimented at 100*g* for 5 min. The nuclear and 'cytoplasmic' fractions were separately incubated with native coelenterazine to reconstitute aequorin. Transgenic tobacco protoplasts expressing aequorin in

cytoplasm were included as control. The supernatant represents the cytoplasmic fraction and the nuclear fraction contained more than 90% of the total nuclei as seen after 4',6-diamidino-2-phenylindole (DAPI) staining (data not shown). The aequorin activities of the 100g and supernatant fraction are related to that of lysed protoplasts. Almost 60% of the total aequorin activity was found in the pelleted nuclei of nucleoplasmin aequorin transgenic protoplasts compared to 20% in aequorin transgenic protoplasts (Fig. 3.6).
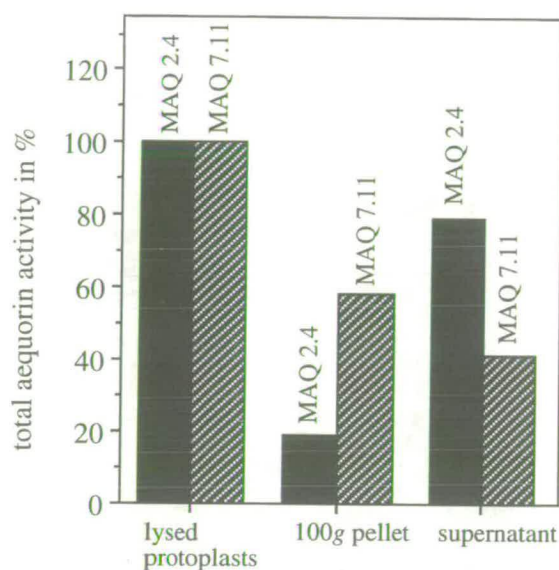
Figure 3.6: Simple subcellular fractionation of apoaequorin distribution in MAQ 2.4 and MAQ 7.11 after gently lysis of protoplasts and centrifugation. Protoplasts isolated from transgenic plant leaves expressing apoaequorin (MAQ 2.4) and nucleoplasmin-apoaequorin (MAQ 7.11). For each fraction the aequorin activity was measured *in vitro* and calculated as percentage of total aequorin activity.

Immunolocalisation

Figure 3.7 shows a comparison of protoplasts obtained from wild type *Nicotiana plumbaginifolia*, MAQ 2.4 containing cytoplasmic apoaequorin (Knight et al., 1991) and MAQ 7.11 containing nucleoplasmin apoaequorin fusion protein. Protoplasts with central dominant vacuoles were prepared from green leaves of all three plants and stained with DAPI (top row) to reveal the location of the nuclei and with the anti-apoaequorin followed by FITC-labelled second antibodies (bottom row) to locate the apoaequorin. Comparison of MAQ 2.4 (Fig 3.7B and E) with MAQ 7.11 (Fig 3.7C and F) clearly demonstrates an abundant cytoplasmic location of apoaequorin in the MAQ 2.4 in the thin cytoplasmic ring surrounding the vacuole, but an obvious nuclear location of apoaequorin only in the MAQ 7.11. Precipitation of antibodies was

sometimes observed and is shown as artifacts of light outside the protoplasts of the wild type.
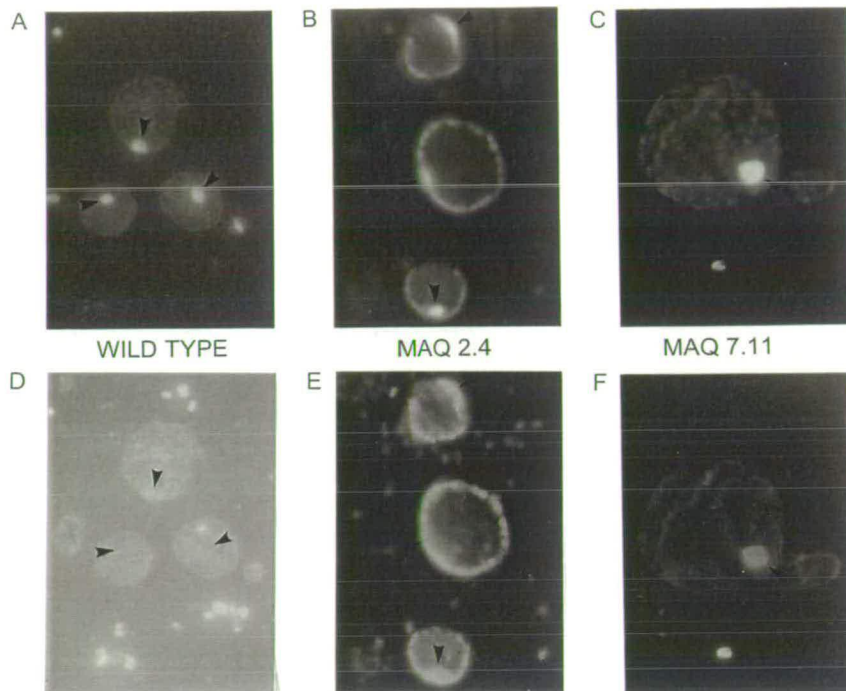

Figure 3.7: Immunolocalisation using FITC-labelled antibodies of the targeting of a nucleoplasmin apoaequorin fusion protein in transformed *Nicotiana plumbaginifolia*. Leaf mesophyll protoplasts were stained with DAPI (A-C) or with anti-apoaequorin (D-F). A and D, protoplasts from wild type. B and E, transgenic tobacco expressing cytoplasmic apoaequorin (MAQ 2.4). C and F, transgenic tobacco expressing the nucleoplasmin apoaequorin fusion proteins (MAQ 7.11). Nuclei are indicated by arrowheads.

Immuno-Electron Microscopy

Because immunolocalisation using FITC-labelled antibodies only provide qualitative data for targeting immunogold-labelled secondary antibodies were used to estimate the degree of targeting of apoaequorin. In figure 3.8A a transgenic protoplast is shown that expresses the nuclear targeted apoaequorin (MAQ 7.11). A fraction of an intact protoplast is shown with the intact nucleus surrounded by cytoplasm and on either side the presence of a chloroplast. Most of the silver-enhanced gold particles were found inside the nuclear profile providing firm evidence for successful nuclear translocation of the fusion protein.
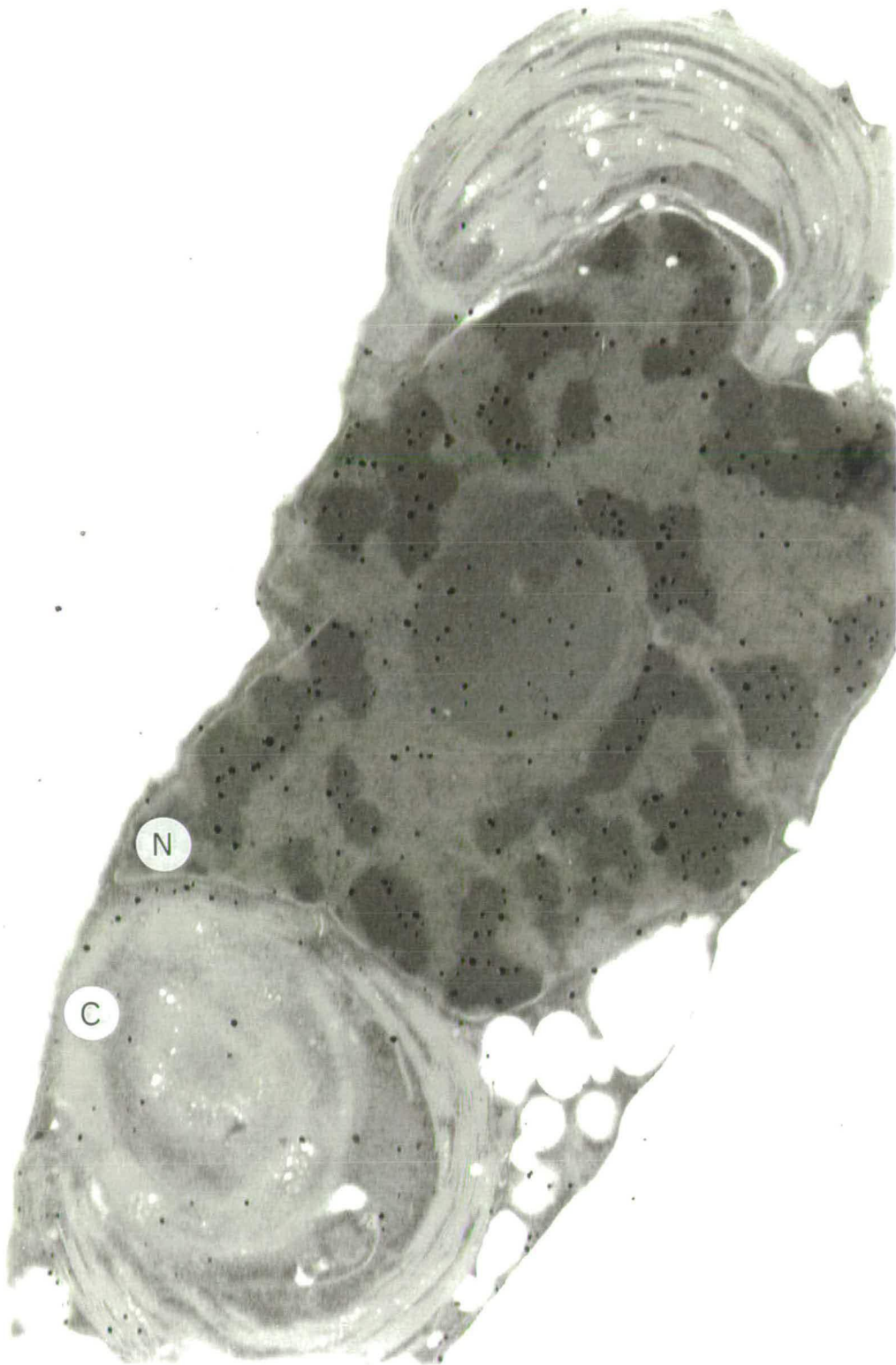
Figure 3.8: Immunolocalisation using immunogold-labelled antibodies of the targeting of a nucleoplasmin apoaequorin fusion protein in transformed *Nicotiana plumbaginifolia*. Leaf mesophyll protoplasts expressing the nucleoplasmin apoaequorin fusion proteins (MAQ 7.11) were fixed, embedded, sectioned and treated with immunogold-labelled antibodies and silver-enhanced; bar indicates 1 μm; C, cytoplasm; N, nucleus.

3.4 Wind and Cold shock Induce Changes in Cytosolic and Nuclear Calcium Concentrations

Wind, touch, cold, salt/drought and osmotic pressure result in changes in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in aequorin transgenic tobacco seedlings (Trewavas and Knight, 1994; Haley et al., 1995; Knight et al., 1996, 1997; Takahashi et al., 1996). Furthermore apoaequorin has also been targeted to chloroplasts (Johnson et al., 1995) and the vacuole membrane (Knight et al., 1996) and provided evidence that Ca^{2+} signals are strictly compartmentalised with cells. With nucleoplasmin-apoaequorin transgenic tobacco plants and their successful targeting shown here, measurements can now be made on changes in nuclear calcium levels and used as tool to study the relation between cytosolic and nuclear calcium levels. The effect of cold shock and wind stimulation on cytosolic and nuclear calcium levels were determined respectively by using MAQ 2.4 expressing aequorin in the cytoplasm (Knight et al., 1991) and MAQ 7.11 expressing nuclear targeted aequorin. Prior to calcium measurements, a single drop of 3 μL of 2 μM coelenterazine, the hydrophobic luminophore necessary for aequorin reconstitution, was placed between the cotyledons and kept for at least 4 hr in the dark. Wind stimulation was achieved by injecting 5 mL of air instantly from above into the sampling housing of the luminometer. Cold shock was simulated by a gently injecting 1 mL ice-cold water from above into the sampling housing at a low rate to prevent mechanical disturbance. The light emitted by the seedling is a measure of the change in the $[Ca^{2+}]_{int}$ and was recorded every 0.2 seconds using a cooled photomultiplier tube. Conversions of emitted luminescence to free Ca^{2+} levels were performed using the calibration curve for the coelenterazines (Chapter 2).

Figure 3.9 shows the effect of wind signalling on cytoplasmic and nuclear $[Ca^{2+}]$. With native coelenterazine, the same luminophore used in previous experiments (Knight et al., 1991, 1992), mean peak increases in cytoplasmic Ca^{2+} level of 1.08 ± 0.23 μM (mean ± s.e., n=7) were obtained after one injection of 5 mL air onto MAQ 2.4 seedlings at the 10 seconds point (Fig. 3.9). Since nuclear $[Ca^{2+}]$ changes were found to be generally smaller than those found in the cytoplasm, the accuracy of nuclear Ca^{2+} measurements was improved by using cp-coelenterazine instead. This form of coelenterazine is more useful for detecting smaller changes in $[Ca^{2+}]_{int}$ (Shimomura et al., 1993), since light emission is greater than native coelenterazine in the lower $[Ca^{2+}]$ ranges. Figure 3.9 shows that nuclear $[Ca^{2+}]$ peaks at 0.80 ± 0.16 μM (mean ± s.e., n=8) after wind stimulation.

The characteristics of the signals in the nucleus and cytoplasm differ. The average time to reach maximal levels for cytoplasmic $[Ca^{2+}]$ was 0.31 ± 0.04 s and

0.60 s ± 0.04 for nuclear [Ca^{2+}]. The average time the Ca^{2+} signal remained above resting levels, (T_A) was similar in both compartments, 13.6 ± 2.1 s for the cytoplasm and 13.6 ± 1.4 s for the nuclear compartment. However, a small but consistent change is to be seen in the resting levels after signalling. In the cytoplasm the estimated resting levels are 24 ± 4 nM before signalling and 44 ± 8 nM after, an approximate doubling. These altered resting levels were observed in all experiments and continue throughout the duration of the experimental periods investigated. This did not occur with nuclear [Ca^{2+}] values before and after signalling that were 8 nM.
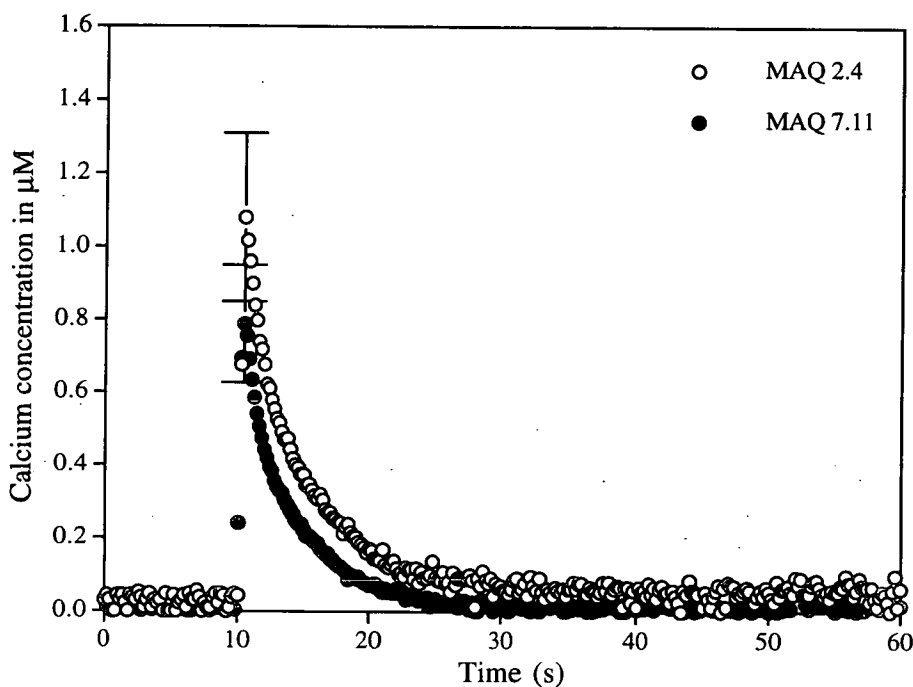

Figure 3.9: Wind-induced changes in cytosolic and nuclear free calcium concentrations. Ca^{2+} changes are shown in cytoplasm (MAQ 2.4) and nucleoplasm (MAQ 7.11) after being stimulated with one injection of 5 mL air.

Figure 3.10 shows the effect of cold shock signalling on cytoplasmic and nuclear [Ca^{2+}]. With native coelenterazine, the same luminophore used in previous experiments (Knight, et al., 1991, 1992), mean peak increases in cytoplasmic Ca^{2+} level of 1.25 μM ± 0.12 (n=7) were obtained after gently injecting 1 mL ice-cold water onto MAQ 2.4 seedlings after 10 seconds (Fig. 3.10). Under similar conditions no clear change in chemiluminescence was observed in MAQ 7.11 (data not shown). Therefore, the more sensitive luminophore, cp-coelenterazine was used to measure changes in nuclear [Ca^{2+}], which reached a maximal value of 0.55 ± 0.07 μM (n=8) after cold shock stimulation.

58

The characteristics of the signals in the nucleus and cytoplasm after cold shock treatment differ from those measured for wind stimulation. The time required to reach maximal values was 4.8 ± 0.3 s and 9.0 s ± 1.1 for cytoplasmic and nuclear $[Ca^{2+}]$ respectively. The average time the Ca^{2+} signal needed to return to resting levels, (T_Δ) was also different in both compartments, 23.6 ± 1.2 s for the cytoplasm and 30.7 ± 1.9 s for the nuclear compartment. As in the case for wind stimulation a consistent change is to be seen in the resting levels after signalling. In the cytoplasm the estimated resting levels are 11 ± 2 nM before signalling and 35 ± 3 nM after it; a 3 fold increase. In contrast to wind stimulation, changes in the resting levels in nuclear Ca^{2+} were also observed. In the nucleus the estimated resting levels are 8 ± 4 nM before signalling and 35 ± 12 nM after, a 4 fold increase. These altered resting levels were observed in all experiments and continue throughout the duration of the experimental periods investigated. Whether these are significant changes in longer term signalling will be the subject of a continuing study.
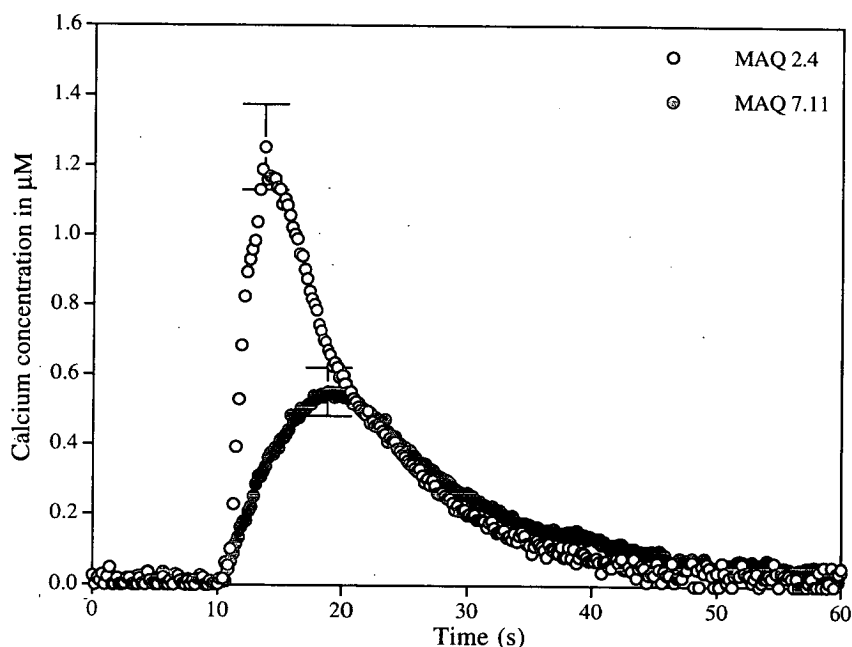

Figure 3.10: Cold shock-induced changes in cytosolic and nuclear free calcium concentrations. Ca^{2+} changes are shown in cytoplasm (MAQ 2.4) and nucleoplasm (MAQ 7.11) after being stimulated by slow injection of 1 mL ice-cold water.

3.5   Discussion

In this chapter the successful nuclear translocation was shown of a fusion protein consisting of the calcium-sensitive luminescent protein, aequorin and a protein that is known for its nuclear translocation namely nucleoplasmin. Plants transformed with a

construct encoding HMG1 fused to apoaequorin produced a functional protein. A band with predicted size was observed after western blot analysis with active apoaequorin properties. Whether HMG1 has the ability to target apoaequorin to plant nuclei awaits further investigation and is the subject of an ongoing study. HMG1 has features in its sequence closely resembles those found in cellular proteins that are imported in the nucleus (compare **KKAETKLAVNKKGAPATKGGKK** with amino acid sequences shown in Box 3.1). In addition, the fact that HMG1 is a putative nuclear protein with binding affinity to DNA might facilitate translocation of the protein inside the nucleus like was suggested to occur for nucleoplasmin (Paine, 1993; Vancurova et al., 1993). Proteins fused to the nuclear localisation signal SV40 large T-antigen were not successful. As no aequorin activity was produced by the fusion protein and no band was observed after western blot analysis, this might be the result of a non-functional protein. This is likely the result of presence of a stop codon in the 5' region introduced during preparations of the construct with PCR amplification to produce the chimaeric gene. At the time this construct was prepared no proof-reading DNA polymerases were available as they are known today and the production of long fragments such as 1.8 kb for SV40-GUS by using Taq polymerase for PCR amplification has a misreading probability factor of $1.2 \cdot 10^{-5}$ which can lead to such complications (Cline et al., 1996).

From subcellular fractionation, immunolocalisation and immuno-electron microscopy, it can be concluded that apoaequorin was sufficiently targeted to plant nuclei by fusion to nucleoplasmin. The centrifugal speeds used during subcellular fractionation are known to result in sedimentation of approximately 2/3rds of nuclear DNA (Trewavas, 1979). This figure is close to the 60% of the aequorin activity found in the nuclear fraction. Nucleoplasmin is a soluble nuclear protein and soluble nuclear proteins are known to be rapidly lost during cell fractionation particularly if the nuclear membrane loses integrity. This was also observed with COS7 cells transfected with nucleoplasmin-aequorin constructs, where the fusion proteins was found to be lost by the nuclei (Badminton et al., 1995). In addition, even gentle lysis of protoplasts can cause damage to nuclei and result in the loss of the protein. Immunolocalisation with FITC-labelled antibodies revealed successful targeting of the fusion protein to plant nuclei. Immuno-electron microscopy using gold-labelled antibodies revealed that approximately 80% of the gold particles were in the nucleus. Greber and Gerace (1995) showed that *Xenopus* oocytes accumulated nucleoplasmin upto 5 fold into the nuclei under physiological conditions. Reasons for not observing more than 80% targeting could be ascribed to (1) aspecific binding of the label, cross-reactivity or affinity of the gold particles to the specimen, (2) the fact that polyclonal antibodies also

bind other soluble proteins like RuBisCo, an enzyme located in the stromal surface of thylakoid membrane and other proteins located inside the cytoplasm as was observed after western blot analysis (data not shown), (3) the loss of protein by the nucleus (Badminton et al., 1995) (4) loss of soluble proteins during fixation.

Nucleoplasmin is a mammalian protein but is recognised by the nuclear import machinery of plants. Increasing evidence suggest that the nuclear protein translocation is highly conserved among higher eukaryotes. Van der Krol and Chua (1991) showed that the short stretch of basic amino acids of the nuclear localisation signal from the large T-antigen of the simian 40 virus that infects mammalian cells can be used to target normally cytosolic proteins to the nuclei of plants. Microinjection of a transcriptional activator protein from *Saccharomyces cerevisiae*, Mcm1 with a predicted molecular weight of 40 kDa, into *Xenopus laevis* frog oocytes resulted in the import of Mcm1 into the oocyte nucleus indicating that nuclear protein transport is functionally conserved between yeast and higher eukaryotes (Wagner and Hall, 1993). Recent evidence suggests that plants like mammalian cells contain docking proteins such as importin α that binds to NLS containing peptides (Hicks et al., 1996; Smith et al., 1997). In addition, plant docking proteins recognise all three classes of NLS described including bipartite NLS (Smith et al., 1997). For that reason plant importin might recognise NLS like nucleoplasmin. Nucleoplasmin is known to be a phosphorylated nuclear-accumulating protein (Vancurova et al., 1995). The kinetics of its cytoplasm-nucleus transport is affected by its degree of phosphorylation by casein kinase II (Vancurova et al., 1995), a protein which has also been identified in plants (Brusa et al., 1994).

The kinetics of the Ca^{2+} response is clearly different upon each environmental stimulus and for the cytosolic and nuclear compartment as shown in 3.9 and 3.10. The average time to reach maximal values after wind stimulation was for cytosolic and nuclear $[Ca^{2+}]$, 0.31 s and 0.60 s, respectively, whereas after a cold shock, 4.8 s and 9.0 s, respectively. The length of time it remained above resting levels was similar in both compartments after wind stimulation, 13.6 s, but was significantly different after cold shock treatment, 24 and 31 s for cytosolic and nuclear $[Ca^{2+}]$, respectively. The distinct temporal dynamics of the calcium response upon the two stimuli in both compartments provide addition evidence for successful targeting of aequorin to tobacco nuclei. The distinct Ca^{2+} response between wind and cold shock might reflect the difference in the origin of Ca^{2+} release and the induction of different signalling pathway upon each environmental stimulus. The physiological significance of these differences will be discussed in the following chapters.

Chapter 4

Isolation of a Wind- and Cold Shock-Inducible Calmodulin Gene

The accumulation of calmodulin mRNA was greatly increased after touch stimulation of leaves from *Arabidopsis thaliana* (Braam and Davis, 1990; Chapter 1, Fig. 1.5). The expression of calmodulin genes in plants is induced by mechanical, temperature and other environmental stress stimuli. In all plants studied so far, calmodulin is represented by a multigene family and the individual calmodulin members exhibit both tissue-specific and developmental stage-specific expression (Ling et al., 1991; Takezawa et al., 1995). These isoforms of mRNA differ with respect to length and sequence of the 3' untranslated regions or 3' UTR (Takezawa et al., 1995). The use of RACE PCR instead of screening cDNA libraries is advantaguous. To obtain individual cDNA clones, the analysis of clones and determination whether the desired sequence is present by cDNA library screening is labour intensive and time consuming. With the PCR technique and proper primers the 3' UTR sequence can be obtained within a few days. PCR is used to amplify the cDNA representing the region between a single point in the transcript and its 3' end. A short internal stretch of sequence is needed from the mRNA of interest and gene-specific primers are chosen that are oriented in the direction of the missing sequence. Using RACE PCR, enrichments on the order of 10^6- to 10^7-fold can be obtained. As a result, relatively pure cDNA "ends" are generated that can be easily cloned or rapidly characterised using convential techniques (Frohmann et al., 1988). To generate the cDNA clones, mRNA is reverse-transcribed using a "hybrid" primer (Q_T) that consists of 17 nucleotides of oligo (dT) followed by a unique 35-base oligonucleotide sequence (Q_i-Q_o; Fig. 4.1; Frohmann, 1995). Amplification is performed using a primer containing part of this sequence (Q_o) that now binds to each cDNA at its 3' end, and using a primer derived from the gene of interest (E086). A second set of amplification cycles can be carried out using "nested" primers (E086-Qi) to quench the amplification of non-specific products.
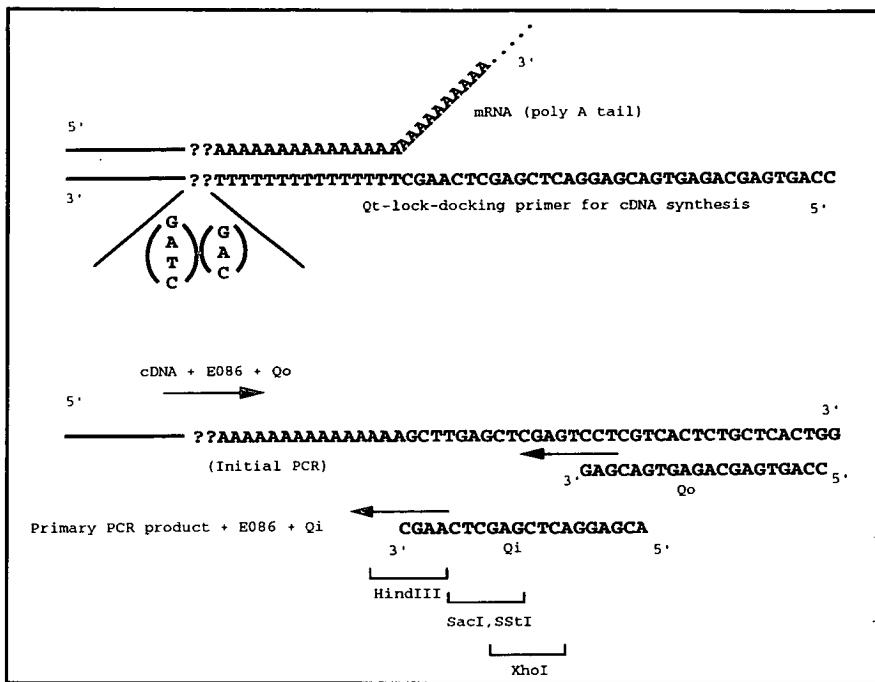
Figure 4.1: The strategy for 3' RACE PCR. cDNA is synthesised by using the Q_T lock-docking primer, RNA is treated with RNasin to remove RNA. Subsequent PCR is performed using gene-specific primer, E086 and Q_o, followed by a final round of amplification using E086 and Q_i. Restriction sites were introduced to the primers to facilitate subsequent cloning of the fragments.

N.B. NiCaM in pBluescript (SK) was cloned and sequenced by Anne Taylor (1993) and the primers Q_T, Q_i and Q_o were kindly provided by Ian Oliver.

4.1   3' RACE Identifies Calmodulin Transcripts

An original full length calmodulin cDNA clone, NiCaM (377 bp *SmaI-SalI* fragment in pBluescript SK; Fig. 4.2) was obtained from Anne Taylor (University of Aberdeen, UK). The region 130-266 had been sequenced in both directions. From this sequence a primer was chosen, E086, that anneals half-way through the sequence encoding the first Ca^{2+} binding site of calmodulin. The reason this sequence was chosen is that it is the region that is most conserved among calmodulin genes (Shepherd, 1997).

Total RNA was extracted from unstimulated seedlings (T_0), at 1 hr after wind stimulation (T_1W) and 2 hr after cold shock stimulation (T_2CS). 3' RACE was carried out as described (Chapter 2). Subsequent PCR was performed at two different annealing temperatures, 52°C and 56°C (Fig. 4.3). Using this technique, 7 potential calmodulin transcripts were identified. On close inspection, one of these putative calmodulin transcripts designated *NpCaM-1* clearly appeared to be induced by wind and cold shock whilst another, designated *NpCaM-2*, was not. In addition, *NpCaM-1*

appeared to be induced more strongly by cold shock signalling then after wind signalling.

```
     ATGGCGGATCAGTTGACGGATGACCAGATCTCTGAGTTCAAGGAGGCCTTTAGCCTATTT
  1  --------+---------+---------+---------+---------+---------+  60
     TACCGCCTAGTCAACTGCCTACTGGTCTAGAGACTCAAGTTCCTCCGGAAATCGGATAAA
      M  A  D  Q  L  T  D  D  Q  I  S  E  F  K  E  A  F  S  L  F

     GACAAGGACGGAGATGGTTGCATCACGACTAAGGAGCTTGGAACAGTGATGAGATCACTG
 61  --------+---------+---------+---------+---------+---------+  120
     CTGTTCCTGCCTCTACCAACGTAGTGCTGATTCCTCGAACCTTGTCACTACTCTAGTGAC
      D  K  D  G  D  G  C  I  T  T  K  E  L  G  T  V  M  R  S  L

     GGACAGAACCCCACTGAAGCAGAGCTTCAAGACATGATAAACGAGGTGGATGCAGATGGT
121  --------+---------+---------+---------+---------+---------+  180
     CCTGTCTTGGGGTGACTTCGTCTCGAAGTTCTGTACTATTTGCTCCACCTACGTCTACCA
      G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A  D  G

     AACGGAACCATTGACTTCCCTGAGTTTCTAAACCTCATGGCCAGGAAAATGAAGGATACT
181  --------+---------+---------+---------+---------+---------+  240
     TTGCCTTGGTAACTGAAGGGACTCAAAGATTTGGAGTACCGGTCCTTTTACTTCCTATGA
      N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K  D  T

     GACTCCGAGGAGGAGCTGAAAGAAGCGTTCAGAGTGTTCGACAAGGATCAAAATGGCTTC
241  --------+---------+---------+---------+---------+---------+  300
     CTGAGGCTCCTCCTCGACTTTCTTCGCAAGTCTCACAAGCTGTTCCTAGTTTTACCGAAG
      D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q  N  G  F

     ATCTCTGCTGCTGAGCTTCGTCATGTGATGACTAACCTTGGGGAGAAACTCACTGATGAA
301  --------+---------+---------+---------+---------+---------+  360
     TAGAGACGACGACTCGAAGCAGTACACTACTGATTGGAACCCCTCTTTGAGTGACTACTT
      I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T  D  E

     GAGGTCGACGAAATGAT
361  --------+------- 377
     CTCCAGCTGCTTTACTA
      E  V  D  E  M  *
```

Figure 4.2: The full length cDNA clone obtained by PCR by Anne Taylor (NiCaM as 377 bp *SmaI-SalI* fragment in pBluescript SK; Fig. 4.2). Nucleotide residues 130-266 have been sequenced in both directions.

Figure 4.3: 3' RACE of RNA extracted from unstimulated (T_0), wind stimulated (T_1W) and cold shock stimulated seedlings (T_2CS) using calmodulin primers. Wind stimulation was one 5 mL air injection onto seedlings and RNA was extracted after one hour. Cold shock stimulation was a gentle 1 mL ice-cold water injection onto seedlings and RNA was extracted after two hours. Subsequent PCR was carried out at two different annealing temperatures: 52°C and 56°C. Bands subcloned for further analyses are indicated and were called *NpCaM-1* and *NpCaM-2*. Water control and DNA marker, a *PhiX*174 DNA *HinfI* digest are indicated.

4.2   Sequence Analysis of NpCaM-1 and NpCaM-2

The band that appeared to be induced by wind and cold shock stimulation as shown after 3' RACE was called *NpCaM-1* and another non-induced was called *NpCaM-2* (Fig. 4.3), both were cloned and sequenced. In figure 4.4, the partial sequence of these two calmodulin isoforms is shown starting half-way the first Ca^{2+} binding site of calmodulin. *NpCaM-1* and *NpCaM-2* were subcloned using a pCR-Script Amp SK(+) Cloning Kit (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 as described in Chapter 2. After sequence analysis it appeared that *NpCaM-1* encodes the calmodulin cDNA that was originally cloned by Anne Taylor. The sequence was identical apart from nucleotide residues 147 (T for a C), 363 (G for an A), 366 (C for a T), 369 (C for a T), and 372 (A for a G). The partial sequences of *NpCaM-1* and *NpCaM-2* are different in nucleotide sequence; they encode, however, polypeptides with the same amino acid sequence (Fig. 4.4B).
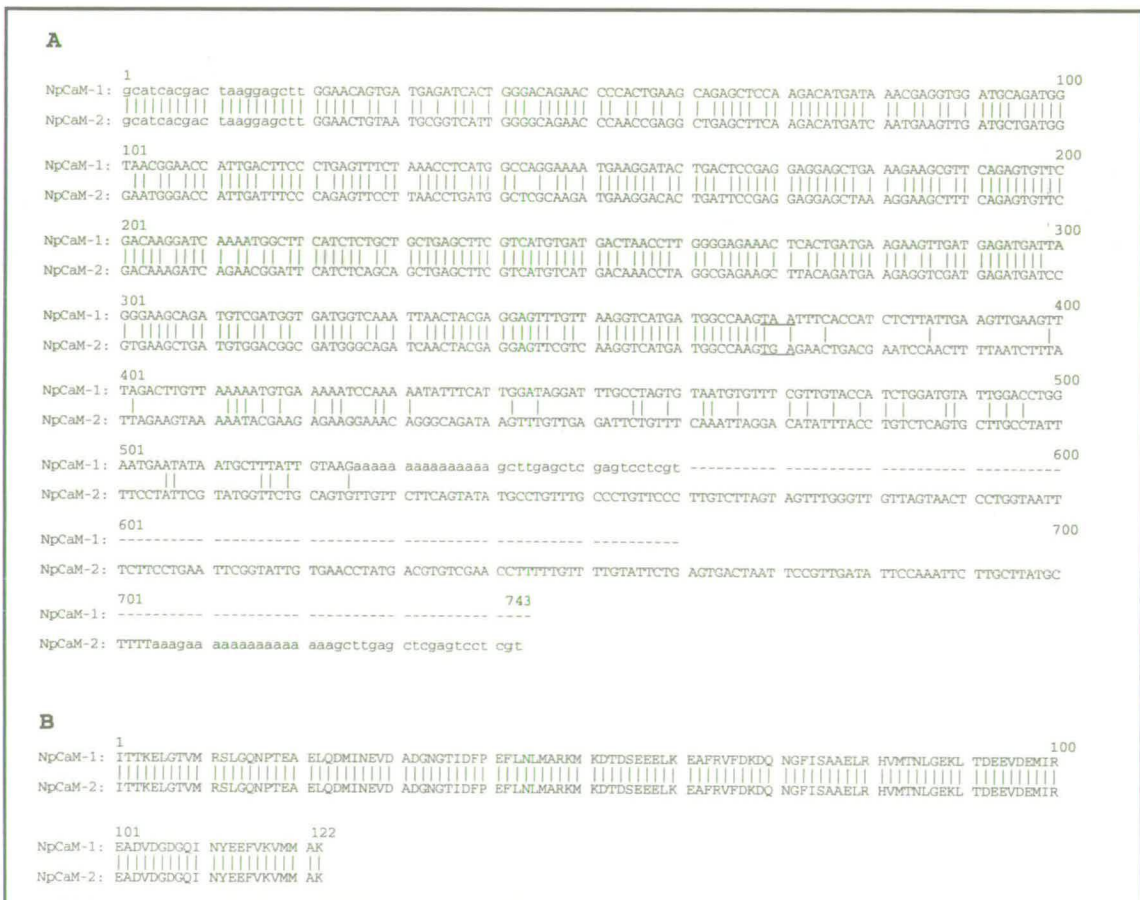
Figure 4.4: Partial cDNA sequence of *NpCaM-1* and *NpCaM-2* showing nucleotide and predicted amino acid homologies. (**A**) Nucleotide sequence (**B**) amino acid sequence. Primers used for 3' RACE and subsequent PCR are indicated in lowercase, stop codons are underlined. Homology is indicated with bars.

4.3   Wind and Cold Shock Induce NpCaM-1 but not NpCaM-2

The 3' untranslated regions from *NpCaM-1* and *NpCaM-2* were subcloned and used as DNA hybridisation probes to study the expression kinetics of *NpCaM-1* and *NpCaM-2* using northern blot analysis (Fig. 4.5). As shown in figure 4.3, this type of analysis indicates that *NpCaM-1* mRNA accumulates after wind and cold shock signalling whereas *NpCaM-2* does not. The accumulation of *NpCaM-1* mRNA is more apparent after cold shock then after wind stimulation substantiating the data obtained with 3' RACE (Fig. 4.3). In addition, the kinetics of *NpCaM-1* induction is different; maximal levels are reached 60 minutes after wind stimulation, whereas 90 minutes are required after cold shock stimulation. In a control, water of room temperature was gently placed onto the seedlings which resulted in a slight increase in *NpCaM-1* mRNA accumulation.
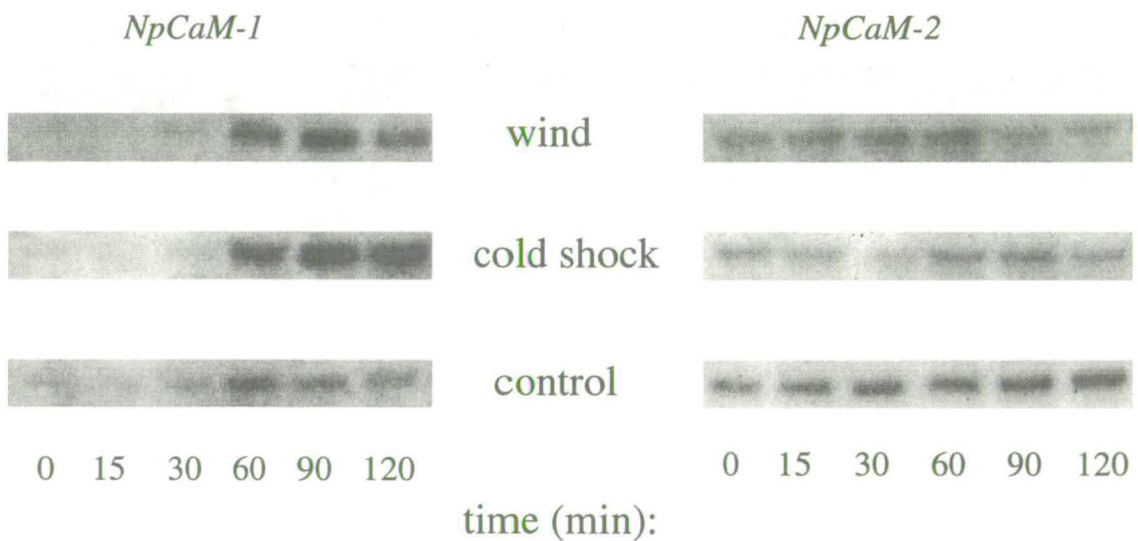
Figure 4.5: Expression kinetics after wind and cold shock stimulation of *NpCaM-1* and *NpCaM-2* determined by northern blot analysis. The 3' untranslated regions of *NpCaM-1* and *NpCaM-2* were used as DNA hybridisation probes in order to study the expression kinetics of *NpCaM-1* and *NpCaM-2* using northern blots.

4.4   Discussion

After 3' RACE 7 putative calmodulin transcripts were identified. Two of them, *NpCaM-1* and *NpCaM-2* were cloned and sequenced. It can only be speculated that the addition bands observed after 3' RACE encode other calmodulin isoforms than NpCaM-1 and NpCaM-2. These fragments might be the result of transcripts of different length originating from the same genes containing different sites for polyadenylation. A third band that was cloned and sequenced (clone X4.4) appeared to be encoded by the same gene as *NpCaM-1* but had a slightly longer 3' end tail (data not shown). The transcripts *NpCaM-1* and *NpCaM-2* are encoded by distinct genes as the sequence of each transcript is different.

After 3' RACE and northern blot analysis it can be concluded that the transcripts for isozymes of calmodulin are differential accumulated after wind and cold shock stimulation. The accumulation of *NpCaM-1* transcript is induced by wind and cold whereas *NpCaM-2* transcript is not as determined by northern blot analysis using the 3' untranslated regions of *NpCaM-1* and *NpCaM-2* as hybridisation probe. These data are therefore in agreement with the data of Takewaza et al. (1995) who demonstrated that only one calmodulin isozyme accumulated after touch stimulation. In addition, the kinetics of induction of *NpCaM-1* mRNA accumulation is different for each environmental stimulus. Maximal levels of induction of *NpCaM-1* are reached 60 minutes after wind stimulation, whereas 90 minutes are required after cold shock stimulation. The kinetics of the Ca^{2+} response was also shown to be clearly different upon each environmental signal (Fig. 3.9 and 3.10). Whether these two can be correlated will be discussed in later chapters.

Taken together, during early developmental stages of tobacco seedlings, at least two different calmodulin genes are actively transcribed and their expression is differently regulated. The cellular location or particular cellular function of these two isozymes remains speculative but further work isolating the promoters and the use of reporter genes such as green fluorescent protein (GFP) might clarify this situation. The presence of different 3' regions of the mRNA encoding calmodulin isoforms suggests that these two might have different regulatory factors controlling their expression.

Chapter 5

Wind Stimulation Induce Changes in Cytosolic, Nuclear Calcium Levels and Calmodulin Gene Expression

Spraying with water or simply touching the leaves of *Arabidopsis thaliana* led to the induction of so-called touch (*TCH*) genes (Braam and Davis, 1990). One of these genes was shown to encode calmodulin. An increase in extracellular Ca^{2+} led to an increase in calmodulin gene expression, therefore, it was hypothesised that Ca^{2+} regulates calmodulin gene expression (Braam, 1992). The fact that wind and touch induce changes in cytosolic Ca^{2+} is in line with this hypothesis (Knight et al., 1992). Mechanically induced cytosolic Ca^{2+} could bind cytosolic calmodulin that is translocated into the nucleus where it regulates its own expression, or alternatively, nuclear calcium could activate nuclear localised calmodulin which could then directly regulate the expression (Fig. 1.9 in Chapter 1).

It is not known which particular characteristics of the Ca^{2+} signal are interpreted by plant cells which leads to changes in downstream processes including gene expression. Consequently all the obvious characteristics and parameters of the wind-induced Ca^{2+} signals were measured with the intention of uncovering which characteristic(s) best relate to subsequent downstream processes. In this chapter, a correlation is sought between wind-induced levels of cytosolic Ca^{2+}, nuclear Ca^{2+} and calmodulin gene expression in *Nicotiana plumbaginifolia* with the use of modulators of calcium-channel action. The calcium response was studied with MAQ 2.4 expressing cytosolic aequorin to study changes in cytosolic calcium levels, $[Ca^{2+}]_{cyt}$ and MAQ 7.11 expressing nuclear targeted aequorin to study changes in nuclear calcium levels, $[Ca^{2+}]_{nuc}$. The spatio-temporal calcium dynamics were related to changes in the levels transcript accumulation of *NpCaM-1* and *NpCaM-2*.

5.1   Wind-Induced Changes in Cytosolic, Nuclear Calcium and NpCaM-1 mRNA Levels

Tobacco seedlings were given precisely similar wind signals (one treatment of 5 mL air) as those used for figure 3.9 (Chapter 3). 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 5.1. Changes in nuclear and cytoplasmic Ca^{2+} are included for comparison. The total increase of *NpCaM-1* mRNA was about 5.5 fold after 90

minutes. *NpCaM-1* accumulates with kinetics which are clearly and unexpectedly S-shaped. *NpCaM-2* exhibits only slight increases throughout the experimental period.
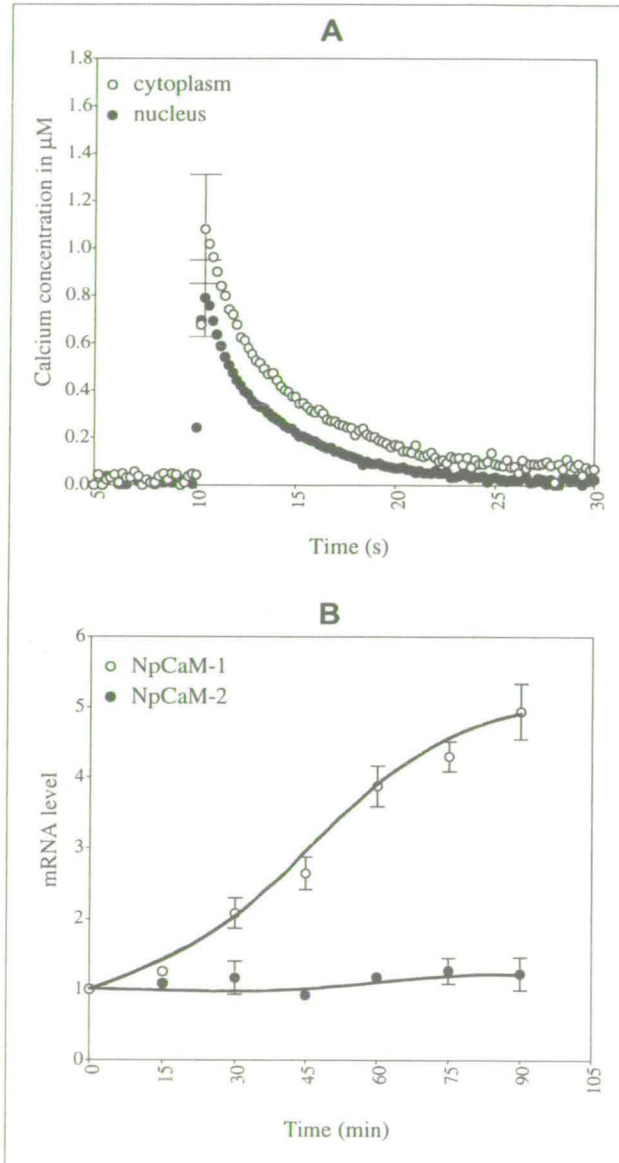

Figure 5.1: Wind-induced changes in cytoplasmic and nuclear free calcium concentration and *NpCaM-1* and *NpCaM-2* mRNA accumulation. (**A**) Ca^{2+} changes are shown in cytoplasm (MAQ 2.4) and nucleoplasm (MAQ 7.11) after wind stimulation by one injection of 5 mL air. (**B**) Wind-induced changes in mRNA levels of *NpCaM-1* and *NpCaM-2* are indicated. Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0. The means ± s.e. for the peak values from 8 experiments are indicated.

5.2 The Effect of Ruthenium Red, BAPTA-AM and Thapsigargin on Wind-Induced Cytoplasmic, Nuclear Calcium and NpCaM-1 mRNA Levels

In order to investigate whether the expression of *NpCaM-1* is regulated by changes in cytoplasmic Ca^{2+}, by nuclear Ca^{2+}, or both seedlings were treated with known agonists/antagonists of Ca^{2+} signalling. The effects of these pharmacological compounds on wind-induced changes in $[Ca^{2+}]_i$ in both compartments have been quantified and compared with the overall change in accumulation of *NpCaM-1* mRNA (Table 5.I). Data on ruthenium red, internally loaded BAPTA and thapsigargin is shown in figures 5.2-4. Each figure contains successively the effects of the inhibitor on cytoplasmic Ca^{2+} (A), nuclear Ca^{2+} (B) and *NpCaM-1* mRNA accumulation (C). Eight replicates of Ca^{2+} signalling have been performed and duplicate time courses for mRNA accumulation were taken to ensure adequate statistical analysis.

The inhibitory effect of ruthenium red on wind-induced cytosolic calcium levels has been reported (Knight et al., 1992). Ruthenium red may be a putative inhibitor of mitochondrial and endoplasmic reticulum Ca^{2+} channels although this is by no means well established (Campbell, 1983). The effect of 50 μM ruthenium red on wind-induced cytoplasmic Ca^{2+} levels were studied on MAQ 2.4 plants. Pretreatment of seedlings with 50 μM ruthenium red significantly decreased wind-induced peak cytoplasmic Ca^{2+} levels from 1.08 ± 0.23 μM to 0.39 ± 0.04 μM (Fig. 5.2A) a reduction of 64%. Nuclear Ca^{2+} treatment peak values dropped from 0.79 ± 0.16 μM to 0.68 ± 0.16 μM although this difference is not significant (Fig 5.2B). In both compartments ruthenium red did not significantly alter either the time to reach maximal values (T_p), nor the duration of the calcium response (T_Δ) but resting levels were slightly elevated prior to wind stimulation.

There are indications of a slight acceleration in the accumulation of *NpCaM-1* mRNA as a result of ruthenium red treatment (Fig 5.2C) but the plateau values are not significant statistically from the control mRNA data shown in figure 5.1. Levels of mRNA of *NpCaM-2* were not significantly affected by ruthenium red treatments. The apparent lack of inhibition of ruthenium red on *NpCaM-1* mRNA accumulation correlates better with the absence of changes in the maximal nuclear Ca^{2+} levels than the pronounced inhibition of the maximal cytoplasmic Ca^{2+} levels.
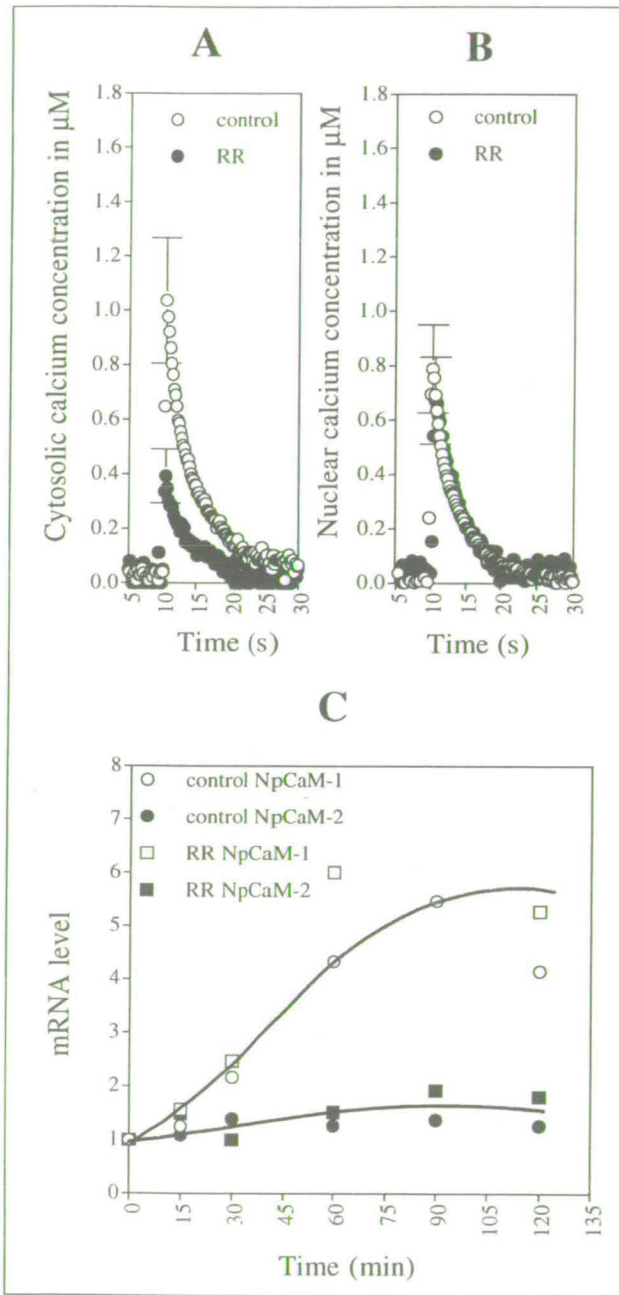
Figure 5.2: Effect of ruthenium red (**RR**) on wind-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 50 μM ruthenium red on wind-induced changes in calcium free concentration in the cytoplasm (**A**) and nucleoplasm (**B**). The effect of 50 μM ruthenium red on wind-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0. The means ± s.e. for the peak values from 8 experiments are indicated.

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Figure 5.3 shows data in which seedlings have been incubated in BAPTA-AM in order to load a calcium-chelator inside the cells of the seedlings and thus provide a possible Ca^{2+} sink which can modify subsequent Ca^{2+} mobilisation and mRNA accumulation. To help improve possible uptake, BAPTA-AM was loaded under acidic conditions (Bush and Jones, 1987), so that if external ester hydrolysis occurred it would still permit further entry of the unesterified BAPTA. In both nuclear and cytoplasmic compartments, wind-induced Ca^{2+} increases were substantially reduced by the BAPTA treatment but to differing extents. In the cytoplasm the peak was reduced from 1.08 ± 0.23 μM to 0.61 ± 0.08 μM a reduction of 44%. The nuclear Ca^{2+} peak height was reduced from 0.79 ± 0.43 μM to 0.34 ± 0.07 μM, a reduction of 57%. The time to reach maximal levels in the cytoplasm remained unchanged at 0.33 ± 0.04 s but was lengthened to 0.83 ± 0.09 s in the nucleus. The total length of the Ca^{2+} transient in the cytoplasm was increased from 13.6 s to 24.5 ± 2.3 s but remained unchanged in the nucleus. Resting levels were elevated slightly but only in the cytoplasmic compartment prior to wind stimulation. In a control experiment using the solvents alone and the acidic conditions used to load BAPTA-AM inside the cells no effects were observed on the calcium signal in either compartment (data not shown).

Calmodulin mRNA accumulates to a different extent after pretreatment with 1 mM BAPTA-AM. Maximal levels of *NpCaM-1* are lowered by 60 % (Fig. 5.3C) and accumulated only up to 2.5 fold. Levels of mRNA of *NpCaM-2* were not affected. Again BAPTA-induced reductions in peak values of nuclear Ca^{2+} correlate much better with the reduction in *NpCaM-1* than with those in cytoplasmic Ca^{2+}.
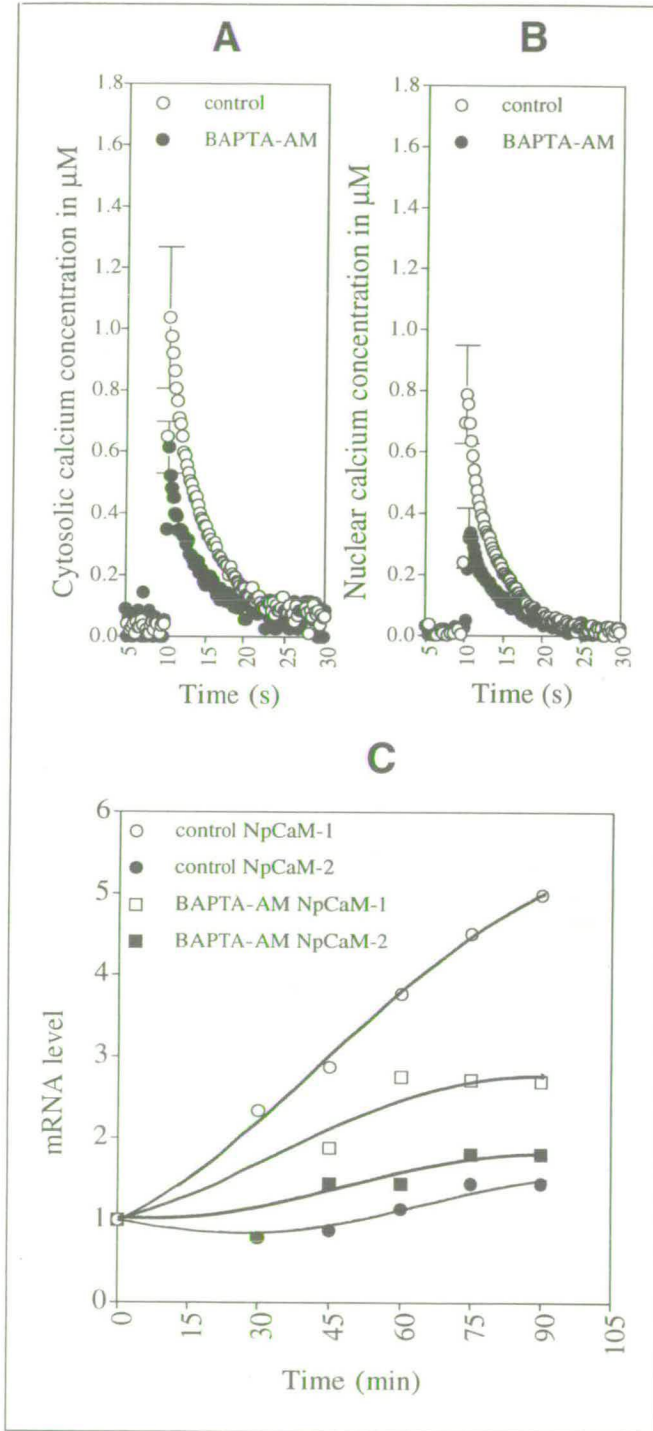
Figure 5.3: Effect of BAPTA-AM on wind-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 1 mM BAPTA-AM on wind-induced changes in calcium concentration in cytoplasm (**A**) and nucleoplasm (**B**). The effect of 1 mM BAPTA-AM on wind-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0. The means ± s.e. for the peak values from 8 experiments are indicated.

Thapsigargin is an effective inhibitor of the Ca^{2+}-ATPase located on the endoplasmic reticulum membranes (Thastrup et al., 1990). Thapsigargin has been used before in plants in order to artificially increase cytosolic Ca^{2+} and induce gene expression in the absence of a normal inductive stimulus (Raz and Fluhr, 1992).

Pretreatment of MAQ 2.4 seedlings with 200 µM thapsigargin only slightly increased wind-induced peak cytoplasmic Ca^{2+} levels but this effect was not significant, 1.08 ± 0.23 µM to 1.12 ± 0.25 µM (Fig. 5.4A). In contrast, nucleoplasmic Ca^{2+} levels were substantially and significantly increased from 0.79 ± 0.16 µM to 1.20 ± 0.10 µM (Fig. 5.4B) an increase of 52%. Thapsigargin did not affect the rise times in either compartment or the length of the transients in the cytoplasm but the transient length in the nucleus was increased by about 5 seconds.

NpCaM-1 mRNA accumulations were increased by 200 µM thapsigargin treatment. Maximal levels of *NpCaM-1* are increased by about 50 % (Fig. 5.4C) and the total accumulation was up to 7.5 fold. Levels of mRNA of *NpCaM-2* were not affected. Again the peak values of the nuclear Ca^{2+} correlate with the changes in *NpCaM-1* and those of the cytoplasmic Ca^{2+} do not.

Table 5.I: Overview of inhibitors used and their effects on wind-induced calcium kinetics in the cytoplasm and nucleus and the levels of wind-induced *NpCaM-1* mRNA accumulation. T_p and T_Δ indicate the time to reach maximal levels and the duration of the response respectively.

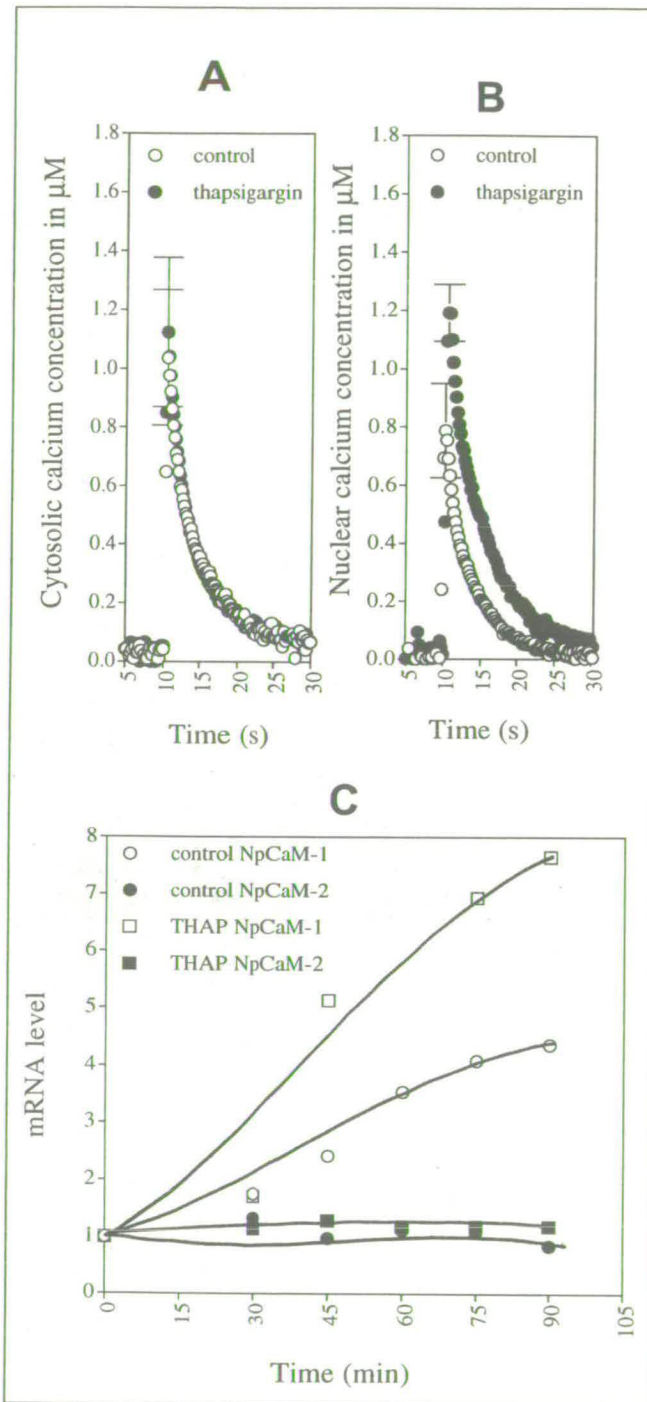|	Cytoplasm	Cytoplasm	Nucleus	Nucleus	*NpCaM-1*
Treatment	Inhibition of Ca^{2+} response	T_p/T_Δ	Inhibition of Ca^{2+} response	T_p/T_Δ	Inhibition of mRNA levels
ruthenium red	64%	none/none	none	none/none	none
BAPTA-AM	44%	none/increased	57%	increased/none	60%
thapsigargin	none	none/none	152%	none/increased	150%

Figure 5.4: Effect of thapsigargin on wind-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 200 μM thapsigargin on wind-induced changes in free calcium concentration in cytoplasm (**A**) and nucleoplasm (**B**). The effect of 200 μM thapsigargin on wind-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T$_0$. The means ± s.e. for the peak values from 8 experiments are indicated.

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5.3   Discussion

The particular features of the changes in nuclear Ca^{2+} i.e. the peak values correlate much better with the subsequent changes in *NpCaM-1* mRNA accumulation than the peak values of cytoplasmic Ca^{2+}. This perhaps is not surprising if there are transduction elements able to interpret the Ca^{2+} signal directly in the nucleus. The presence of nuclear calmodulin has been reported on a number of occasions (Bachs et al., 1992; Gilchrist et al., 1994; Zimprich et al., 1995). Calmodulin, an acidic protein, might bind directly to histones thus mediating changes in gene expression. Alternatively, transcription factors able to bind calmodulin or calcium and/or calmodulin dependent protein kinases in the plant nucleus might be responsible for regulating expression of *NpCaM-1*. Data presented here does not preclude involvement of cytoplasmic Ca^{2+} concentrations in the responses; it merely suggests that changes in nuclear Ca^{2+} may be more important.

Now that good quantitative measurements of Ca^{2+} can be made by using aequorin, important elements of Ca^{2+} signal transduction are beginning to emerge. Conversion of aequorin-emitted light to $[Ca^{2+}]_{cyt}$ was first described by Knight and co-workers (1996). The Ca^{2+} signals can now be quantified and the significance of changes in nuclear Ca^{2+} levels can be determined. The relationship of light emission to Ca^{2+} is not linear and thus simple measurements of luminescent light on its own may lead to confusing interpretations unless care is taken. An important tool used here is *cp*-coelenterazine. When reconstituted, the *cp*-aequorin responds more readily to lower elevations of Ca^{2+} thus clarifying the kinetics and response of nuclear Ca^{2+} concentrations.

A number of pharmacological compounds were used to try and correlate changes in Ca^{2+} with inhibition of gene expression. Other researchers have used external calcium and calcium chelators of external Ca^{2+} (EGTA and BAPTA) and examined the effects on the mRNA levels of heat and cold shock-induced *TCH* genes (Braam, 1992; Polisensky and Braam, 1996). In *Arabidopsis*, the channel blockers lanthanum and gadolinium affected cold-induced expression of *TCH* genes (Polisensky and Braam, 1996). In alfalfa, external Ca^{2+}, BAPTA, lanthanum and calmodulin inhibitors partially inhibited the cold induction of cold acclimation-specific genes (Monroy et al., 1993; Monroy and Dhindsa, 1995). In maize seedlings, ruthenium red inhibited the expression of *adh1* and *sh1* genes in maize seedlings under anaerobic conditions (Subbaiah et al., 1994) and in *Nicotiana tabacum*, blocking of Ca^{2+} fluxes with EGTA inhibited and ionomycin and thapsigargin stimulated ethylene-dependent induction of chitinase accumulation (Raz and Fluhr, 1992). In addition, recently

reported from studies in *Arabidopsis*, lanthanum or EGTA causes a partial inhibition of both cold shock $[Ca^{2+}]_{cyt}$ elevation and cold-dependent *kin1* gene expression (Knight et al., 1996). Measurement of nuclear Ca^{2+} in these above cases might help clarify and improve the particular relationships between signal transduced Ca^{2+} and gene expression. The data presented here show that cytoplasmic Ca^{2+} generally responds in the same direction as nuclear Ca^{2+} to the inhibitors that were used, but a more exacting quantitative relationship with the accumulation of *NpCaM-1* mRNA suggest a more profound role for nuclear Ca^{2+} then for cytosolic Ca^{2+}.

A key question unanswered in previous studies is the relationship between cytosolic Ca^{2+} and nuclear Ca^{2+} levels. The data in this chapter suggests that there must be at least partial independence between the two compartments. For example, thapsigargin elevated nuclear Ca^{2+} in response to wind treatments but it failed to cause an elevation of cytoplasmic Ca^{2+}. With a different signal this situation could well change and in both compartments Ca^{2+} might be elevated to the same degree or the cytoplasmic response might even be higher. At the present time the relationship between cytoplasmic Ca^{2+} and nuclear Ca^{2+} is a controversial question in mammalian cells (Bustamante et al., 1994). Many investigations have suggested that increases in cytosolic Ca^{2+} subsequently manipulate nuclear Ca^{2+} by direct movement of Ca^{2+} through nuclear pores (Brini et al., 1993; Al-Mohanna et al., 1994; O'Malley, 1994; Meyer et al., 1995; Gerasimenko et al., 1996). Thus it can be envisaged that Ca^{2+} waves generated by signals in the cytoplasm continue in a truncated form through the nuclear membrane. The data presented here show that after wind stimulation, cytoplasmic Ca^{2+} peaks first within 0.3 seconds whilst nuclear Ca^{2+} peaks after 0.6 seconds. On the basis of on these kinetics it could be speculated that the same holds true for plant cells.

However alternative evidence suggests that nuclear Ca^{2+} is regulated independently from cytosolic Ca^{2+} levels (Williams et al., 1985; Shankar et al., 1993; Badminton et al., 1996). There are also signal transduction elements in the nuclear membrane which involve the synthesis of inositol phosphates and which could mobilise Ca^{2+} from intranuclear stores (Divecha et al., 1994; Stehno-bittel et al., 1995; Humbert et al., 1996). Alternatively cytoplasmic Ca^{2+} might induce Ca^{2+}-induced Ca^{2+} release from the nuclear membrane. The data described here suggests that cytoplasmic Ca^{2+} and nuclear Ca^{2+} are indirectly related in plant cells; they do behave in semi-independent fashion and thus the latter view is favoured at present.

Key features which underpin the above controversy concerning the relationship between nuclear and cytoplasmic Ca^{2+} are that (1), Ca^{2+} is thought to act in a very local fashion due to its very low diffusion rate of calcium inside the cytoplasm (Rose and

Loewenstein, 1976; Clapham, 1995) and (2), the apparent impermeability of the nuclear envelope for cations (Loewenstein and Kanno, 1963). In addition, further difficulties in accurately measuring nuclear Ca^{2+} levels have resulted from the limitations of using Ca^{2+}-sensitive fluorescent dyes to measure changes in nuclear calcium (Al-Mohanna et al., 1994; Rand et al., 1994). Fluorescent dyes have been shown to behave differently in the two compartments (Perez-Terzic et al., 1997). Much more investigation is needed on the origins of free Ca^{2+} in the nucleus and the technology described by using aequorin should be helpful in resolving some of these issues because aequorin as a reporter may be less ambiguous.

Chapter 6

Cold Shock Induce Changes in Cytosolic, Nuclear Calcium Levels and Calmodulin Gene Expression

Cold shock-induced *cas* (cold-acclimation specific) gene expression correlates with the uptake of extracellular Ca^{2+} in alfalfa protoplasts and mRNAs are accumulated encoding a putative Ca^{2+}-dependent protein kinases (Monroy and Dhindsa, 1995). However, mRNA that encoded calmodulin were not found to be induced after cold shock. It was hypothesised that protein kinases/phosphatases and/or their substrates lead to *cas* gene expression without the mediation of increased levels of calmodulin. In *Arabidopsis*, Polisensky and Braam (1996) reported a correlation between cold shock-induced changes in cytosolic Ca^{2+} and the induction of *TCH-2* and *TCH-3* genes that are related to calmodulin, but not calmodulin itself.

In this chapter, a correlation is sought between cold shock-induced levels of cytosolic Ca^{2+}, nuclear Ca^{2+} and calmodulin gene expression in *Nicotiana plumbaginifolia* with the use of modulators of Ca^{2+}-channel action. The Ca^{2+} response was studied with MAQ 2.4 expressing cytosolic aequorin to study changes in cytosolic calcium levels, [Ca^{2+}]$_{cyt}$ and MAQ 7.11 expressing nuclear targeted aequorin to study changes in nuclear calcium levels, [Ca^{2+}]$_{nuc}$. The spatio-temporal calcium dynamics were related to changes in the levels transcript accumulation of *NpCaM-1* and *NpCaM-2*.

6.1   Cold Shock-Induced Changes in Cytosolic, Nuclear Calcium and NpCaM-1 mRNA Levels

Tobacco seedlings were given precisely similar cold shock signals (one treatment of 1 mL ice-cold water) as those used for figure 3.10 (Chapter 3). Cold shock-induced changes in cytosolic and nuclear Ca^{2+} are shown in Fig. 6.1A and the kinetics can be compared to wind-induced changes in cytosolic and nuclear Ca^{2+} shown in Fig. 6.1B. RNA was extracted and the levels of *NpCaM-1* and *NpCaM-2* mRNAs estimated from northern blots.   Intensities of hybridising bands were quantified using Imagequant™ from Molecular Dynamics ('s-Hertogenbosch, NL). These data (n=3) are shown in figure 6.1C. The total increase of *NpCaM-1* mRNA was about 10-12 fold after 90 minutes which are in apparent conflict with results previously described in alfalfa (Monroy and Dhindsa, 1995) but are in line with the observation that cold shock

induces the expression of calmodulin-related (*TCH*) genes (Polisensky and Braam, 1996). As observed after wind stimulation, cold shock-induced *NpCaM-1* accumulates with kinetics which are clearly S-shaped (Fig. 6.1C). Whereas wind-induced mRNA levels of *NpCaM-1* start to plateau after 75 minutes, cold shock-induced *NpCaM-1* mRNA levels continue to increase upto 90-120 minutes after which it declines. *NpCaM-2* exhibits only slight increases throughout the experimental period for both stimuli.

The kinetics of the Ca^{2+} response is clearly different upon each environmental stimulus and for the cytosolic and nuclear compartment. The average time to reach maximal values after wind stimulation was for cytosolic and nuclear $[Ca^{2+}]$, 0.31 s and 0.60 s, respectively, whereas after a cold shock, 4.8 s and 9.0 s, respectively. The length of time it remained above resting levels was similar in both compartments after wind stimulation, 13.6 s, but was significantly different after cold shock treatment, 24 and 31 s for cytosolic and nuclear $[Ca^{2+}]$, respectively. In the cytosol the estimated Ca^{2+} resting levels are 11 ± 2 nM before cold signalling and 35 ± 3 nM after, a 3 fold increase. In the nucleus the estimated resting Ca^{2+} levels are 8 ± 4 nM before cold signalling and 35 ± 12 nM after, a 4 fold increase. These altered resting levels were observed in all experiments and continue throughout the duration of the experimental periods investigated.
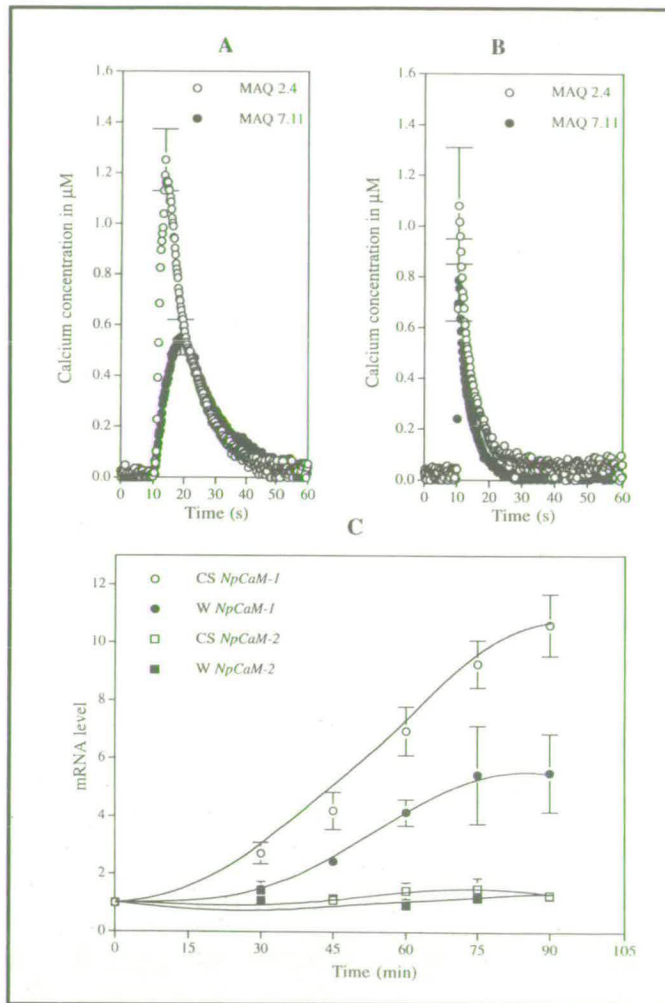
Figure 6.1: Cold shock-induced changes in cytosolic and nuclear free calcium concentration and *NpCaM-1* and *NpCaM-2* mRNA accumulation. (**A**) Ca^{2+} changes are shown in cytoplasm (MAQ 2.4) and nucleoplasm (MAQ 7.11) after cold shock stimulation by a gently injection of 1 mL ice-cold water. (**B**) Ca^{2+} changes are shown in cytoplasm (MAQ 2.4) and nucleoplasm (MAQ 7.11) after wind stimulation by an instant injection of 5 mL air. (**C**) Cold shock- and wind-induced changes in mRNA levels of *NpCaM-1* and *NpCaM-2* are indicated. Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0.

6.2 The Effect of LaCl₃ and Neomycin on Cold Shock-Induced Cytoplasmic, Nuclear Calcium and NpCaM-1 mRNA Levels

It has been reported (Knight et al., 1991, 1992), that gadolinium, a mechanosensitity Ca^{2+}-channel blocker and lanthanum, a Ca^{2+}-channel blocker at the plasma membrane resulted in weaker Ca^{2+} response after cold shock treatment. It has been suggested that Ca^{2+} originates from extracellular sources. The uptake of Ca^{2+} of cold-shocked alfalfa protoplasts are in line with this prediction (Monroy and Dhindsa, 1995). In more recent experiments the effect of neomycin, that blocks metabolism of

polyphosphoinositides (Atkinson et al., 1993) and phospholipase C (Toyada et al., 1992 and Légendre et al., 1993) was determined on the cold shock-induced cytosolic Ca^{2+} concentration (Knight et al., 1996) which suggested Ca^{2+} release from internal stores that are IP$_3$ mediated in the cold shock response although its contribution was found to be minute.

The role of Ca^{2+} in the cold shock response has been implicated in gene regulation, as it has been shown that the expression of calmodulin-related genes (*TCH*) are effected by the calcium agonists/antagonists and calcium chelators, BAPTA, LaCl$_3$ and GdCl$_3$ (Polisensky and Braam, 1996). In addition, Ca^{2+} has been implicated in *cas* gene regulation in cold-treated alfalfa (Monroy and Dhindsa, 1995) and in *kin1* gene regulation in cold-shocked *Arabidopsis* (Knight et al., 1996). The effects of calcium modulators were studied here on cold shock-induced nucleoplasmic calcium levels and were compared to cytoplasmic Ca^{2+} levels and *NpCaM-1* and *NpCaM-2* mRNA levels.

- Data on LaCl$_3$ and neomycin is shown in figures 6.2 and 6.3. Each figure contains successively the effects of the inhibitor on cytoplasmic Ca^{2+} (A), nuclear Ca^{2+} (B) and *NpCaM-1* mRNA accumulation (C). Eight replicates of Ca^{2+} signalling have been performed, so that adequate statistical analysis can be made, and duplicate time courses for mRNA accumulation. Table 6.I shows an overview of all inhibitors used and their effect on cold shock-induced cytosolic and nuclear calcium dynamics and *NpCaM-1* mRNA accumulation.

Lanthanum is an inhibitor of Ca^{2+}-channels located at the plasma membrane and its effect was studied previously (Knight et al., 1992) where it inhibited the cold shock-induced increases in cytosolic Ca^{2+} levels. Only recently it has been possible to quantify the inhibition effect by using calibration curves for the Ca^{2+} response and was found to be more than 50% in *Arabidopsis* (Knight et al., 1996). In addition, lanthanum has been described to affect cold shock-induced *TCH* gene expression (Polisensky and Braam, 1996). Here, the effect of lanthanum is studied on the cold shock Ca^{2+} response in the cytoplasm, nucleus and the mRNA levels of *NpCaM-1* and *NpCaM-2* of tobacco seedlings.

Pretreatment of MAQ 2.4 seedlings with 10 mM LaCl$_3$ significantly lowered the cold shock-induced cytosolic Ca^{2+} response from 1.25 ± 0.12 μM to 0.37 ± 0.05 μM, a reduction of 70% (Fig. 6.2A). Identical treatment of MAQ 7.11 seedlings with 10 mM LaCl$_3$ significantly lowered the cold shock-induced nuclear Ca^{2+} response from 0.55 ± 0.07 μM to 0.14 ± 0.03 μM, a reduction of 75% (Fig. 6.2B). Pretreatment of seedlings with 10 mM LaCl$_3$ did not affect the time to reach peak height, T$_p$, but prolonged the response in cytoplasm and nucleus significantly from 23.6 ± 1.2 s to

30.2 ± 2.6 s and from 30.7 ± 1.9 s to 42.6 ± 2.2 s, respectively. Lanthanum did not affect resting levels in either compartments. Lanthanum almost completely abolished the cold shock-induced accumulation of *NpCaM-1*. Levels were decreased from 11 fold down to 1.5 fold (Fig. 6.2C). Levels of mRNA of *NpCaM-2* were not affected.
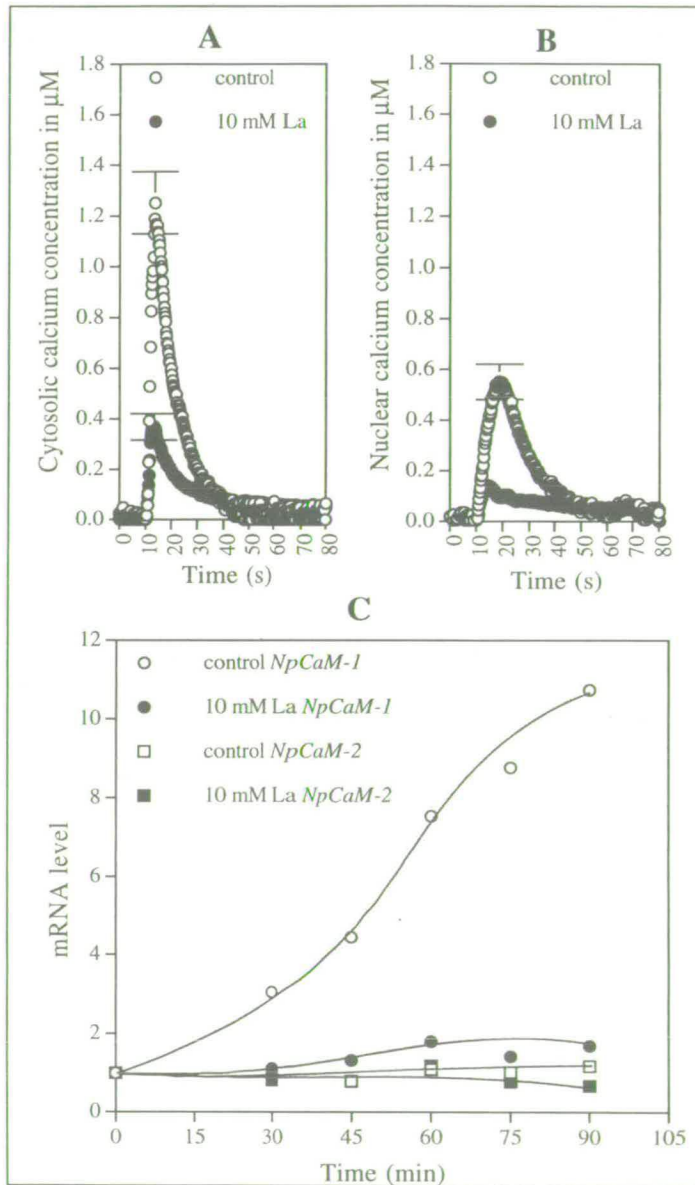

Figure 6.2: Effect of $LaCl_3$ (**La**) on cold shock-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 10 mM $LaCl_3$ on cold shock-induced changes in calcium free concentration in the cytoplasm (**A**) and nucleoplasm (**B**). The effect of 10 mM $LaCl_3$ on cold shock-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0.

Neomycin inhibits the metabolism of polyphosphoinositides and phospholipase C and affects cold shock-induced increase slightly in cytosolic Ca^{2+} levels in *Arabidopsis* (Knight et al., 1996). Here, the effect of neomycin is studied on the cold shock calcium response in the cytoplasm, nucleus and of calmodulin gene expression. Pretreatment of MAQ 2.4 seedlings with 200 µM neomycin significantly lowered the cold shock-induced cytoplasmic Ca^{2+} levels from 1.25 ± 0.12 µM to 0.88 ± 0.02 µM (Fig. 6.3A), a reduction of 30%. Identical treatment of MAQ 7.11 inhibited the cold shock-induced nuclear Ca^{2+} increase from 0.55 ± 0.07 µM to 0.33 ± 0.03 µM (Fig. 6.3B), a reduction of 40%. Neomycin did not affect the time to reach peak heights T_p, nor the duration of the transient in both compartments. A higher resting level in nucleoplasmic Ca^{2+} level was observed prior to cold shock treatment and a lower returning level in the nucleus.

Calmodulin mRNA accumulates to a different extent when tobacco seedlings were treated with 200 µM neomycin. Maximal levels of mRNA of *NpCaM-1* were lowered by 70% from 11 fold to 4 fold (Fig. 6.3C). Levels of mRNA of *NpCaM-2* were not affected.

Table 6.I: Overview of inhibitors used and their effects on cold shock-induced calcium kinetics in the cytoplasm and nucleus and the levels of cold shock-induced *NpCaM-1* mRNA accumulation. T_p and T_Δ indicate the time to reach maximal levels and the duration of the response respectively.

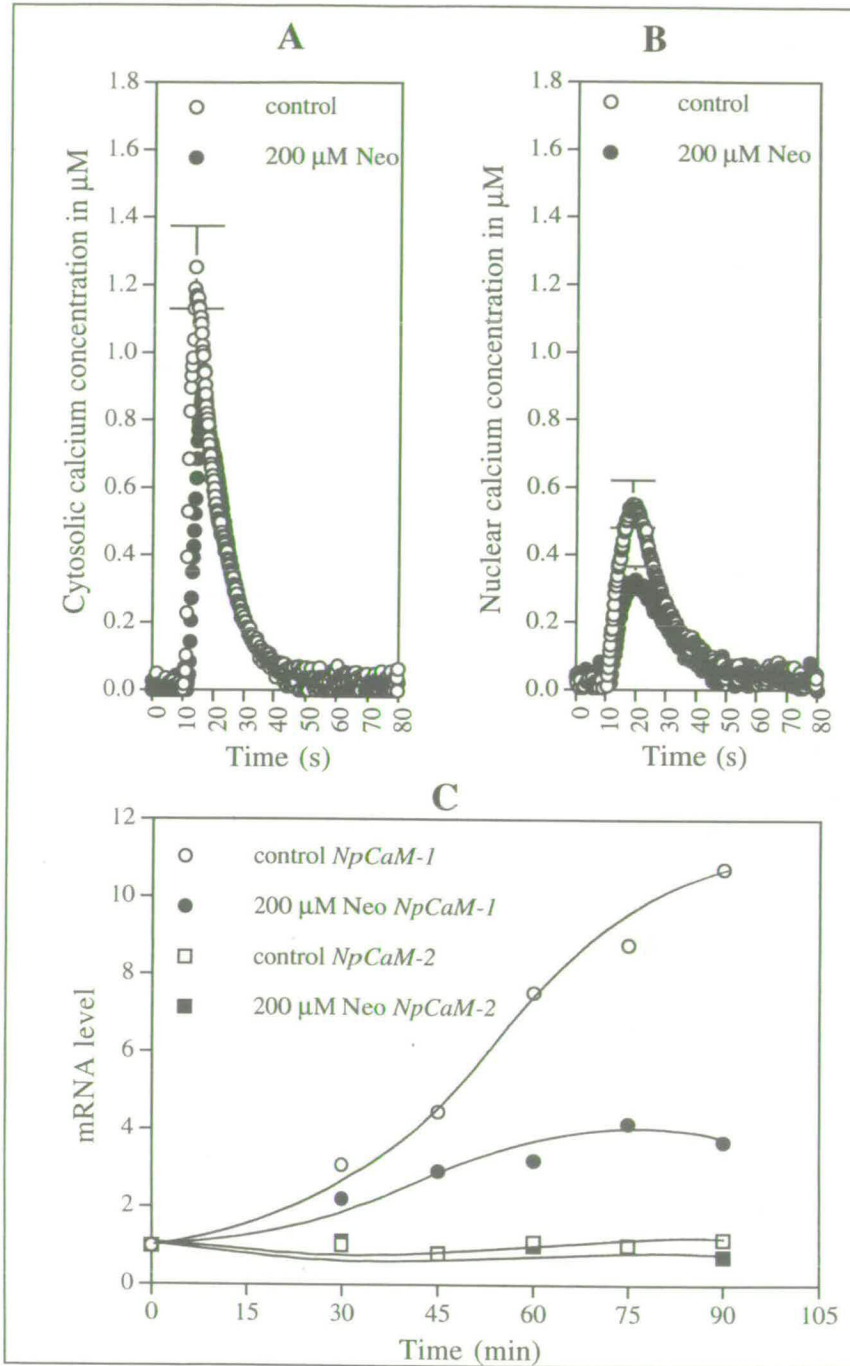|	Cytoplasm	Cytoplasm	Nucleus	Nucleus	*NpCaM-1*
Treatment	Inhibition of Ca^{2+} response	T_p/T_Δ	Inhibition of Ca^{2+} response	T_p/T_Δ	Inhibition of mRNA levels
lanthanum	70%	none/increased	75%	none/increased	90%
neomycin	30%	none/none	40%	none/none	70%
BAPTA-AM	120%	none/none	135%	none/none	50%
thapsigargin	129%	none/none	160%	none/none	50%

Figure 6.3: Effect of neomycin (**Neo**) on cold shock-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 200 µM neomycin on cold shock-induced changes in calcium free concentration in the cytoplasm (**A**) and nucleoplasm (**B**). The effect of 200 µM neomycin on cold shock-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0.

6.3 The Effect of BAPTA-AM and Thapsigargin on Cold Shock-Induced Cytoplasmic, Nuclear Ca^{2+} and NpCaM-1 mRNA Levels

Figure 6.4 shows data in which seedlings have been incubated in BAPTA-AM in order to load a Ca^{2+}-chelator inside the cells of the seedlings and thus provide a possible Ca^{2+} sink which can modify subsequent Ca^{2+} mobilisation and mRNA accumulation. To help improve possible uptake, BAPTA-AM was loaded under acidic conditions (Bush and Jones, 1987), so that if external ester hydrolysis occurred it would still permit further entry of the unesterified BAPTA. In a control experiment using the solvents alone and the acidic conditions used to load BAPTA-AM inside the cells no effects were observed on the calcium signal in either compartment (data not shown). In both nuclear and cytoplasmic compartments, cold shock-induced Ca^{2+} increases were significantly increased by the BAPTA treatment. The effect of 1 mM BAPTA-AM on cold shock-induced cytoplasmic Ca^{2+} levels was significant increased from 1.25 ± 0.12 μM to 1.50 ± 0.09 μM (Fig. 6.4A), an increase of 20%. The effect on cold shock-induced nuclear Ca^{2+} was significant from 0.55 ± 0.07 μM to 0.74 ± 0.10 μM (Fig 6.4B), 35% increase. BAPTA-AM did not affect the time to reach peak height nor the length of the transient significantly. However, lower resting levels in nucleoplasmic Ca^{2+} level after the cold shock treatment was observed (Fig. 6.4B).

Calmodulin mRNA accumulates with distinct kinetics after a pretreatment of tobacco seedlings with 1 mM BAPTA-AM. Maximal levels of *NpCaM-1* are lowered by 50 % (Fig. 6.4C) by pretreatment with 1 mM BAPTA-AM. Levels of mRNA of *NpCaM-2* were not affected.

The effect of BAPTA-AM on wind stimulated tobacco seedlings in chapter 5 showed a close relation in the reduction in wind-induced nuclear Ca^{2+} level and the reduced accumulation in *NpCaM-1* mRNA (Fig. 5.3). Cold-shocked tobacco seedlings treated with BAPTA-AM showed increased cytosolic and nuclear Ca^{2+} levels but clearly reduced the cold shock-induced *NpCaM-1* mRNA accumulation, from 12 to 6 fold.
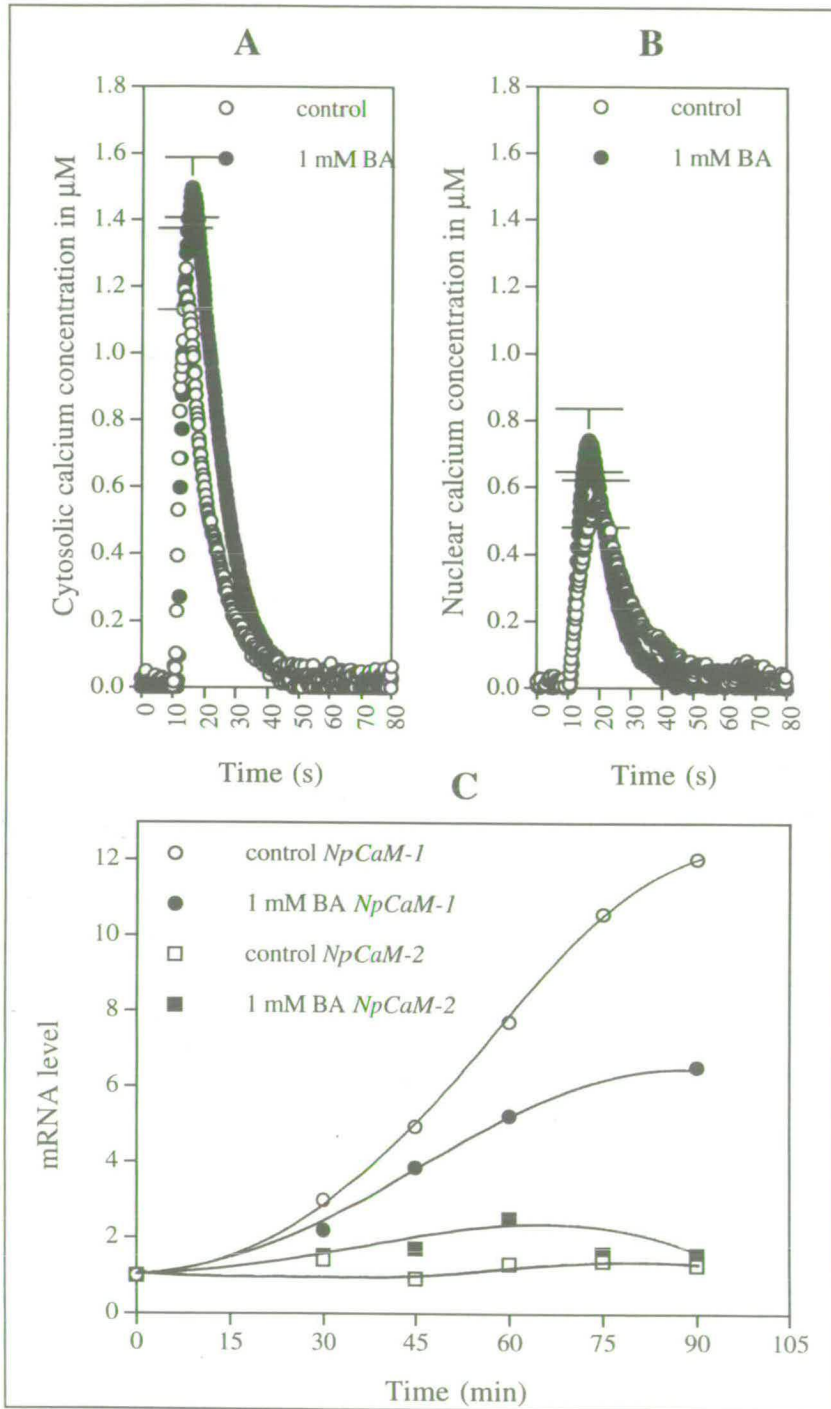
Figure 6.4: Effect of BAPTA-AM (**BA**) on cold shock-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 1 mM BAPTA-AM on cold shock-induced changes in calcium concentration in cytoplasm (**A**) and nucleoplasm (**B**). The effect of 1 mM BAPTA-AM on cold shock-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0.

Thapsigargin is an effective inhibitor of the Ca^{2+}-ATPase located on the endoplasmic reticulum membranes (Thastrup et al., 1990). Thapsigargin has been used before in plants in order to artificially increase cytosolic Ca^{2+} and induce gene expression in the absence of a normal inductive stimulus (Raz and Fluhr, 1992).

Pretreatment of MAQ 2.4 seedlings with 200 µM thapsigargin only slightly but significantly increased cold shock-induced peak cytoplasmic Ca^{2+} levels, 1.25 ± 0.12 µM to 1.61 ± 0.16 µM (Fig. 6.5A), an increase of 29%. In contrast, nucleoplasmic Ca^{2+} levels were substantially increased from 0.55 ± 0.07 µM to 0.88 ± 0.12 µM (Fig. 6.5B) an increase of 60%. Thapsigargin did not affect the rise times in either compartment or the length of the transients in the cytoplasm or the nucleus. There was, however, a higher resting level in nucleoplasmic Ca^{2+} level shortly before the cold shock treatment by thapsigargin (Fig. 6.5B).

Calmodulin mRNA accumulates to a different extent after a pretreatment of tobacco seedlings with 200 µM thapsigargin. Maximal levels of *NpCaM-1* are lowered by 50 % (Fig. 6.5C) by pretreatment with 200 µM thapsigargin. Levels of mRNA of *NpCaM-2* were not affected.

The effect of thapsigargin on wind-stimulated tobacco seedlings in chapter 5 showed a close relation in the increase in wind-induced nuclear calcium level and the increased accumulation of *NpCaM-1* mRNA (Fig. 5.4C). Cold shock tobacco seedlings treated with thapsigargin showed increased cold shock-induced cytosolic and nuclear calcium levels but clearly reduced the levels of cold shock-induced *NpCaM-1* mRNA accumulation, from 9 to 5 fold.
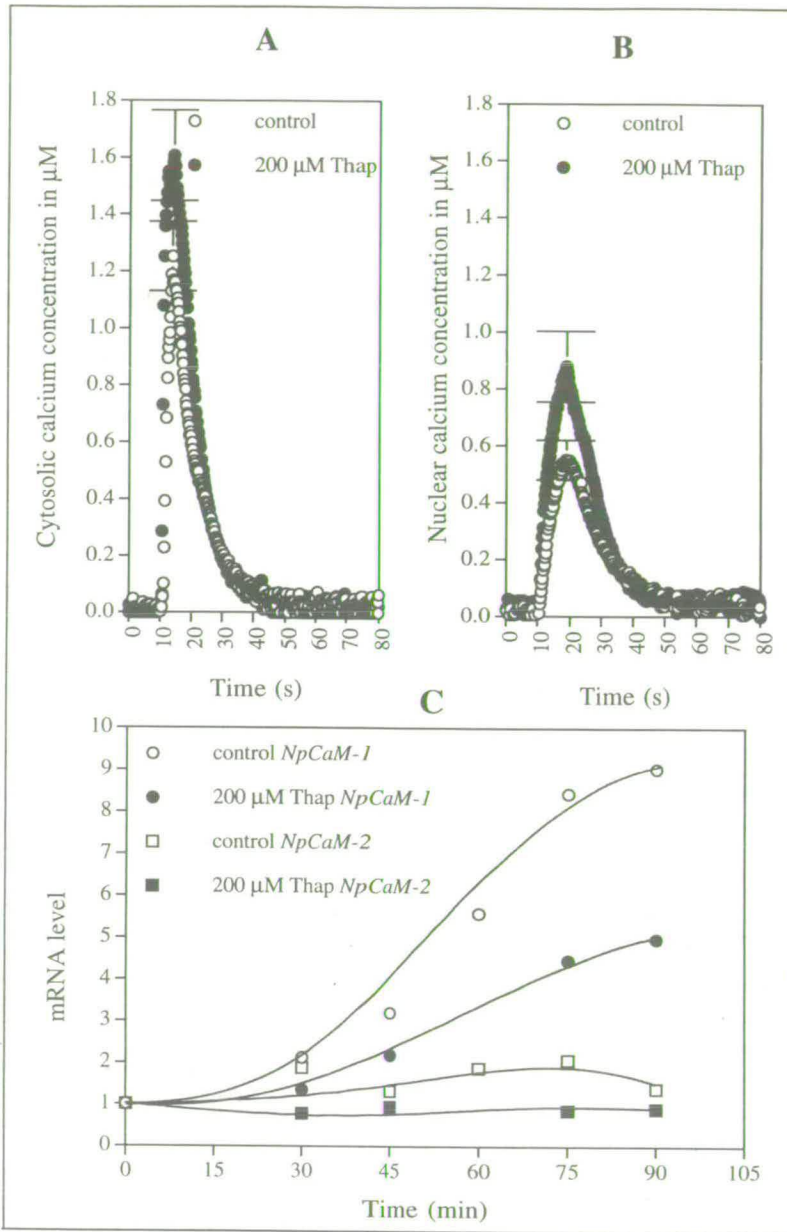
Figure 6.5: Effect of thapsigargin (**Thap**) on cold shock-induced cytosolic and nuclear free calcium concentrations and mRNA accumulation of *NpCaM-1* and *NpCaM-2*. The effect of 200 µM thapsigargin on cold shock-induced changes in free calcium concentration in cytoplasm (**A**) and nucleoplasm (**B**). The effect of 200 µM thapsigargin on cold shock-induced changes in *NpCaM-1* and *NpCaM-2* mRNA levels (**C**). Data are shown as hybridisation relative to non-induced mRNA levels (given a value of 1) and plotted against time in minutes after stimulation at time T_0.

6.4   Discussion

The first important observation described here is cold shock stimulation differentially accumulates transcripts for isozymes of calmodulin in *Nicotiana plumbaginifolia* like it was observed after wind stimulation (Chapter 4: Fig. 4.3 and Fig. 4.5; Chapter 5: Fig.

5.1). However, the kinetics of this induction is not the same for each stimulus. The induction of cold shock-induced gene expression is slightly delayed compared to wind stimulation in that maximal levels of *NpCaM-1* mRNA are reached after 90 minutes, whereas 75 minutes were required after wind stimulation, a difference of 15 minutes. Moreover, maximal levels of induction are clearly different for each stimulus. Wind led to a 5 fold induction whereas cold shock led upto a 11 fold induction (Fig. 6.1C).

The upregulation of calmodulin gene expression by cold is in apparent conflict with observation in alfalfa, where calmodulin mRNA levels were clearly not affected by cold shock (Monroy and Dhindsa, 1995). However, the kinetics of induction of *NpCaM-1* reached maximal levels 90 minutes after stimulation when it declines steadily back to resting levels that occurred after 120-180 minutes (data not shown). Because of the time course chosen by Monroy and Dhindsa (1995); 0, 3, 6, 24 hours, the elevation and decline back to resting levels could have occurred between 0 and 3 hours. The upregulation of calmodulin gene expression by cold is in line with observation done in *Arabidopsis*. The kinetics of cold shock-induced mRNA accumulation of calmodulin-related genes, *TCH2* and *TCH3* was different from the data presented here in that maximal induction levels were reached 30 minutes after stimulation when it declined back to resting levels 2.5 hr after stimulation (Polisensky and Braam, 1996). The differences in cold shock perception of different plant species, tobacco and *Arabidopsis* have been reported before (Knight et al., 1996). Tobacco and *Arabidopsis* also show a different Ca^{2+} signal in response to blue light (G. Baum, pers. commun.). In chapter 5, wind induced the accumulation of *NpCaM-1* 5 fold over resting levels 75 minutes after stimulation whereas 10-100 fold increase of *TCH* gene expression was observed 30 minutes after stimulation (Braam and Davis, 1990). This suggests that each plant species has evolved in an different manner to adapt to environmental changes and reflects which habitats it populates.

After wind stimulation a close correlation could be made between wind-induced nuclear Ca^{2+} levels and the level of *NpCaM-1* mRNA accumulation as shown by Ca^{2+} agonists/antagonists on transgenic seedlings expressing cytosolic apoaequorin, nuclear targeted apoaequorin and the accumulation of *NpCaM-1* mRNA (Chapter 5). From these data it was suggested that changes in nuclear $[Ca^{2+}]$ play a crucial role in the regulating levels of *NpCaM-1* mRNA. A close relation was clearly absent between intracellular Ca^{2+} levels and *NpCaM-1* mRNA levels after cold shock stimulation. No quantitative correlation could be detected between levels of either cytosolic $[Ca^{2+}]$ and/or nuclear $[Ca^{2+}]$ and *NpCaM-1* mRNA production. Lanthanum and neomycin affected cold shock-induced Ca^{2+} levels in the cytosol and the nucleus to a different extent than the levels of *NpCaM-1* mRNA accumulation (Fig. 6.2 and 6.3). Treatment

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of BAPTA-AM substantial increased cold shock-induced cytosolic and nuclear Ca^{2+} levels. The same was observed with thapsigargin, an inhibitor of Ca^{2+}-ATPase that prevent re-uptake of Ca^{2+} and increased the Ca^{2+} response. On the other hand BAPTA-AM lowered the wind-induced Ca^{2+} response. One plausible explanation for this is that the depletion of internal stores of Ca^{2+} could somehow stimulate the influx of Ca^{2+} across the plasma membrane. It has been suggested that the release and re-uptake of Ca^{2+} stores are highly regulated inside cells (Berridge, 1993, 1995, 1997). Inhibition of the Ca^{2+} re-uptake pathway into intracellular Ca^{2+} stores in BAPTA-AM or thapsigargin-loaded cells might result in an increased influx of Ca^{2+} from the extracellular apoplast leading to increase responses in cold shock-induced cytoplasmic and nuclear Ca^{2+} levels.

Whereas cold shock Ca^{2+} response in cytoplasm and nucleus were increased by the BAPTA treatment, cold shock-induced mRNA levels of *NpCaM-1* were decreased. The same was observed with thapsigargin, an inhibitor of Ca^{2+}-ATPase that prevent re-uptake of Ca^{2+} and led to an increase in the Ca^{2+} response but also led to a decreased cold shock-induced mRNA levels of *NpCaM-1*. BAPTA-AM and thapsigargin could replenish the nuclear envelope from Ca^{2+} that could block nuclear transport by closure of the nuclear pore complexes (Perez-Terzic et al., 1996; 1997; Clapham, pers. commun.). Closure of the pores could precent entry of transcription factor into the nucleus that are involved in active transcription of *NpCaM-1*.

Not only is the kinetics of induction of *NpCaM-1* mRNA accumulation different upon wind and cold shock stimulation, also the kinetics of the Ca^{2+} response is clearly different upon each environmental stimulus as shown in Fig. 6.1A-B. The average time to reach maximal levels for cytosolic [Ca^{2+}] is 0.31 s after exposure to wind (Fig. 3.9) and 4.8 s after a cold shock (Fig. 3.10). For nuclear [Ca^{2+}] the average time to reach maximal levels is 0.60 and 9 s after exposure to wind and cold shock respectively (Fig. 3.9-10). The time the Ca^{2+} remained elevated was similar in both compartments after wind stimulation, 13.6 s, but was different after cold shock stimulation, 24 and 31 s for cytosolic and nuclear [Ca^{2+}] respectively. The distinct Ca^{2+} response upon each stimulus might reflect the difference in the origin of Ca^{2+} release. As Ca^{2+} is thought to be released from internal stores upon wind stimulation it will affect Ca^{2+} levels instantly in both compartments. Upon cold shock Ca^{2+} is thought to originate from the extracellular apoplast. This is more likely to affect cytosolic Ca^{2+} levels first which are then followed by changes in nuclear Ca^{2+} levels. It has been suggested that in HeLa cells, a mammalian model system, global intracellular Ca^{2+} signals can be constructed from elementary building blocks, designated as 'Ca^{2+} puffs' and 'Ca^{2+} sparks' (Bootman and Berridge, 1995). It was

demonstrated that the spatio-temporal recruitment of these elementary Ca^{2+} release events underlies Ca^{2+} wave initiation and propagation in HeLa cells (Bootman et al., 1997). It has been argued that only 'Ca^{2+} puffs' localised near the nucleus will cause the nucleus to respond whereas 'Ca^{2+} puffs' appearing further away from the nucleus leave the nuclear Ca^{2+} levels unaffected. Ca^{2+} release near the nucleus after wind stimulation affects cytosolic and nuclear Ca^{2+} levels instantly and almost simultaneously in the two compartments, whereas Ca^{2+} release further away from the nucleus, such as release from the extracellular apoplast could lead to a delayed and truncated increase in nuclear Ca^{2+} levels. The relatively slower response of nuclear [Ca^{2+}] could only in part reflect the delay in the accumulation of *NpCaM-1*.

Perhaps the lack of a correlation between the intracellular Ca^{2+} response and the accumulation of *NpCaM-1* is not so unexpected. Monroy and Dhindsa (1995) showed the cold shock-induction of *cas* (cold-acclimation specific) genes correlate well with the uptake of extracellular Ca^{2+} in alfalfa protoplasts. The uptake of Ca^{2+} was suggested to occur over a long period of time, i.e. hours. In addition, they found that cold shock enhanced the accumulation of mRNA encoding a putative calcium-dependent protein kinase. It was hypothesised that yet uncharacterised protein kinases/phosphatases and/or their substrates that are regulated by Ca^{2+} and/or cold lead to *cas* gene expression without the mediation of increased levels of calmodulin. It has been suggested that phosphorylation of proteins by protein kinases will lead to an amplification of the signal through protein kinase cascades (Trewavas and Mahló, 1997). Cold shock-induced Ca^{2+} transients could activate such protein kinase cascades that could in turn activate transcription factors by phosphorylation and act then together with slight sustained increases in intracellular Ca^{2+} levels to activate other cellular processes. These processes might include the opening of nuclear pore complexes to translocate the by phosphorylation activated transcription factors into the nucleus which in turn could regulate the expression of *NpCaM-1*. In the cytoplasm the estimated Ca^{2+} resting levels are 11 ± 2 nM before cold signalling and 35 ± 3 nM after, a 3 fold increase. In the nuclear the estimated resting Ca^{2+} levels are 8 ± 4 nM before cold signalling and 35 ± 12 nM after, a 4 fold increase. These altered resting levels were observed in all experiments and continue throughout the duration of the experimental periods investigated. Whether these are significant changes in longer term signalling remain speculative. A role for a biphasic Ca^{2+} response has been described previously in B lymphocytes (Dolmetsch et al., 1997). Different part of the biphasic Ca^{2+} signal activates distinct cellular processes. The initial Ca^{2+} spike observed after activation induced persistent activation of proflammatory transcription regulator, NF-κB and c-Jun N-terminal kinase (JNK), whereas it only caused a transient nuclear translocation

of nuclear factors of activated T cells (NFATc) (Dolmetsch et al., 1997). Alternatively, different *cis*-acting response elements in the promoter of the *NpCaM-1* gene might be activated by different signal transduction pathways initiated by wind and cold shock stimulation. The promoter region of *NpCaM-1* might have several response elements as described for the promoter of the *c-fos* gene (Hardingham et al., 1997). The expression of c-*fos* is regulated by two characterised response elements, a serum-response element (SRE) and a cAMP responsive element (CRE). Deletion studies of the promoter region of c-*fos* and microinjection of dextran-BAPTA into nuclei to lower nuclear Ca^{2+} have revealed that an increase in nuclear $[Ca^{2+}]$ is critical for CRE-dependent Ca^{2+}-activated transcription of c-*fos*, whereas a second signalling pathway, activating transcription through the SRE is triggered by a rise in cytosolic $[Ca^{2+}]$ and does not require an increase in nuclear $[Ca^{2+}]$. Whether cold shock and wind stimulation initiate distinct signalling pathway that leads to the activation of distinct *cis* acting elements in the promoter of *NpCaM-1* remains to be determined and is the subject of an ongoing study.

In alfalfa, the requirement for elevated levels of intracellular Ca^{2+} for the induction of cold shock-induced *cas* gene expression appears to be temporary, since if EGTA was added after the on-set of cold acclimation to lower intracellular Ca^{2+} levels the expression of the cold-induced *cas* genes was not affected. When intracellular Ca^{2+} levels were artificially increased with a Ca^{2+} ionophore, A23187, a transient expression was obtained without any cold shock treatment, indicating that the influx of extracellular Ca^{2+} can induce the expression transietly but is not to sustain it (Monroy and Dhindsa, 1995). It was suggested that yet undetermined low temperature-promoted processes were involved in sustaining the Ca^{2+}-induced *cas* gene expression. These processes may include low-temperature signalling pathways other than that of Ca^{2+}, leading to either the inhibition of RNases or the expression of mRNA-stabilizing proteins. Indication that such mechanisms play a role after cold shock treatment comes from work on whole rat hypothalamus. Northern blot analysis on the hypothalamic somatostatin mRNA levels revealed a 2.0-fold increase after 15 min at 4°C (Rage et al., 1994). Cold shock-induced stabilisation of the *NpCaM-1* transcript could explain, at least in part, the lack of the relation between cold shock-induced cytoplasmic and nuclear Ca^{2+} response and levels of *NpCaM-1* mRNA accumulation. However, whether this holds true for *NpCaM-1* mRNA in tobacco seedlings after cold shock treatment awaits further investigation.

Chapter 7

Heat Shock-Induced Changes of Intracellular Ca^{2+} Level in Transgenic Tobacco Seedlings in Relation with Thermotolerance

Exposure of plants to elevated temperatures results in a complex set of changes in gene expression that induce thermotolerance and improve cellular survival to subsequent stress. In this section, a relationship is sought between intracellular Ca^{2+} levels and thermotolerance in *Nicotiana plumbaginifolia*.

N.B. This work was carried out in collaboration with Prof. Ming Gong.

7.1   Effect of Exogenous Calcium or EGTA on the Development of Thermotolerance of Tobacco Seedlings

To examine whether an involvement of cytosolic Ca^{2+} can be invoked during the development of thermotolerance tobacco seedlings were pretreated with either 10 mM Ca^{2+} or 10 mM EGTA or water as described in Chapter 2. The seedlings were divided into two batches one of which was maintained at 25°C whilst the other was incubated at 38°C for two hours to acquire heat shock-induced thermotolerance. After a further four hours at 25°C both seedling batches were incubated at 48°C for four hours to induce heat jury. Seedling viability was estimated eight days later. These results are shown in figure 7.1A.

In a further similarly constructed experiment, seedlings pretreated with Ca^{2+} or EGTA or water were first incubated at 40°C to acquire heat-induced thermotolerance and subsequently treated at 50°C for 140 min to induce heat injury. Again viabilities were estimated eight days later. These data are shown in figure 7.1B.

A prior treatment of seedlings at 38°C or 40°C substantially increased the percentage surviving the subsequent severe heat shocks of 48°C or 50°C (Fig 7A and B, $P<0.05$). Thermotolerance of tobacco seedlings is therefore induced by several hours of incubation at these lower temperatures as it has been reported for other plants including maize (Gong et al., 1997a,b). Pretreatment of the seedlings with 10 mM $CaCl_2$ enhanced the survival percentage under heat stress at 48 or 50°C as compared to controls ($+H_2O$, $P<0.1$). The effect was observed when the seedlings were transferred directly from 25°C to 48 or 50°C for heat treatment (therefore as a result of intrinsic thermotolerance). Moreover, the effect was apparent after the seedlings were first prehardened at 38 or 40°C to induce the prior development of thermotolerance

(therefore as a result of heat shock-induced thermotolerance, Fig. 7.1A and B). In contrast, pretreatment of the seedlings with the Ca^{2+} chelator, EGTA (+EGTA), led to a greater loss of viability compared to the treatment with water (+H$_2$O, $P<0.05$). If these two treatments, Ca^{2+} or EGTA have their anticipated effects on [Ca^{2+}]$_{cyt}$ then these results suggest the possible involvement of the Ca^{2+} signal transduction chain in the subsequent development of thermotolerance. Pretreatment of the seedlings with 10 mM CaCl$_2$ or 10 mM EGTA overnight had little effect on the growth or survival of the seedlings at 25°C with a 16-hr photoperiod during 2 weeks (data not shown).

38°C HS	38°C	25°C	48°C	Recovery at 25°C
	2 h	4 h	4 h	8 days

Non-HS	25°C	48°C	Recovery at 25°C
	6 h	4 h	8 days

40°C HS	40°C	25°C	50°C	Recovery at 25°C
	2 h	4 h	140 min	8 days

Non-HS	25°C	50°C	Recovery at 25°C
	6 h	140 min	8 days
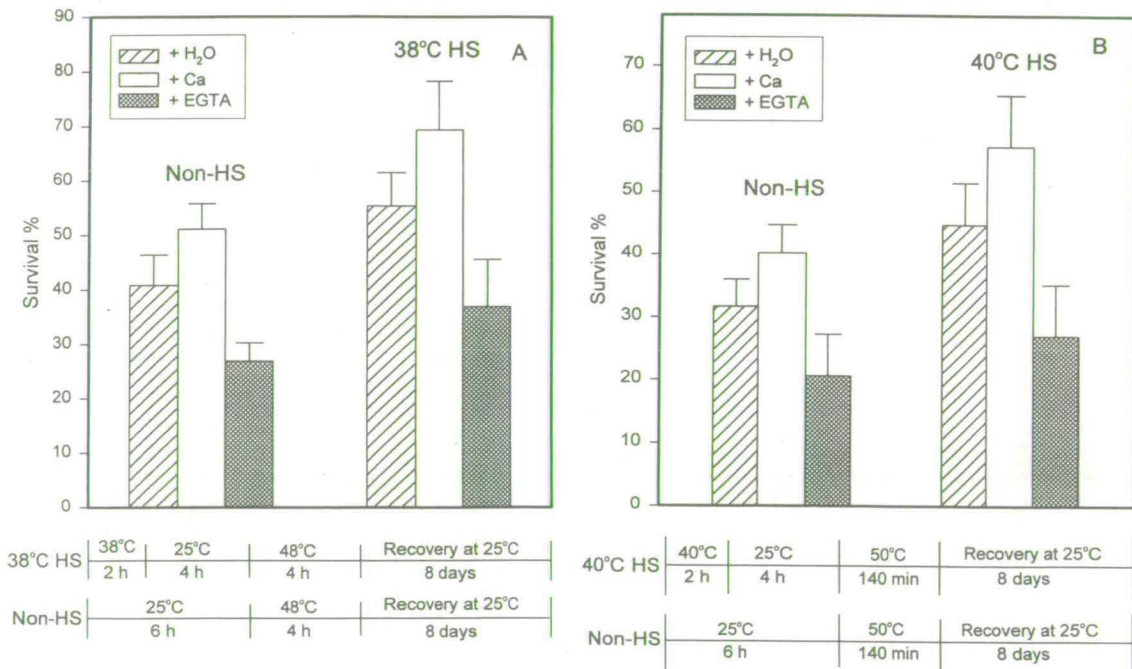
Figure 7.1: Effects of external Ca^{2+} and the Ca^{2+} chelator EGTA on intrinsic and heat shock-induced thermotolerance in tobacco seedlings. Overnight pretreatment of seedlings with 10 mM Ca^{2+} (+Ca), 10 mM EGTA (+EGTA) or sterile water (control, +H$_2$O), subsequent heat treatment and investigation of survival percentage were carried out. In each case the schedule of treatments for fig 7.1A and B are indicated below the figure. Each data bar represents means ± SE of 3 replicates, and 180 to 240 seedlings were investigated for each replicate. HS: heat shock.

7.2   Changes in Cytosolic and Nuclear Calcium Levels during Heat Shock

The effect of heat shock on aequorin transformed tobacco seedlings has been studied before (Knight et al., 1991). Irrigation with hot water at temperatures up to 55°C only induced slight or evoked no detectable changes in luminescence. At the commencement of this investigation this was repeated and those observations were confirmed (data not shown). However, when the transformed tobacco seedlings cultured in cuvettes were heat-shocked at 39, 43 or 47°C, for periods up to 35 min, a significant increase of $[Ca^{2+}]_{cyt}$ from these seedlings was observed (Fig. 7.2). This increase in $[Ca^{2+}]_{cyt}$ lasted for 10 to 20 min depending on the temperature used and was followed by a gradual decrease which approached but did not quite achieve original resting levels (Fig. 7.2). Continued heat shock treatment did not elicit further increases in $[Ca^{2+}]_{cyt}$. Measurement of luminescence required removal of the seedlings from the heat shock treatment bath for the 15 s luminescence measurements. To assess the effect of transient removal on the temperature of the seedlings, thermocouples were introduced into blank tubes containing the requisite volume of agar and temperatures recorded continuously. The quoted heat shock temperatures, 39°C, 43°C and 47°C, are therefore the weighted average of the temperatures experienced by the seedlings throughout the whole 35 min measurement period. The temperature fluctuation in the cuvette during 15 s luminescence measurement was about 2 to 3°C lasting 80 to 90 s (data not shown). In addition, heat shock treatment at 39, 43 and 47°C for 35 min did not lead to any lethal injury to the seedlings and all of the seedlings could survive after the treatment (data not shown).

Seedlings transgenic for nuclear localised aequorin, MAQ 7.11 cultured in cuvettes were heat-shocked at 43 or 47°C, for periods up to 60 min. A sustained increase of $[Ca^{2+}]_{nuc}$ in these seedlings was observed with a different kinetics in comparison with $[Ca^{2+}]_{cyt}$ (Fig. 7.3A and B). The increase in $[Ca^{2+}]_{nuc}$ was delayed by 10 to 20 min depending on the temperature used and reached maximal levels after 40 min for 43°C and 25 min for 47°C. The increase in $[Ca^{2+}]_{nuc}$ induced by a 43°C heat shock did not gradually decreased but remained elevated upto 60 min (Fig 7.3A). Conversely, the increase in $[Ca^{2+}]_{nuc}$ induced by the 47°C heat shock was followed by a gradual decrease which approached but did not quite achieve original resting levels (Fig. 7.3B).

A new calibration curve was constructed for the particular isoform of aequorin which has been used for transformation (Knight et al., 1996). The apoaequorin was over-expressed in *E. coli* and calibration determined using standard mixtures of Ca^{2+}/EGTA after reconstitution with coelenterazine. Fortunately the isoform used for

transformation is among the most sensitive of the isoforms and the dose response curve commences below a pCa of 7 (about 100 nM) and is saturated at about 10 μM (pCa 5). This calibration curve has been used to estimate the putative increases in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ resulting from heat shock treatment. From the luminescence data, pCa values in heat-treated tobacco seedlings were calculated, plotted and are shown in figure 7.2 and 7.3. In every case pCa increases substantially due to the heat treatment although it takes 10 and 25 min at least before peaks are reached for cytosolic and nuclear Ca^{2+} respectively and the transient starts to decline. The higher the temperature the bigger the increase in pCa for $[Ca^{2+}]_{cyt}$ whereas $[Ca^{2+}]_{nuc}$ reached similar Ca^{2+} levels. We have also measured resting levels at 25°C to be pCa values of 7. Upon heat shock, cytosolic Ca^{2+} levels, are increased two fold at 39°C, about a 3-fold increase at 43°C, 7-fold at 47°C. Conversely, nuclear Ca^{2+} levels, are increased about 2 fold independently of the heat shock temperature applied. The final resting pCa levels vary between 6.9 and 6.95.
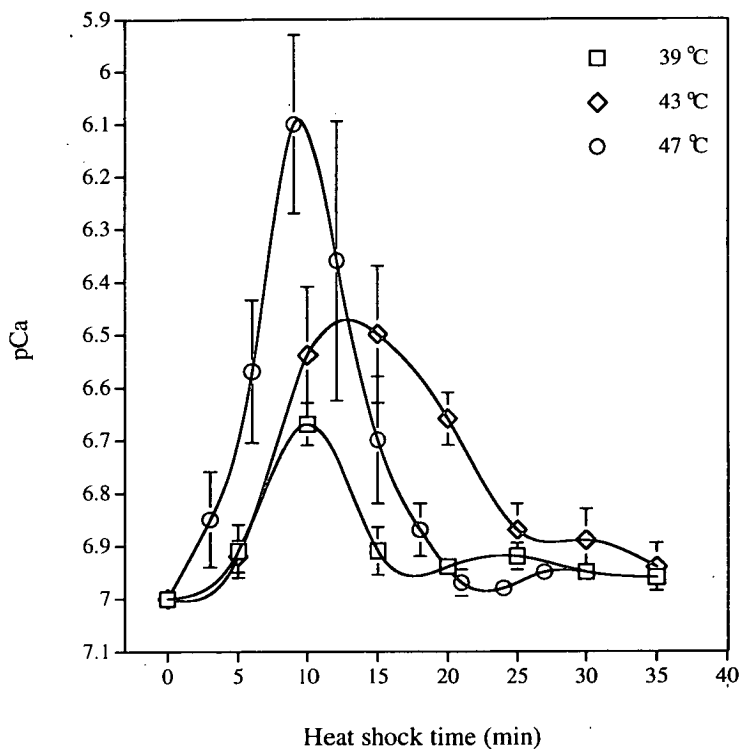
Figure 7.2: Changes of cytosolic free Ca^{2+} levels in transgenic tobacco (MAQ 2.4) seedlings containing cytoplasmic aequorin during heat shock at 39°C, 43°C and 47°C. Each datum point represents the mean ± SE of 10 measurements. When no error bar is indicated, the standard error was within the size of the symbol.

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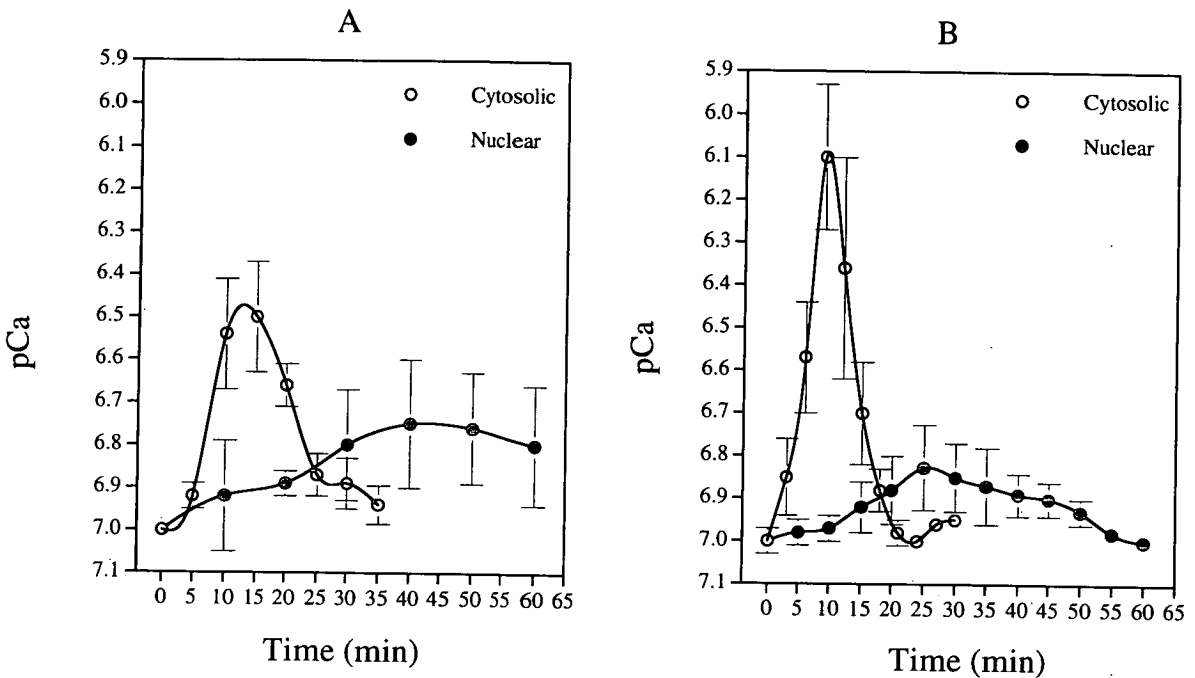
Figure 7.3: Changes of cytosolic and nuclear free Ca^{2+} levels in transgenic tobacco (MAQ 2.4 and MAQ 7.11, respectively) containing cytoplasmic and nuclear localised aequorin during heat shock at 43°C (A) and 47°C (B). Each datum point represents the mean ± SE of 10 measurements.

To demonstrate that these changes were not the result of non-specific discharge of aequorin luminescence, the stability of aequorin was tested to heat treatment. Purified aequorin was incubated in reconstitution buffer at 45°C and 50°C and then discharged with an excess of Ca^{2+}. As shown in table 7.1, the total luminescence of aequorin at 45 or 50°C for 0, 20, 40 or 60 min remained unchanged. Purified aequorin is therefore stable to high temperatures and the changes in figure 7.2 and 7.3 represent genuine changes in intracellular calcium.

Table 7.1. Effect of heat shock on Ca^{2+}-dependent luminescence of purified aequorin (counts·s^{-1}). Solutions of purified aequorin were heat treated at a given temperature for the time as shown and then discharged with excess $CaCl_2$. The values are means SE of 5 measurements.

Heat shock (min)	45°C (counts·s^{-1})	50°C (counts·s^{-1})
0	107160 ± 987	108317 ± 741
20	105516 ± 1662	109200 ± 1008
40	103673 ± 1280	109788 ± 860
60	106831 ± 1036	109928 ± 1788

Since the data in figure 7.1 indicated that pretreatments with exogenous Ca^{2+} or EGTA modified thermotolerance, the effects of these two pretreatments were also tested upon the subsequent changes in $[Ca^{2+}]_{cyt}$ induced by heat shock. The pretreatments were carried out for four hours in the dark at 25°C before heat shock was applied by transferring the seedlings to 43°C. This temperature was chosen as the best compromise between the shorter time period for these experiments compared to the two hour heat shock treatment at 38°C and 40°C used in figure 7.1. Heat shock effect, like many other plant processes, is dependent upon both the experimental temperature and the time of exposure (Nover et al., 1989; Gong et al., 1997a). In addition, this temperature (43°C) also gave a higher heat shock-induced increase $[Ca^{2+}]_{cyt}$ (Fig. 7.2) but did not lead to detectable injury to the tobacco seedlings during the heat treatment (data not shown). The results of these experiments are shown in figure 7.4.

Pretreatment with exogenous Ca^{2+} clearly increased both the rate of elevation of the pCa signal and it peaked at a pCa of about 6.35 compared to 6.52 for the control Fig. 7.4). Treatment with Ca^{2+} also shortened the time taken to reach the peak. In contrast, the EGTA treatment severely limited the capacity of heat shock to increase pCa and also delayed the onset of the peak at a pCa of 6.88 (Fig. 7.4). These data therefore support the hypothesis deduced from the data of figure 7.1 that regulation of $[Ca^{2+}]_{cyt}$ might represent part of the signal transduction process that leads to thermotolerance.
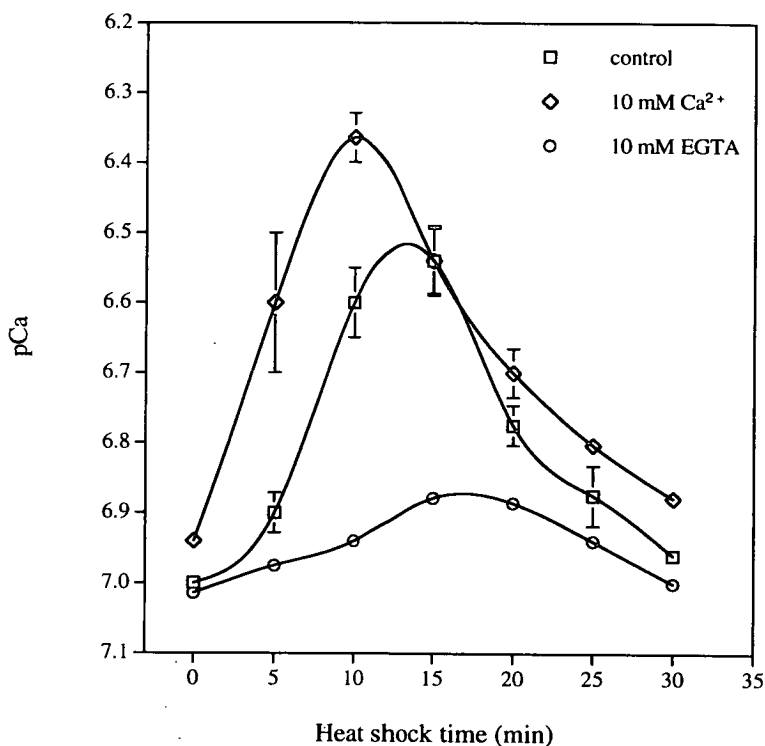
Figure 7.4: Effect of Ca^{2+} and EGTA pretreatments on the heat shock-induced changes of cytosolic free Ca^{2+} level in transgenic tobacco (MAQ 2.4) seedlings containing aequorin during heat shock at 43°C. The seedlings were pretreated with 10 mM Ca^{2+}, sterile water (control) or 10 mM EGTA in the dark for four hours and subsequent heat-shocked at 43°C. Each data point represents the mean ± SE of 8 to 10 measurements.

Although seedlings from the MAQ 2.4 line contain 95-99% of their aequorin in the soluble fraction of the cell (Johnson et al., 1995), modification of the Ca^{2+} relations of organelles could contribute to the final cell response. In particular heat shock is known to modify subsequent photosynthetic rates (Quinn and Williams, 1985). By using tobacco seedlings with aequorin targeted to the chloroplast, MAQ 6.3a (Johnson et al., 1995) any possible relationship of chloroplast Ca^{2+} to photosynthetic alterations was tested. However, the data in figure 7.5 showed there is little change in free Ca^{2+} level of the chloroplasts from these seedlings when they were heat-shocked at 43°C or 47°C and therefore intrachloroplast Ca^{2+} levels do not seem to respond to heat shock, unlike $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$. These observations also confirm that aequorin is stable to the high temperatures used for heat shock.
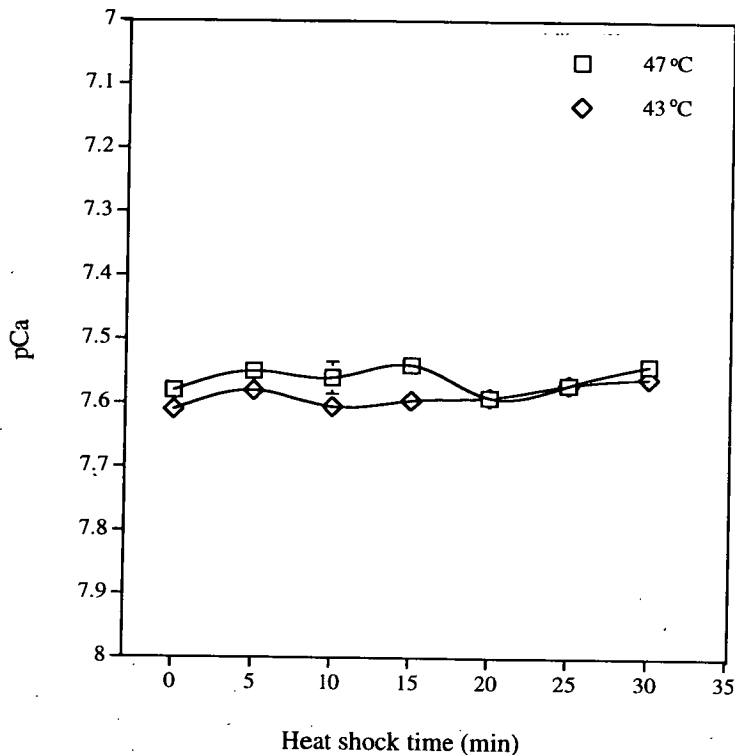
100

Figure 7.5: Changes in intrachloroplast free Ca^{2+} level in transgenic tobacco (MAQ 6.3a) containing chloroplast-located aequorin during heat shock at 43°C or 47°C. Each data point represents the mean ± SE of 7 or 8 measurements.

7.3   Recovery of $[Ca^{2+}]_{cyt}$ Responsiveness from Heat Shock

The changes of $[Ca^{2+}]_{cyt}$ shown in figure 7.2 indicated that heat shock-induced increases of $[Ca^{2+}]_{cyt}$ only lasted 15-20 minutes even though the seedlings continued to be stimulated by high temperatures. This implies that a possible refractory period follows heat shock in which no further heat shock-induced change of $[Ca^{2+}]_{cyt}$ can be elicited. We investigated this possibility by application of heat shock followed by a return to 25°C and subsequently heat shocks every hour. Only after a further 5 hour period at 25°C could a recovery in sensitivity to heat shock be detected as shown in figure 7.6 and full recovery required eight hours. This time course is similar to the loss of the refractory period induced by exogenously added hydrogen peroxide to induce oxidative stress (Price et al., 1994).
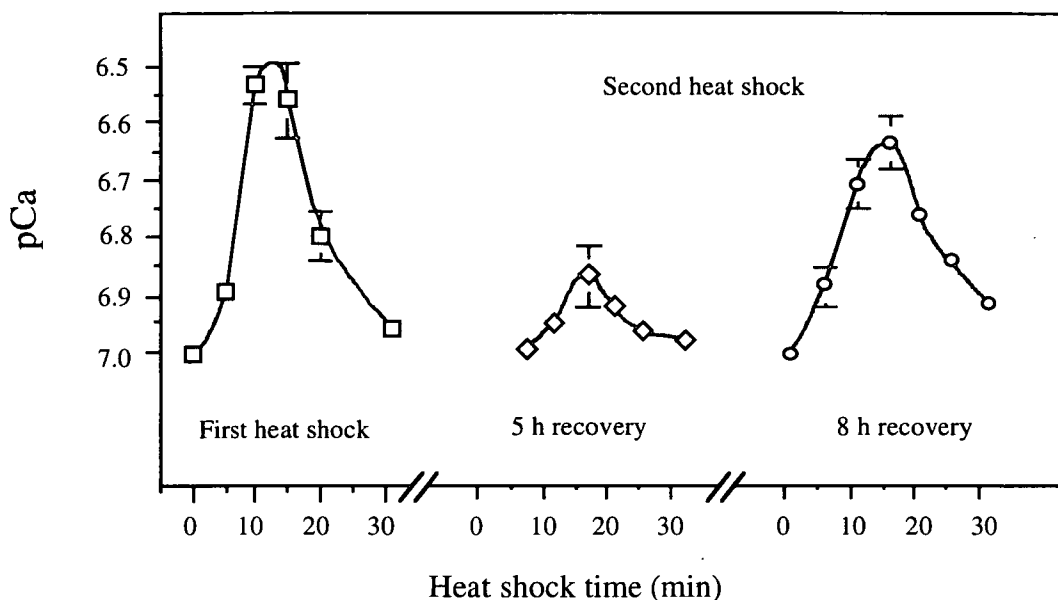
101

Figure 7.6: Changes in the cytosolic free Ca^{2+} level in transgenic tobacco (MAQ 2.4) seedlings when given a second heat shock either five or eight hours after the first. Three two-week-old tobacco seedlings cultured in a cuvette were first heat-shocked at 43°C for 30 min and luminescence numerically integrated for 15 s at 5 min intervals. These seedlings were kept in the dark at 25°C for either 5 or 8 hr and then heat-shocked again at 43°C. Each data point represents the mean ± SE of 8 measurements.

On the other hand, seedlings refractory to heat shock still responded well to cold shock, wind or touch stimulation. Transient spikes of $[Ca^{2+}]_{cyt}$ were observed when seedlings which had just been heat-shocked at 39°C or 43°C were instantly challenged with ice-cold water or stimulated by wind or touch (Table 7.2), indicating that the heat-shocked seedlings still retained a responsiveness to these other stimuli. As before, cold shock increased pCa about 10 fold (from 100 nM to 1 µM) whilst wind increased pCa 7-8 fold. The seedlings heat-shocked at 47°C for 30 min showed a much lower cold shock-induced increase in $[Ca^{2+}]_{cyt}$ (Table 7.2), most likely due to the fact that heat shock at 47°C led to the eventual injury of the seedlings. These data do indicate that the refractory period does not directly involve modification of aequorin or an inability of the cells to regulate $[Ca^{2+}]_{cyt}$.

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Table 7.2. Effects of prior heat shock of transformed tobacco seedlings (MAQ 2.4) on subsequent responses to cold shock and wind signalling. All values are either resting levels of pCa or transient increases in pCa resulting from cold shock or wind stimulation. Three tobacco seedlings in a cuvette were first heat-shocked in a waterbath at a given temperature for the given time as shown, taken out and cooled down for 5 min at 25°C. Then the cuvette was placed into the sample chamber of the chemiluminometer. For cold shock 1 mL ice-cold water was injected gently into the cuvette and the cold shock-induced Ca^{2+}-dependent luminescence of the seedlings was determined numerically for 15 s. For wind stimulation, 10 mL of air was injected rapidly over the seedlings by a port in the sample chamber with a syringe and the wind-induced Ca^{2+}-dependent luminescence of the seedlings was determined numerically for 15 s. Remaining aequorin was estimated at the end of the treatment and pCa calculated. The values are means ± SE of 6 to 10 replicates.

Heat shock	25°C Control	39°C 35 min	43°C 35 min	47°C 30 min
Background	7.0 ± 0.02	7.01 ± 0.01	7.0 ± 0.02	7.0 ± 0.01
Cold shock-induced increase in $[Ca^{2+}]_{cyt}$	6.0 ± 0.1	6.10 ± 0.1	5.9 ± 0.07	6.4 ± 0.03
Wind-induced increase in $[Ca^{2+}]_{cyt}$	6.3 ± 0.03		6.1 ± 0.03	

7.4   Possible Cellular Origin for Heat Shock-Induced Increase of $[Ca^{2+}]_{cyt}$

To investigate the sources for the increased $[Ca^{2+}]_{cyt}$ under heat shock, the transformed tobacco seedlings containing reconstituted cytosolic aequorin were pretreated with several Ca^{2+}-signalling inhibitors. As shown in figure 7.7, seedlings pretreated with 1 mM $LaCl_3$, a putative plasma membrane Ca^{2+}-channel blocker demonstrated a much lower heat shock-induced increase in $[Ca^{2+}]_{cyt}$.

Similarly, pretreatment of these transformed seedlings containing reconstituted aequorin with the putative intracellular Ca^{2+}-channel blocker ruthenium red (25 μM) or phospholipase C inhibitor neomycin (200 μM) also greatly lowered heat shock-induced luminescence as compared with the control although the luminescence levels from these seedlings were a little higher than those of seedlings pretreated with La^{3+} (Fig. 7.7).
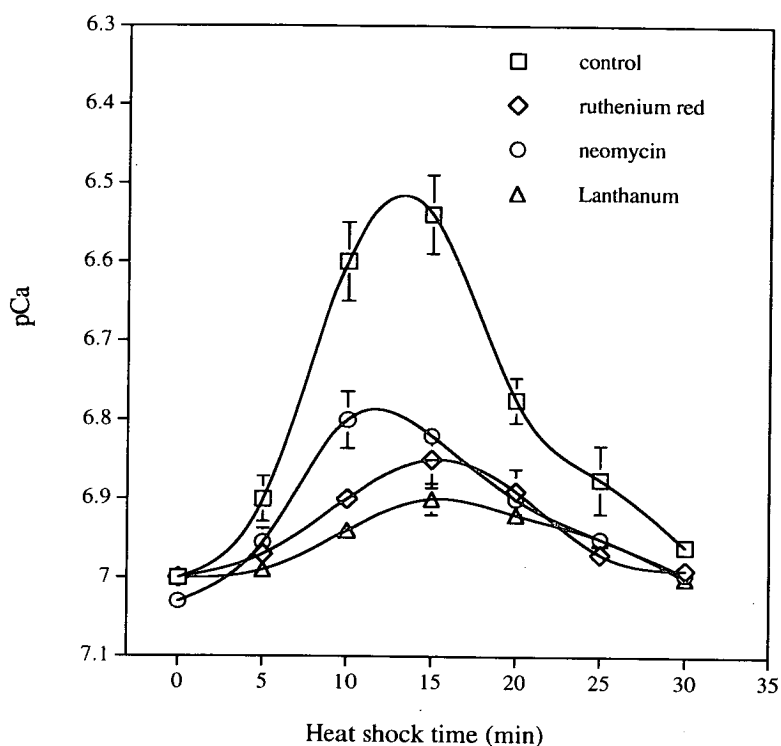
Figure 7.7: Effects of LaCl₃, ruthenium red (RR) and neomycin on the heat shock-induced changes of cytosolic free Ca^{2+} level in transgenic tobacco (MAQ 2.4) seedlings during heat shock at 43°C. 3 µL of 1 mM LaCl₃, 25 µM ruthenium red (RR), 200 µM neomycin solution or of sterile water was added onto the two opened cotyledons of each seedling, and these seedlings were kept in the dark at 25°C for 4 hr. After removal of the solution heat shock was conducted at 43°C. Each data point represents the mean ± SE of 8 to 10 measurements.

7.5   Discussion

The data presented here clearly shows that heat shock results in a sustained increase of $[Ca^{2+}]_{cyt}$ in tobacco seedlings (Fig. 7.2). This increase in cytosolic Ca^{2+} level was followed by an increase in nuclear Ca^{2+} levels and was not mirrored in the chloroplasts, suggesting that heat shock does not modify the free Ca^{2+} level in chloroplasts (Fig. 7.5). Thus, presumably the effects of heat shock on chloroplast activity, *e.g.* photosynthetic rates (Quinn and Williams, 1985), are not mediated by chloroplast calcium, although measurement using *cp*-coelenterazine for measuring small changes in Ca^{2+} levels might provide more exclusive evidence. The stability of aequorin also was not affected under the heat shock conditions that was employed in these experiments (Table 7.1).

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The heat shock-induced increases in $[Ca^{2+}]_{cyt}$ gradually returned to resting levels even while heat shock continued (Fig. 7.2). Heat-shocked seedlings required recovery at 25°C for 8 hr to allow a full heat shock-induced $[Ca^{2+}]_{cyt}$ response (Fig. 7.6). These results suggest a refractory period following heat shock. Price et al. (1994) proposed that the refractory period of $[Ca^{2+}]_{cyt}$ which resulted from oxidative stress was the result of a strong regulation of the prooxidant/antioxidant ratio. However, since refractory periods have also been observed in the responses of $[Ca^{2+}]_{cyt}$ to wind, touch, cold shock (Knight et al., 1991, 1992, 1996) and heat shock presented here, it indicates that other mechanisms are also likely to be involved in the refractory periods to these other signals. A sustained high $[Ca^{2+}]_{cyt}$ disturbs the intracellular phosphate-based energy metabolism and causes cytotoxicity (Hepler and Wayne, 1985). A refractory period following a stimulus-induced increase of $[Ca^{2+}]_{cyt}$ might prevent cells from damage which would otherwise be caused by a prolonged increase in $[Ca^{2+}]_{cyt}$.

Although the heat-shocked seedlings were refractory to a subsequent second heat shock treatment, they retained full responsiveness (with respect to $[Ca^{2+}]_{cyt}$ elevation) to other stimuli such as cold shock and touch stimulation (Table 7.2). This is very similar to the situation with oxidative stress (Price et al., 1994) and mechanical signalling (Knight et al., 1992). These observations indicate that plant cells can distinguish between different stimulus-induced increases of $[Ca^{2+}]_{cyt}$ and yet retain a full responsiveness of $[Ca^{2+}]_{cyt}$ to other stimuli; whilst remaining refractory to the same signal. In addition, this retention of full sensitivity to other stimuli after heat shock indicates that heat shock-induced changes in $[Ca^{2+}]_{cyt}$ were a positive response of tobacco seedlings to heat shock, not a consequence of heat injury (as also indicated in Fig. 7.1).

Nelles (1985) reported that in corn coleoptile cells, heat shock led to an initial increase in membrane potential which was followed by a steep decrease. It is known that a rapid decrease in plasma membrane potential and depolarisation of the membranes will lead to opening of plasma membrane Ca^{2+}-channels and influx of extracellular Ca^{2+} into cells (Poovaiah and Reddy, 1987; 1993). In the experiments described here, external Ca^{2+} treatment enhanced the heat shock-induced increase of $[Ca^{2+}]_{cyt}$. Conversely the Ca^{2+} chelator EGTA and plasma membrane Ca^{2+} channel blocker La^{3+} (Tester, 1990; Monroy and Dhindsa, 1995) both significantly lowered heat shock-induced increase of $[Ca^{2+}]_{cyt}$ (Figures 7.4 and 7.7). These data suggest that extracellular Ca^{2+} may enter cells across plasma membranes during heat shock to increase $[Ca^{2+}]_{cyt}$ level. Additionally, the putative intracellular Ca^{2+} channel inhibitor, ruthenium red (Kreimer et al., 1985; Subbaiah et al., 1994b) significantly lowered the heat shock-induced increase of $[Ca^{2+}]_{cyt}$, implying that mobilisation and redistribution

of intracellular Ca^{2+} are also involved in heat shock-induced changes of $[Ca^{2+}]_{cyt}$ (Fig. 7.7). Therefore, we suggest that the increased $[Ca^{2+}]_{cyt}$ observed in transformed tobacco seedlings during heat shock arises from both extracellular and intracellular sources. However, since ruthenium red-sensitive channels also occur in the plant plasma membranes (Marshall et al., 1994) and lanthanum may enter into plant cells (Quiquampoix et al., 1990), these conclusions must be made tentatively.

Intracellular Ca^{2+} mobilisation is often mediated by another second messenger, IP_3 (Cote and Crain, 1993; Bush, 1995; Allen et al., 1995). The data show that the phospholipase C inhibitor, neomycin, reduces the magnitude of the heat shock-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 7.7). Neomycin is believed to inhibit the hydrolysis of phosphoinositides, thereby preventing the production of IP_3 and IP_3-mediated mobilisation of intracellular Ca^{2+} (Phillippe, 1994). IP_3 could therefore be involved in the heat shock-induced mobilisation and redistribution of intracellular Ca^{2+} in plant cells. It is found that the heat shock responses of cultured animal cells also involve altered mobilisation of IP_3 (Calderwood et al., 1988).

Although many environmental stresses are known to increase $[Ca^{2+}]_{cyt}$ (see introduction), these changes in $[Ca^{2+}]_{cyt}$ exhibit enormous variability in amplitude, kinetics and spatial distribution of $[Ca^{2+}]_{cyt}$. For example, touch, wind stimulation and cold shock all cause sharp spikes of $[Ca^{2+}]_{cyt}$ in tobacco seedlings within 15 s (Knight et al., 1991, 1992, 1996; Chapter 5 and 6), oxidative and salt stresses cause relatively lower transients of $[Ca^{2+}]_{cyt}$ lasting for several minutes (Price et al., 1994; Bush, 1996; Okazaki et al., 1996), whilst anoxia induces increases in $[Ca^{2+}]_{cyt}$ lasting several hours (Subbaiah et al., 1994b; Sedbrook et al., 1996). Data presented here show that in tobacco seedlings, heat shock induces a lower but sustained increase in $[Ca^{2+}]_{cyt}$ lasting 10 to 20 minutes and peaks after 10-15 minutes (Figures 7.2 and 7.5-7). This response is then followed be an increase in nuclear $[Ca^{2+}]$ that lasts 40-60 minutes and peaks after 15-25 minutes (Fig 7.3). These temporal, spatial and amplitude variation in stress-induced increases of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ under different environmental stresses may allow plant cells to distinguish one kind of stress from another and to induce distinct gene expression to adapt to a particular stress.

Recently reported is that Ca^{2+} and calmodulin may be involved in the acquisition of the heat shock-induced thermotolerance in maize seedlings. The acquisition of the heat shock-induced thermotolerance requires the entry of extracellular Ca^{2+} into cells across plasma membrane and the mediation of intracellular calmodulin (Gong et al., 1997a). In addition, it has been found previously that external Ca^{2+} treatments enhanced intrinsic thermotolerance in maize seedlings, which was associated with increased activities of antioxidative systems during heat stress, and EGTA

treatments had the opposite effect (Gong et al., 1997b). Braam (1992) found that heat shock treatment strongly up-regulated expression of calmodulin-related *TCH* genes in cultured *Arabidopsis* cells, and furthermore that external Ca^{2+} was required for maximal heat shock-induction of these genes. Conversely EGTA treatment inhibited the heat shock-induced expression of *TCH* genes. *TCH* genes are considered to play some important role in the perception, response and adaptation of plants to various environmental stresses (Xu et al., 1995, 1996; Braam et al., 1996).

In the present experiments, modification of $[Ca^{2+}]_{cyt}$ in tobacco seedlings led to a change of thermotolerance. External Ca^{2+} treatments, which enhanced the heat shock-induced increases in $[Ca^{2+}]_{cyt}$ (Fig. 7.4), also enhanced intrinsic and heat shock-induced thermotolerance in tobacco seedlings (Fig. 7.1). In contrast, EGTA treatment, which chelates extracellular Ca^{2+}, and which greatly lowered the heat shock-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 7.4), also decreased the intrinsic and heat shock-induced thermotolerance as compared with the controls (Fig. 7.1). These results imply the physiological importance of Ca^{2+} in generating thermotolerance in tobacco seedlings. As discussed above these increases are a positive response of tobacco seedlings to heat stress rather than being due to injury. Heat shock-induced increases in $[Ca^{2+}]_{cyt}$ therefore seems to act as a signal to trigger some of the biochemical and physiological events enabling plants to adapt following heat stress.

Chapter 8

Discussion

8.1   Regulation of Nuclear Calcium Levels

The relation between cytosolic Ca^{2+} and nuclear Ca^{2+} levels has remained controversial in previous studies. The nuclear membrane may provide a barrier for Ca^{2+} ions because concentration differences as well as temporal differences are observed (Birch et al., 1992; Shankar et al., 1993; Kocsis et al., 1994; Badminton et al., 1995). There might be free diffusion of Ca^{2+} from the cytoplasm towards the nucleus as molecules with molecular weights under 10 kDa can diffuse freely through the nuclear pores (Brini et al., 1993; Al-Mohanna et al., 1994).

Wind stimulation led to transient increases in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$, respectively 0.3 and 0.6 s after stimulation (Chapter 5). In both compartments the Ca^{2+} signal lasted for about 13 s. In previous experiments wind resulted in release of Ca^{2+} from internal stores like the endoplasmic reticulum (Knight et al., 1992). In this project this hypothesis was enforced as ruthenium red, a putative Ca^{2+}-channel blocker at mitochondrial and/or endoplasmic reticulum membranes, inhibited wind-induced increases in $[Ca^{2+}]_{cyt}$. Ruthenium red did not affect wind-induced $[Ca^{2+}]_{nuc}$ which suggests that both compartments have different pools of Ca^{2+} release. Ca^{2+} depletion of internal stores by BAPTA-AM decreased the response in both compartments to different extents. Thapsigargin led to an increase Ca^{2+} response in the nucleus but not in the cytoplasm.

These observations fit in a model that predicts that wind leads to the release of Ca^{2+} from at least two internal stores, namely the endoplasmic reticulum and the nuclear envelope which is continuous with the endoplasmic reticulum but differs with respect to membrane composition and protein content. At the time of stimulation the Ca^{2+} levels are affected almost simultaneously (Fig. 8.1A). Subsequently Ca^{2+} levels in both compartments rise and decline by the re-uptake of Ca^{2+} into the endoplasmic reticulum and nuclear envelope. Stretch-activated Ca^{2+}-release channels have been localised to the endoplasmic reticulum of higher plants (Klüsener et al., 1995; 1997). Alternatively, Ca^{2+}-channels localised in the nuclear envelope may be associated with actin filaments as shown in amphibian epithelial cells (Prat and Cantiello, 1996). Due to wind stimulation these may be activated and increase the levels of cytoplasmic and nuclear calcium.

In chapter 6 cold shock was shown to induce a transient increase in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ respectively 4.8 and 9 s after stimulation. The Ca^{2+} signal remained elevated for 24 s in cytoplasm and 30 s in the nucleus. It has been suggested that cold shock releases Ca^{2+} from the extracellular apoplast and the vacuole, although the contribution of the latter was found to be minute (Knight et al., 1991, 1996). The Ca^{2+}-channel blocker, lanthanum and the inhibitor of IP_3 production, neomycin, led to a decreased response in Ca^{2+} signal in both the cytoplasm and the nucleus. The esterified form of BAPTA, BAPTA-AM loaded inside cells to deplete internal stores by sequestering Ca^{2+} and thapsigargin, an inhibitor of Ca^{2+} re-uptake into the internal stores, led to an increase of the Ca^{2+} response in both compartments. As it was suggested that the release and re-uptake pathway of Ca^{2+} are highly regulated inside cells (Berridge, 1993, 1996, 1997) this could be explained by the fact that depleted internal Ca^{2+} stores lead to an enhanced influx of Ca^{2+} across the plasma membrane. Lanthanum was shown to have the most pronounced effect and affected the two compartments in a similar manner. Neomycin affected the nuclear Ca^{2+} response more strongly than the cytosolic Ca^{2+} signal suggesting an additional role for an IP_3 signalling pathway operational inside the nucleus (Divecha et al., 1994). These observations fit in a model that predicts that cold shock leads to the release of Ca^{2+} from the extracellular apoplast with the partial mediation of an IP_3-sensitive Ca^{2+} pool, like the vacuole and possibly the nuclear envelope (Fig. 8.1B). The Ca^{2+} response in the cytoplasm reaches its maximal levels after 5 s and declines by the re-uptake or extrusion of Ca^{2+} outside the cells. Nuclear Ca^{2+} levels could then be affected through two pathways. The increase in cytosolic Ca^{2+} levels could either diffuse through the nuclear pores (stippled route in Fig. 8.1B) according to observations in HeLa cells (Brini et al., 1993; Al-Mohanna et al., 1994). Alternatively, Ca^{2+} release might be triggered in the nuclear envelope with the mediation of IP_3 leading to increased nuclear Ca^{2+} levels (broken line route in Fig. 8.1B). In this way nuclear Ca^{2+} levels might be regulated in a partial independent fashion. The nuclear Ca^{2+} increase declines by the re-uptake of Ca^{2+} into internal stores or by extrusion outside cells. This model is very speculative and awaits further verification.

An independent regulation of nuclear Ca^{2+} levels was perhaps most upon continuous heat shock treatment (Chapter 7). The cytoplasmic Ca^{2+} level returned to resting levels after 20-25 minutes while nuclear Ca^{2+} levels continued to rise. The physiological relevance for this discrepancy remains to be determined and is the subject of an ungoing study.
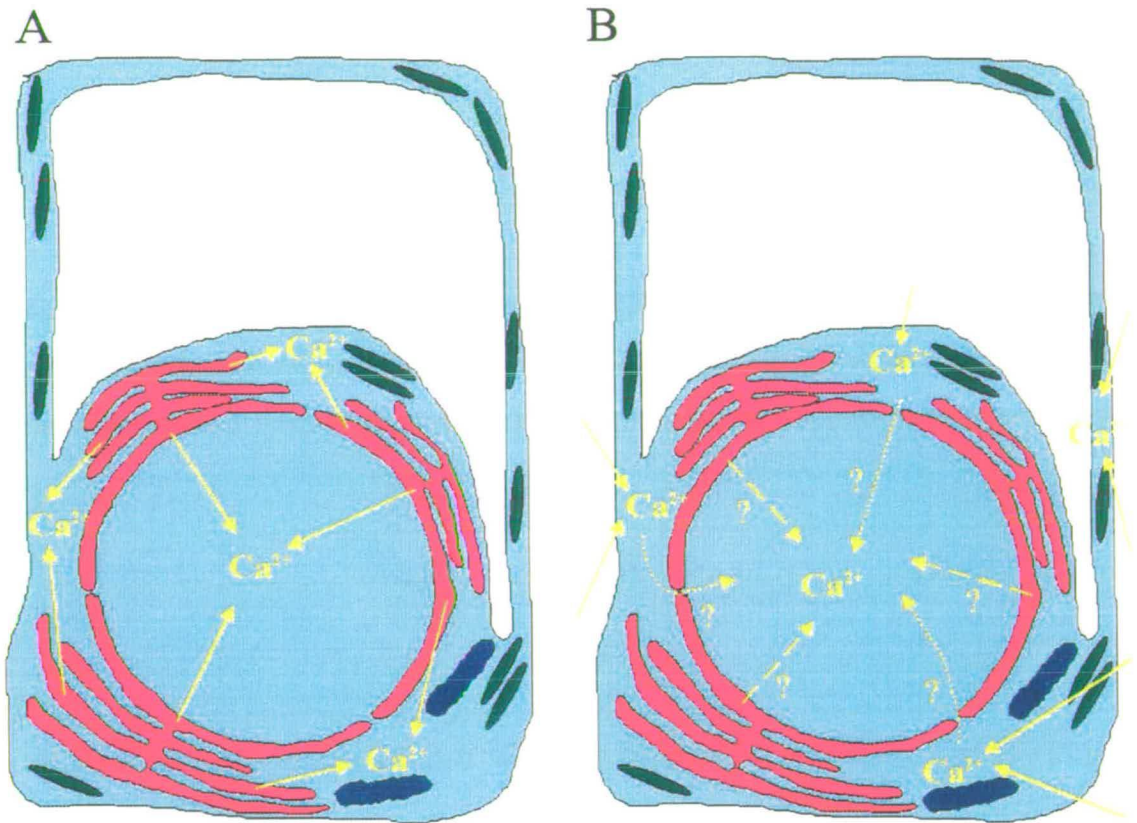
Figure 8.1. Two models explaining the temporal and spatial Ca^{2+} dynamics after wind (**A**) and cold shock (**B**) stimulation. Cytoplasm and nucleoplasm are displayed in light blue, the vacuole is white, mitochondria are shown in dark blue, chloroplasts are in green, the endoplasmic reticulum with the nuclear envelope shown as a continuum are shown in red. The Ca^{2+} signalling pathway is displayed in yellow and shown as stippled or broken lines where they remain highly speculative.

8.2   Does an Increase in the Nuclear Calcium Level Co-localise with an Increase in NpCaM-1 *mRNA Accumulation?*

Wind and cold shock induce the accumulation of *NpCaM-1* mRNA but not *NpCaM-2*. In chapter 5 it was described that wind-induced elevated changes in nuclear Ca^{2+} levels correlate closely with wind-induced *NpCaM-1* mRNA levels, but not *NpCaM-2* mRNA levels. It was suggested nuclear Ca^{2+} levels regulate *NpCaM-1* mRNA levels upon wind signalling. Whether the increase in *NpCaM-1* mRNA levels co-localises with increased nuclear Ca^{2+} levels in the individual cells of the stimulated seedling remains academic. An experimental test of this hypothesis would be to image the distribution of nuclear Ca^{2+} signals amongst individual cells after signalling. Calcium response of individual cells can be pronouncedly non-uniform as it has been shown with cold shock (Knight et al., 1993; Campbell et al., 1996). A further test using reporter genes such as luciferase and green fluorescent protein (GFP; Chalfie et al., 1994) coupled to the *NpCaM-1* promoter would help to indicate the subsequent

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expression of *NpCaM-1* amongst individual cells. Imaging localisation of the wind-induced nuclear Ca^{2+} response with aequorin reconstituted with *cp*-coelenterazine and that of *NpCaM-1* gene expression with GFP under the control of the *NpCaM-1* promoter with an Image Intensifier camera would help to clarify this.

8.3   Subcellular Localisation of mRNA Encoding Calmodulin Isoforms

Sequence analysis of partial clones of the genes *NpCaM-1* and *NpCaM-2* revealed two identical calmodulin isoforms with differences in the 3' untranslated regions of the transcripts (3' UTR; Chapter 4). That the 3' UTR plays a role in the localisation of mRNA to defined subcellular areas is well documented. In oocytes and early embryos of *Xenopus* and *Drosophila*, mRNAs are located in defined subcellular areas, the same phenomenon occurs in differentiated somatic mammalian cells (Hesketh, 1996, St. Johnston, 1996). This specific localisation of mRNAs might be a general phenomenon which mediates protein targeting. In addition, elements involved in the translation of mRNAs co-localise with actin and microtubules in plant cells (Durso and Cyr, 1994, Bokros et al., 1995, Clore et al., 1996). Reasons for localisation of mRNA are logical: i) As protein translocation is relatively slow and energy demanding process, accumulation of mRNAs to defined sites may be an efficient first step to direct protein to their destiny. ii) By having mRNA and the translational machinery tied down at one site, its specific localisation enables the cell to regulate this process more effectively. For example, in response to synaptic activity in neurones mRNAs encoding both the cytosketetal protein MAP2 and the subunit of Ca^{2+}/calmodulin-dependent protein kinase II have been found to localise to dendrites, the regions of the cell where synaptic inputs are received (Torre and Steward, 1992). iii) It may prevent interactions of proteins towards different cellular components. iv) By limiting the synthesis of the different isoforms to separate compartments of the cell, this may impede the formation of heteromultimers. For example, the composition of actin filaments in differentiating myoblasts, where ß-actin mRNA localises to the leading lamellae at the cell periphery, while α and γ-actin transcripts show a perinuclear distribution (Hill and Gunning, 1993, Kislauskis et al., 1993).

A common characteristic of all the localised mRNAs identified is that they contain *cis*-acting sequences required for localisation in their 3' UTR. It is not clear whether these localisation signals derive from primary or secondary structures of the transcripts. However, it is known that this sequence can be >100 nucleotides in length and sometimes exists in several patches. Several mechanisms are known to cause localisation of mRNAs: i) Spatial control of mRNA stability. A simple but efficient

way of localising mRNA for the cell is to stabilise the mRNA at the correct position and to degrade the unlocalised transcripts. ii) Anchoring transcripts to localised binding sites. Another way by which mRNAs can become concentrated in a particular region of a cell without being actively transported is if they are sequestered by localised binding sites. Cytoplasmic streaming is probably sufficient to circulate mRNAs that can be efficiently sequestered by localised binding sites. iii) Vectorial nuclear export as observed in Drosophila eggs where transcripts are localised to the cytoplasm on one side of blastoderm nuclei as a result of directed export from the organelle. iv) Active transport of specific mRNAs along the cytoskeletal elements.

Could localisation of mRNA encoding calmodulin be a feature in tobacco plant cells? It is known that Ca^{2+} signals are highly spatially and temporally localised inside single cells and that other components mediating in signal transduction pathways, e.g. calmodulin co-localises with the rhizoid pole (Love et al., 1997). If the mRNA encoding isoforms are defined to specific areas of the cell, they could be translated locally and act in concert with a localised elevated Ca^{2+} signal. These signals could arise from stimulation or a sustained local elevated Ca^{2+} levels after an initial Ca^{2+} response. Translation of calmodulin could be regulated by locally-activated initiation and elongation factors that may be under control of a localised Ca^{2+} signal. Some of factors of the translational initiation machinery have been described to be under control of phosphorylation (Deharo et al., 1996; Gallie et al., 1997), localised in the cell (Bokros et al., 1995), and might therefore be under the control of a Ca^{2+} signalling pathway (Durso and Cyr, 1994). Localised translation of calmodulin isoforms might either directly, or after activation by a localised Ca^{2+} elevation, in turn, trigger subsequent reactions that comprise a cellular response. A calmodulin-sensitive interaction between microtubules and a higher plant homologue of elongation factor-1α has been observed (Durso and Cyr, 1994). Alternatively, Ca^{2+} has an effect on polymerisation of microtubules (Fisher and Cyr, 1993; Fisher et al., 1996) and could therefore affect the translocation of mRNAs encoding for calmodulin to a specific site inside the cell. This assumption awaits exiting new research.

8.4   NpCaM-1 mRNA Accumulates as an S-shaped Curve

Accumulation of *NpCaM-1* mRNA after wind and cold shock stimulation was not as anticipated, a hyperbolic response but showed an S-shaped curve. Berlin and Schimke (1965) investigated the effects of various signals on protein and macromolecule accumulation and their analyses and measurements showed a simple hyperbolic response. Implicit in their approach is the indication that the change in macromolecule synthesis directly after signalling is extremely fast. The rate of accumulation of the macromolecule is then determined solely by the rate of degradation of the macromolecule. The Berlin and Schimke (1965) model can be applied to much mammalian mRNA accumulation data which often shows simple hyperbolic changes (Harpold et al., 1981). It had been the intention at the outset of this work to apply the analysis of Berlin and Schimke (1965) to *NpCaM-1* mRNA data. It was intended to estimate the synthesis and degradation rates of *NpCaM-1* and then try to correlate these values after signalling with specific events in the kinetics of the changes in $[Ca^{2+}]_{int}$. However the first attempts to apply these analyses to *NpCaM-1* mRNA accumulation revealed that the Berlin and Schimke (1965) model was not applicable to the data because the accumulation of the mRNA was clearly S-shaped and not hyperbolic.

Two possible explanations for this discrepancy can be suggested. Firstly, within individual seedling cells the absolute rates of synthesis of *NpCaM-1* mRNA continue to accelerate in a synchronous fashion throughout the first 45 minutes after wind and cold shock stimulation. Thereafter as figure 6.1C shows the acceleration declines until it levels of at about 75 minutes for wind and 90 minutes for cold shock. However if *NpCaM-1* is a single gene the precise mechanism whereby this acceleration could occur occupying this time period is unclear at the present time. When we plotted the mRNA data in figure 6.1 on probit (probability) paper, however, a straight line was obtained. This suggests that the underlying control which specifies the pattern of mRNA accumulation is some factor which varies in a normal distribution. A plausible explanation for this could be a cell recruitment process. The second possibility is, then, that cells in the seedling do not commence an elevated rate of *NpCaM-1* accumulation immediately after signalling but are progressively recruited into this new mode of activity over the whole period of the response i.e. 75 or 90 minutes for wind or cold respectively. Each cell has a certain probability that it will enhance *NpCaM-1* synthesis after signalling and the probability maximum is located at the midpoint of the accumulation curve i.e. 45 minutes. A similar phenomenon has been observed elsewhere. Hillmer et al., (1993) examined the secretion of amylase by individual protoplasts after gibberellin treatment and observed that protoplasts were progressively

recruited to the synthesis/secretion mode up to a maximum of 50% viable protoplasts over a period of four hours. The recruitment period here is shorter but the basic principle i.e. that of recruitment may be identical.

Novick and Weiner (1957) showed that the induction kinetics of ß-galactosidase in *Escherichia coli* behaves as an "all-or-none" phenomenon, since bacteria grown at low concentrations of inducer were either non-induced or fully induced. The kinetics of induction that was observed therefore reflects changes in the relative number of bacterial cells synthesising ß-galactosidase. The rate of enzyme synthesis rose linearly, because there is a linear rise in the fraction of the population in the induced state. A linear rise lasting for several generations means that the probability of a bacterium becoming induced is constant in time; hence the transition from non-induced to induced is the consequence of a single random event. This event must be the achievement of some critical threshold of the permease content responsible for the uptake of the substratum which assures the rise in permease to its maximum and is in fact the critical factor for recruitment. The induction kinetics of ß-galactosidase with low concentration of induces in *E. coli* follows a S-shaped curve (Novick and Weiner, 1957).

A number of processes in plant development can be described in a similar way to the above using a recruitment basis and these are detailed in Bradford and Trewavas (1994). The analysis applied by Bradford and Trewavas (1994) might be helpful to understanding *NpCaM-1* accumulation described here. Kent Bradford (1990) constructed a simple mathematical model which fits many plant development data. The factor in this model which varies, like the single random event in the induction kinetics of *E. coli*, in a normal distribution amongst the cells of the seedling, would be an intrinsic Ca^{2+} threshold value which is specific to each cell and must be exceeded before downstream events can be initiated. Evidence that such thresholds do exist in Ca^{2+} signalling has been described in Gilroy et al., (1990) although the minimum and the maximum Ca^{2+} thresholds levels in cells of tobacco are currently unknown. If the Bradford model is applicable, the difference between the intrinsic threshold Ca^{2+} and the actual Ca^{2+} signal achieved by wind stimulation or cold shock stimulation determines the time at which the cell commences enhanced *NpCaM-1* synthesis. If the difference is large the cell rapidly commences *NpCaM-1* synthesis; if the difference is small then the cell takes much longer to commence *NpCaM-1* synthesis.

The induction kinetics of a *E. coli* population is an "all-or-none" response when grown at low concentrations of inducer and is therefore determined by a single random event, the permease that ensures that the accumulation follows a normal distribution (Novick and Weiner, 1957). Likewise, the induction kinetics of *NpCaM-1*

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accumulation may be controlled by the normally distributed actual Ca^{2+} signal compared to Ca^{2+} thresholds amongst the cell population that will ensure an "all-or-none" response that recruits cells into an *NpCaM-1* accumulation mode and therefore will follow a S-shaped pattern.

8.5   Transcription Activation by Recruitment

An alternative explanation for the fact that the accumulation of *NpCaM-1* mRNA after cold shock and wind stimulation follows S-shaped curves is based on a transmission of a state that is progressively recruited into a new mode of activity. Mechanisms like this are known to occur in anatomy, where incorporation into tissues or regions of cells from elsewhere in the body leads to recruitment. This principle has been applied to explain transcription activation in prokaryotes and eukaryotes (Ptashne and Gann, 1997). In this model for gene activation it was suggest that an activator works by bringing the transcriptional machinery to the DNA. Summarising recent findings in bacteria that characterise a basic mechanism for activation called recruitment, describes how a large variety of activator-target interactions can effect gene activation. Experiments in yeast indicate that a similar mechanism works in eukaryotes. The model that emerges at least partly explains activating region design, the nature of the targets of those peptides, and how disparate activators can work cooperatively and synergistically can affect gene activation. The model also suggests a role for histones in the process.

In bacteria promoters, activator-target interactions can effect gene activation by two mechanisms. In the first, NTRC (nitrogen regulatory protein C) works on a stable pre-bound polymerase (also known as the classic gene induction mechanism) (Fig 8.2A). In the second, the cyclic AMP responsive, CAP (catabolic activator protein) and a lambda repressor works by recruiting polymerase to DNA (Fig. 8.2B). In all bacterial promoters studied so far, polymerases bind in at least two steps: in the first step, it binds to helical DNA to form a 'closed' complex (known as the initial binding step), and in the second step, the complex isomerises to form the 'open' complex in which the DNA strands are locally opened (known as the isomerisation step). There is, however, a difference in the stability of the closed complexes and in the requirements for the transition from the 'closed' to the 'open' complexes, at two types of promoters. In the first type stimulated like NTRC, the polymerase forms a highly stable closed complex with DNA in the absence of the activator (Fig. 8.2A). At the promoter, the activator's role is not to stimulate formation of a stable polymerase-DNA complex, but rather to stimulate isomerisation from one stable complex (closed) to another (open).

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In the second type, stimulation by CAP or lambda repressor, the polymerase is not stably associated with DNA before activation and so it is stated that activation works by recruitment (Fig. 8.2B). Because polymerases are bound more stably only upon formation of the open complex, in these cases isomerisation is part of the recruitment process. For example, CAP working at the *lac* promoter primarily stimulates the initial binding of polymerase, the lambda repressor primarily stimulates isomerisation at its own promoter, and CAP stimulates both steps at the *gal* promoter. The stimulation of either or both steps has the effect of moving the promoter from one state, in which polymerase is largely absent, to another, in which polymerases is bound and can initiate transcription.

A
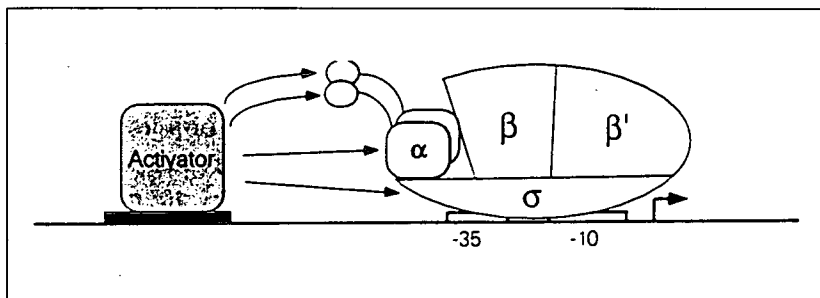
B
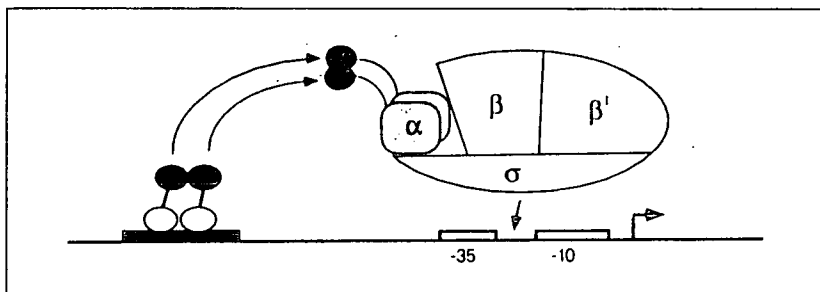
Figure 8.2: (A) Some activators work on a stable pre-bound polymerase. The polymerases bind to the helical DNA to form a 'closed' complex and the complex isomerises to form the 'open' complex in which the DNA strands are locally opened upon interaction with an activator protein. (B) The recruitment model: Activator proteins work by recruiting polymerase to DNA where isomerisation is part of the recruitment process. Taken from Ptashne and Gann, 1997.

Typical eukaryotic promoters are characterised as having an (A+T)-rich sequence (the TATA-element) positioned some 50-70 base pairs upstream of the transcriptional start site. Binding sites for yeast activators are typically found 100-250 base pairs upstream of the genes that they regulate. The proteins that direct transcription are believed to be organised into a small number of complexes (GTFs, or general transcription factors) that work together (Fig. 8.3A). For instance the TFIID contains TBP, the protein that binds to the TATA sequence, and in addition a group of proteins called TAFs (TBF-associated factors). The separated GTF's were found to assemble in a unique order at the promoter; TFIID first, followed by TFIIB, and then, in a defined order, the remaining factors. TBP (transcription binding protein) binds to and distorts DNA bearing the TATA sequence so that a platform is presented for subsequent binding of TFIIB (Fig. 8.3B).
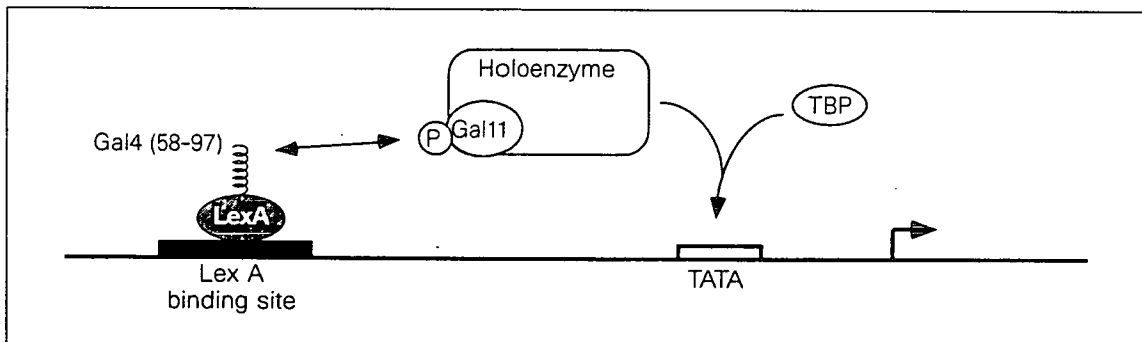
A
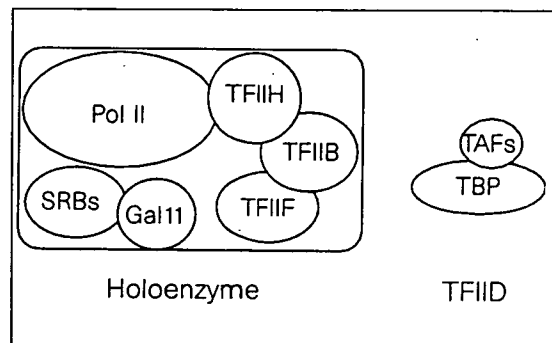
B

Figure 8.3: The yeast machinery that initiates transcription is organised in complexes, PolII, polymerase II; TFIIB, TFIID TFIIF, TFIIH, transcription factor IIB, IID, IIF, IIH, respectively; Gal11, galactosidase activator protein 11, SRBs, suppressor of RNA polymerase B; TAFs, transcription activator factor; TBP, TAF binding protein (A). Gene activation in yeast is affected by recruitment of the holoenzyme. Cooperative binding between the holoenzyme, TFIID and the pre-bound activator protein, LexA recruit the machinery to the helical DNA (B). Taken from Ptashne and Gann, 1997.

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The basic design of eukaryotic activators is similar to that of prokaryotic activators; each bears an activating region and a DNA-binding region. One notable difference is that the activating regions can be physically separated from the DNA binding region. It is suggested that each of the two complexes might be recruited separately leading to the possibility of synergistic effects (Fig 8.3B). Additional obstacles for transcription are presented by nucleosomes. The holoenzyme and activator proteins compete for DNA access with histones (Marsolier et al., 1995; Felsenfeld et al., 1996). Histones would thus constitute an important part of the regulatory apparatus by presenting a barrier which holoenzyme recruitment must be effected. Weakening the histone barrier - through the binding of additional proteins (a function assigned for nucleoplasmin) (Owenhughes and Workman, 1996) or by histone modification - could therefore increase gene expression. Histone modifications can be phosphorylation (Zhang et al., 1996), methylation (Tikoo and Ali, 1997) and recently identified acetylation (Penissi, 1997). Transcription of many genes, in particular of higher eukaryotes, is dependent upon multiple physiological signals. In the simplest elaboration of the recruitment mechanism, multiple activator-machinery contacts would be required for efficient transcription, and these contacts would be provided by disparate DNA-binding activators, each of which is controlled by a different physiological signal. Binding sites for these various activators, suitably positioned, would allow these activators to work together (synergistically) to activate the gene.

Models that described the way by which calmodulin gene expression is regulated are not new (Braam et al., 1996). In *Arabidopsis*, a model has been constructed that illustrates how diverse stimuli could lead to the common response of *TCH* gene regulation of expression (Braam et al., 1996). It has been suggested that each environmental signal might activate a distinct signalling pathway that leads to the regulation of *TCH* genes. Different pathways might affect different activators or even the intactness of nucleosomes that might recruit different part of the functional DNA polymerase holoenzyme and could account for the synergistic transcription observed after cold shock but not after wind stimulation. One activator might be activated through a nuclear Ca^{2+} involving signalling pathway like after wind stimulation whereas an additional one, not involving nuclear Ca^{2+} but perhaps cytosolic Ca^{2+} might be activated in addition to the first upon cold shock. Recruitment of hepatocyte nuclear factor 4 into specific intranuclear compartments depends on tyrosine phosphorylation that affects its DNA-binding and transactivation potential (Ktistaki et al., 1995). In *Arabidopsis* a transcription activator has been identified that responds to low temperatures (Stockinger et al., 1997). A phosphorylation cascade initiated by a cold-induced cytosolic Ca^{2+} signal could affect the phosphorylation status of this low

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temperature-responsive transcription element, that would direct transcription of *NpCaM-1* in addition to nuclear Ca^{2+} signal that regulates transcription of *NpCaM-1* so that a higher accumulation *NpCaM-1* mRNA is obtained. This, of course, is by no means established, remains highly debatable and awaits further exiting exploration.

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