SIGNALING COMPONENTS DURING STOMATAL CLOSURE INDUCED BY METHYL JASMONATE AND ABSCISIC ACID

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By

D. Suhita

Supervisor

Professor A. S. Raghavendra *FNA, FASc, FNASc, FNAAS*

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046 INDIA

December 2004

Enrol. No. 99LPPH09

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046 INDIA

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Signaling components during stomatal closure induced by methyl jasmonate and abscisic acid** " has been carried out by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

D. Suhita
Enrol. No: 99LPPH09
(Candidate)

27/2/04

Professor A. S. Raghavendra
FNA, FASc, FNASc, FNAAS
(Supervisor)

Professor A.S. Raghavendra
Principal Investigator

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046 INDIA

CERTIFICATE

*This is to certify that Miss. Suhita D. has carried out the research work embodied in the present thesis entitled "**Signaling components during stomatal closure induced by methyl jasmonate and abscisic acid**" for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.*

Supervisor 27/12/04
Professor A.S. Raghavendra
Principal Investigator

Head 27/12/04
Department of Plant Sciences

HEAD
DEPT. OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046. INDIA

Dean 27/12/04
School of Life Sciences

Dean, School of Life Science
University of Hyderabad
Hyderabad-500 046

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Suhita D.

ABBREVIATIONS

ABA	=	abscisic acid
BCECF-AM	=	2',7'-bis(2-carboxy-ethyl)-5(6)-carboxyfluorescein-acetoxymethylester
cADPR	=	Cyclic ADP-Ribose
CaM	=	calmodulin
DAF-DA	=	4,5-diamino fluorescein diacetate
DAG	=	diacylglycerol
EGTA	=	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra acetic acid
H_2DCF-DA	=	2',7'-dichlorofluorescein diacetate
IP_3	=	inositol 1,4,5-triphosphate
L-NAME	=	N-nitro-L-arginine methylester
MJ	=	methyl jasmonate
PI3P	=	phosphatidylinositol 3-phosphate
PI4P	=	phosphatidylinositol 4-phosphate
PIP_2	=	Phosphatidylinositol 4,5-bisphosphate
PMA	=	phorbol myristate acetate
SNP	=	sodium nitroprusside
TFP	=	trifluoperazine
U73122	=	1-[6-[17β3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyls]-1H-pyrrole-2,5-dione

All the remaining abbreviations are all standard ones, and as indicated in *Plant Physiology* issue, 2004, Instructions for contributors, website: http://www.aspb.org

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Chapter 1

Introduction and Review of Literature

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1.1 Introduction

The exchange of oxygen and carbon dioxide in the leaf occurs through stomata. Each stoma is constituted by a pair of guard cells, which are modified epidermal cells of the epidermis. Antagonism between the guard cell and epidermal cell turgor control the extent of stomatal aperture. Thus, guard cells regulate the dimensions of stomata, thereby regulating rates of not only transpiration but also photosynthesis. Guard cells are highly differentiated, metabolically active and respond quickly and reversibly to diverse environmental signals. Guard cells have therefore been the focus of intense research in plant biology (Willmer and Fricker, 1996).

Stomatal movement is the result of changes in guard cell turgor, which depends on their osmotic potential. The major contributors to the guard cell osmolarity are K^+, Cl^-, malate, sucrose and a few organic anions (Willmer and Fricker, 1996). This leads to changes in turgor and makes guard cell to swell or shrink, resulting in stomatal opening or closure, respectively (MacRobbie, 1983, Talbott and Zeiger, 1998, Blatt, 2000a,b).

Guard cells have become a popular model systems to study the roles of ion channels, changes in cytosolic calcium (Ca^{2+}) in response to stimuli and provide a platform for understanding signal transduction (Schroeder et al., 1984, Schroeder and Hagiwara, 1989, McAinsh et al., 1990). The ion channels are the targets of several signals. An understanding of the signals that impinge upon guard cells and the mechanisms through which guard cells respond

would allow an integration of stomatal function into the whole plant's physiology.

The environmental (abiotic) stimuli affecting stomatal aperture includes CO_2, light, temperature, water stress, humidity. Among the plant hormones, abscisic acid (ABA) and methyl jasmonate (MJ) cause stomatal closure while auxins and cytokinins promote stomatal opening (Raghavendra and Reddy, 1987, Irving et al., 1992). The effect of different stimuli appears to converge on ion channels, regulating the uptake/influx of K^+ and Ca^{2+} ions. The message of particular signal is transduced through several secondary messengers, and these in turn regulate the ion channels.

The bioenergetics, carbon metabolism and signaling cascades in guard cell have been reviewed frequently in books and excellent reviews. Some of the books are by Zeiger et al. (1987), MacRobbie, (1987) and Willmer and Fricker (1996). Among the recent reviews on stomata are those by Leung and Giraudat (1998), Raymond et al. (1999), Blatt (2000a,b), Assmann and Wang (2001), Hetherington (2001), Medrano et al. (2002), Outlaw (2003), Hetherington and Woodward (2003), Fan et al. (2004) and Gray and Hetherington (2004).

1.2 Major abiotic factors affecting stomatal movements

1.2.1 Carbon dioxide

Stomatal apertures are reduced at increased atmospheric CO_2 (Assmann, 1999). As increase in CO_2 concentrations promotes efflux of K^+ causing depolarization, inhibition of H^+-ATPase and influx of Ca^{2+} (Hedrich et

al., 2001). Guard cells respond also to lowered internal CO_2 concentration within the intercellular spaces resulting from photosynthesis. Stomatal closure is mediated by K^+, anion efflux, removal of sucrose and synthesis of malate (Gaedeke et al., 2001). Elevated CO_2 concentrations arising from respiration in darkness stimulate stomatal closing (Mansfield et al., 1990).

1.2.2 Light

The stomata open as a direct response to light or respond indirectly to light via decreased intercellular CO_2 concentration. Stomatal guard cells can respond to light within few minutes (Parvathi and Raghavendra, 1995). Stomatal opening is promoted strongly by blue light, much more than that by red light. The pattern and mechanism of blue light responses of guard cells to blue light is therefore studied extensively (Willmer and Fricker, 1996). Several secondary messengers like calcium and calmodulin (CaM)-dependent protein kinases of the myosin light chain kinase type (MLCK) type are possibly involved in the transduction of blue light perception by guard cells (Assmann, 1993, Shimazaki et al., 1992). The prominent studies on blue light suggest the involvement of carotenoids, particularly zeaxanthin, in blue light perception by guard cells *in vivo* and *in vitro* (Zeiger et al., 1987, Zeiger and Zhu, 1998, Talbott et al., 2003). Stomatal opening *in vivo* is stimulated by even a single pulse of blue light. The stimulatory effect of blue light on stomatal opening is pronounced at low Ca^{2+} (Parvathi and Raghavendra, 1997a).

1.2.3 Humidity and water stress

Stomata open in response to high humidity (Maier-Maercker, 1983). Stomatal opening as well as transpiration increases as the difference between the leaf humidity and air humidity widens. In a humid environment, plants keep their stomata wide open, quite often during the day. Changes in guard cell metabolism and upregulation of certain selective mRNA levels were observed in guard cells of *Solanum tuberosum* on exposure to water stress (Kopka et al., 1997). The changes in transcript levels were noticed much earlier to the decrease in leaf water potential. It is suggested that ABA could be involved in these responses (Gowing et al., 1993).

1.2.4 Temperature

Stomatal aperture increased with increasing temperature up to an optimal level followed by closure (Willmer and Fricker, 1996). High temperatures lead to irreversible damage of guard cell function. Thus, stomata open increasingly at higher temperatures until guard cells are damaged (Roger et al., 1981). Low temperature may effect the enzymes, ion channels, $[Ca^{2+}]_{cyt}$ oscillations, Ca^{2+} channel activity or Ca^{2+} influx into guard cells (Sanders et al., 1999, Knight, 2000, Wilkinson et al., 2001). Thus, sub- or supraoptimal temperatures induce signaling cascade leading to loss of turgor and eliciting stomatal closure (Giraudat et al., 1994, Allen et al., 2000, Wilkinson et al., 2001).

1.3. Events during stomatal opening and stomatal closure

During stomatal opening the flanking guard cells accumulate K^+ salts (Outlaw, 1983, Zeiger, 1983), malate and sucrose (Talbott and Zeiger, 1998). Osmotic H_2O influx causes increased guard cell turgor, asymmetric guard cell enlargement and consequent increase in stomatal aperture. During stomatal closure, these solutes move out of guard cells. Stomatal closure results from activation of non-specific cation channels, membrane depolarization, and enhanced K^+ efflux via K^+ outward channels. Obviously, the ion uptake phenomenons are activated during stomatal closure. Activation of guard cell plasma membrane H^+-ATPase and hyperpolarization of plasmamembrane are among the initial event in stomatal opening (Zhao et al., 2000). Stomatal closure results to the ion efflux from the guard cells. Depolarization in turn deactivates K^+ inward channels and activates K^+ outward channels, resulting in K^+ efflux from guard cells (Schroeder et al., 1988). Elevation of cytosolic Ca^{2+} inhibits K^+ inward channels, thus limiting K^+ uptake (Grabov and Blatt, 1997, 1999). Obviously, the ion uptake phenomena are activated during stomatal opening and down regulated during stomatal closure.

Inorganic anion fluxes across the guard cell plasma membranes do not neutralize K^+ fluxes completely. The synthesis and accumulation of malate in cytosol helps for balancing the K^+ ions taken up by guard cells. Thus, regulation of malate accumulation is quite important in not only guard cell carbon metabolism but also maintaining ionic relations in guard cells (Outlaw, 1990, 2003).

1.4 Guard cell metabolism and bioenergetics

Guard cells contain numerous mitochondria and are expected to have high metabolic activity (Raghavendra and Vani, 1989, Vani and Raghavendra, 1994, Willmer and Fricker, 1996). The abundance of mitochondria, along with high respiration rates, suggests that oxidative phosphorylation is an important source of ATP to fuel the guard cell machinery (Parvathi and Raghavendra, 1995). Guard cells contain few chloroplasts, which are smaller than those of mesophyll cells (Willmer and Fricker, 1996). It has been shown that guard cell protoplasts (GCP) have high respiration rate compared to mesophyll cell protoplasts (MCP) (Gautier et al., 1991).

The guard cell carbon metabolism appears to be unique. Although at reduced levels, the Calvin cycle enzymes are present in guard cells (Shimazaki et al., 1989, Parvathi and Raghavendra, 1995). Mesophyll chloroplasts accumulate starch as a final product of photosynthesis during the light period and destarched during night. In guard cell chloroplasts starch is present in darkness and is mobilized in light (Ritte et al., 1999, Talbott and Zeiger, 1998). Recently, an accumulation of starch even under light was reported in *Arabidopsis* guard cells (Stadler et al., 2003). Starch content is inversely correlated to the degree of stomatal aperture (Outlaw, 2003), leading to the hypothesis of a starch-to-sugar conversion as osmotic motor to drive guard cell turgor (Willmer and Fricker, 1996, Talbott and Zeiger, 1996, 1998). However, K^+ is the dominant contributor for osmotic potential in guard cells (MacRobbie, 1998, Dietrich et al., 2001).

Recent studies revealed a significant participation of sucrose in building guard cell turgor, particularly under light (Talbott and Zeiger, 1996, 1998). Guard cells are capable of importing glucose and sucrose (Reddy and Das, 1986, Ritte et al., 1999). During high photosynthesis and transpiration, the sucrose content in the apoplast increases, leading to elevation of sucrose in the guard cell symplast (Outlaw, 2003). In the morning phase, stomatal opening is mostly correlated with K^+ uptake, while in the afternoon K^+ content declines and sucrose become the dominant osmoticum, suggesting role of sugar transport during stomatal movement (Talbott and Zeiger, 1996).

K^+ accumulation in guard cells is associated with influx of Cl^- and synthesis of malate, with the help of phosphoenolpyruvate carboxylase (PEPC). The PEPC from guard cells is therefore extensively studied. The guard cell PEPC is highly regulated by cytoplasmic pH, and metabolites, such as malate, glucose-6P and triose-6P (Tarczynski and Outlaw, 1990, 1993). During stomatal opening, PEPC is phosphorylated, leading to an increase in enzyme activity and decrease in sensitivity to malate (Chollet et al., 1996). Fusicoccin (FC), which promoted stomatal opening, induced phosphorylation of guard cell PEPC while ABA dephosphorylates (Du et al., 1997). Cytoplasmic acidification also leads to guard cell PEPC activation, suggesting cytosolic pH as a signal in guard cell PEPC regulation (Meinhard and Schnabl, 2001). Stomatal opening is restricted by a PEPC inhibitor by 3,3-dichloro-dihydroxyphosphinoyl-methyl-2-propenoate (DCDP). The inhibition by DCDP is relived by ribose-5-phosphate indicating that Calvin cycle is important, when PEPC is restricted (Parvathi and Raghavendra, 1997b).

1.5 Secondary Messengers and signaling components in guard cells

1.5.1 Plasma membrane ATPase

The importance of ATPase in guard cells was demonstrated by diminished stomatal function in plants with suppressed H^+-ATPase (Zhao et al., 2000). The guard cell ATPase exhibits cell specific expression (Assmann, 1996). Abundance of the ATPase gives guard cells an increased capacity for H^+ extrusion (Nakajima et al., 1995, Hentzen et al., 1996). Activation of the ATPase is mediated by regulatory interaction of its phosphorylated C-terminus with 14-3-3 protein (Emi et al., 2001, Kinoshita and Shimazaki, 2002). The binding of 14-3-3 proteins to the phosphorylated autoinhibitory C-terminal domain prevents its interaction with the catalytic site leading to a high-activity state of H^+-ATPase and eventually stomatal opening (Blatt, 1988).

1.5.2 K^+ channel

Stomatal opening requires K^+ uptake into guard cells through K^+_{in} channels (MacRobbie, 1998). The guard cell K^+ channels are activated by external high H^+ (Blatt, 1992, Blatt and Armstrong 1993, Ilan et al., 1996) and low ATP concentrations (Wu and Assmann, 1995) mediated by membrane hyperpolarization (Ward and Schroeder, 1994, Assmann and Haubrick, 1996, MacRobbie, 1998). The K^+_{in} channel is modulated by protein phosphorylation (Theil and Blatt, 1994).

There are also K^+_{out} channels, for facilitating the efflux of K^+_{out} of guard cells. The K^+_{out} channel is activated by depolarization and cytosolic alkalinization (Blatt, 2000a). In *Arabidopsis*, *GORK* gene encodes the major K^+_{out} channel of guard cell plasma membrane (Hosy et al., 2003). Luan (2002)

reported that osmotically driven swelling of guard cell protoplasts resulted in a reversible decrease in the conductance of the K^+_{out} rectifying channel.

1.5.3 Calcium channels in guard cell

Ca^{2+} channels on the plasma membrane are subdivided on the basis of their modulation by a variety of secondary effectors (Sanders et al., 1999, White and Broadley, 2003). These include non-selective voltage-gated Ca^{2+}-permeable channels (Schroeder and Hagiwara, 1990, Fairley-Grenot and Assmann, 1992). Vacuolar membrane also have Ca^{2+} channels: slow vacuolar (SV) channels which exhibit Ca^{2+} selectivity, cADPR-activated Ca^{2+} permeable channels and voltage-dependent Ca^{2+} channels (Ward and Schroeder, 1994, McAinsh et al., 1996, MacRobbie, 2000). In addition, plasma membrane Ca^{2+}-ATPases and the Ca^{2+}/nH^+ antiporter in the vacuolar membrane may mediate the efflux of Ca^{2+} from the cytosol (MacRobbie, 1998, Webb and Hetherington, 1997).

In guard cells, reactive oxygen species (ROS) and phosphorylation increase hyperpolarization, activate Ca^{2+} channels and initiate stomatal closure (Köhler and Blatt, 2002, Kwak et al., 2003). Hyperpolarization activated Ca^{2+} channels catalyze Ca^{2+} influx to the cytoplasm and play an important role in modulating cytosolic Ca^{2+} and then cell signaling (Ng and McAinsh, 2003, Hetherington and Brownlee, 2004). Secondary messengers, inositol-3-phosphate (IP_3) and cyclic ADP ribose (cADPR) activate Ca^{2+} channels and increase the influx of Ca^{2+} (Ward and Schroeder, 1994, Leckie et al., 1998).

1.5.4 G Proteins

G protein and the G-protein-coupled receptors (GPCRs) are important components of signal perception. The *Arabidopsis* genome encodes only single canonical Gα and Gβ subunit genes (*GPA1* and *AGB1*, respectively), and two Gγ subunits (*AGG1* and *AGG2*) (Pandey and Assmann, 2004). GTP γ-S is the activator of G protein whereas GDP α-S is an inhibitor of G protein. The G-proteins in guard cells of *Vicia faba* regulate the magnitude of the inward K^+ channels (Fairley-Grenot and Assmann, 1991), and also lead to elevation of cytosolic free calcium (Assmann and Wu, 1994). Studies from *Arabidopsis* reported that small guanosine triphosphatase (GTPase) protein AtRac1 as a central component in the ABA mediated stomatal closure (Lemichez et al., 2001).

ABA increases the activity of sphingosine-1-phosphate, a possible receptor during ABA induced G-protein activation (Coursol et al., 2003). The T-DNA insertional mutant studies suggested that manipulation of G-protein would provide a mechanism for controlling plant water balance (Wang et al., 2001, Zheng et al., 2002). Recent studies, from *Arabidopsis* revealed the signaling cascade of ABA mediated G-protein activation for stomatal closure (Coursol et al., 2003, Pandey and Assmann, 2004).

1.5.5 Phosphoinositides and Phospholipases

Phosphoinositide (PI) metabolism plays an important role in ABA induced cytosolic Ca^{2+} changes and stomatal closure (Gilroy et al., 1991, Staxen et al., 1999, Jung et al., 2002). The binding of an external signal to cell

surface receptors activates phospholipase C (PLC) and hydrolyses phosphatidylinositol (4,5)-bisphosphate generating inositol (1,4,5) triphosphate (IP_3) and 1,2-diacyalglycerol (DAG). IP_3 releases the Ca^{2+} from the internal stores (Leckie et al., 1998, Lemtiri-Chlieh et al., 2003). The elevation of IP_3 in the cytosol can induce $[Ca^{2+}]_i$ increases, followed by a reduction in guard cell turgor (Gilroy et al., 1991). IP_3 can also reversibly inactivate inward K^+ channels, whilst at the same time activating an inward current that depolarizes the plasma membrane (Blatt et al., 1990). Recently Park et al. (2003) reported that the role of PI in ABA induced ROS generation. Stomatal guard cells have been reported to contain PI 3-phosphate (PI3P) and PI 4-phosphate (PI4P), the products of PI 3-kinase (PI3K) and PI 4-kinase (PI4K). Studies including kinase inhibitor tests, biochemical assays, biolistic transformation of guard cells with chimeric genes encoding GFP-tagged phosphoinositide-specific binding proteins, and calcium imaging results consistently showed that PI3P and PI4P in guard cells are involved in normal stomatal movements (Jung et al., 2002, Park et al., 2003).

Phospholipases clearly play a role in early signal transduction events that promote the cell volume changes associated with osmotic stress and osmoregulation in plants. Treatment of ABA marginally elevated IP_3 levels in guard cell protoplasts (Lee et al., 1996, MacRobbie, 1998) and U73122 inhibited the activity of recombinant PLC from tobacco (Staxen et al., 1999). Complete inhibition of ABA induced stomatal closure was achieved with a combination of U73122 and nicotinamide (an inhibitor of cADPR), suggesting that both function in ABA signaling (Jacob et al., 1999, MacRobbie, 2000).

A product of PLD is phosphatidic acid (PtdOH). ABA treatment of *Vicia faba* guard cells caused increased levels to PtdOH (Jacob et al., 1999). PtdOH also promotes stomatal closure and inactivates K^+_{in} currents (Schroeder et al., 2001a). Cytosolic Ca^{2+} was not increased in guard cells following PtdOH treatment, suggesting that PLD acts in a parallel pathway or down stream of Ca^{2+} mobilizing secondary messengers systems (Schroeder et al., 2001a). 1-butanol, an inhibitor of PLD, caused only a partial inhibition of ABA induced stomatal closure. However, addition of 1-butanol together with nicotinamide, completely reversed the ABA induced stomatal closure, suggesting a parallel action of PLD to the cADPR-mediated pathway (Jacob et al., 1999).

1.5.6 Calmodulin

Calmodulin (CaM) is a ubiquitous, calcium binding protein that can bind to and regulate a multitude of different protein targets, thereby affecting many different functions. The levels of CaM were comparatively higher in epidermal and guard cell protoplasts than mesophyll cell protoplasts of leaves (Ling and Assmann, 1992). Further Li et al. (1998) demonstrated the involvement of a Ca^{2+}-dependent protein kinase with a calmodulin like domain (CDPK) in guard cell protoplasts of *Vicia faba*, suggesting CDPK may be an important component of Ca^{2+} signaling in guard cells. CDPK is also found to be involved during activation of Cl^- channels (Pei et al., 1996). ABA activates a myelin basic protein (MBP) kinase in epidermal peels of Argenteum mutant of pea, suggesting that requirement of MAP kinase activation, which is obviously regulated by CaM (Burnett et al., 2000). Recently, Chen et al. (2004) reported that CaM stimulates a cascade of intercellular signaling events to regulate

stomatal movement, due to increase in both H_2O_2 and $[Ca^{2+}]_{cyt}$ levels leading to reduction in stomatal aperture.

1.5.7 Cytosolic pH

The action of the H^+/ATPase at the pHs prevalent in the cytoplasm causes one H^+ to be extruded (Blatt et al., 1998, Palmgren, 2001). This does not necessarily imply an increase in the pH of the well-buffered cytoplasm (Blatt et al., 1998) because the proton is generated during the hydrolysis of ATP rather than by translocating a proton cross the plasma membrane. Cytosolic pH is one of early events during stomatal opening and is believed to precede K^+ influx in to guard cells. Changes in cytosolic pH can be transient and are quickly dampened. Cytosolic alkalization has frequently been observed as a signaling components during the stomatal closure caused by not only plant hormones but also conditions such as stress (Irving et al., 1992, Gehring et al., 1997, Netting, 2000, 2002).

1.5.8 Protein kinases and phosphatases

Pharmacological approaches, using the serine/threonine protein kinase inhibitors and cytosolic replacement of ATP, showed that phosphorylation events are positive regulators in ABA induced stomatal closure (Schroeder et al., 2001a). Two Ca^{2+}-dependent protein kinases (CDPK) have been characterized from *Vicia faba* guard cells (Li et al., 1998, Mori and Muto, 1997). CDPK activated vacuolar Cl^- and malate conductance in guard cells and activation was dependent on Ca^{2+} and ATP (Pei et al., 1996). In addition, MAP

kinases have been also reported to positively control ABA induced stomatal closure (Burnett et al., 2000). Non-MAPkinase, such as AAPK (ABA activated protein kinase) was suspected to function in the ABA regulation of stomatal aperture in *Vicia faba* (Li et al., 2000) as well as with ABA induced reactive oxygen species (ROS) production and stomatal closure (Mustilli et al., 2002).

Inhibitors of PP1 and PP2A protein phosphatases such as Okadaic acid (OA) enhanced S-type anion currents and ABA induced stomatal closure (Schroeder et al., 2001a). OA partially inhibited ABA activation of S-type anion channels, stomatal closure or ABA induction of dehydrin mRNA (Pei et al., 1997, Hey et al., 1997), suggesting that phosphorylation and dephosphorylation can occur in ABA signaling depending on the physiological state of guard cells. The disruption of a guard cell protein phosphatase 2A, subunit RCN, leads to insensitivity of ABA, suggesting that the involvement of PP2A during ABA signaling (Kwak et al., 2002). Suppression of ABA-induced Ca^{2+} increases in guard cells by the dominant protein phosphatases 2C in *Arabidopsis* mutant (Allen et al., 2002). The interaction of AKT2 and AtPP2CA during ABA signaling that allows K^+ transport and membrane polarization (Cherel et al., 2002). The inhibition study of protein tyrosine phosphatases (PTPases) and suggested clear evidence for the involvement of PPTases in a major signaling network in plants (MacRobbie, 2002). Recently, Leonhardt et al. (2004) identified an highly ABA-induced protein phosphatase in guard cells.

1.5.9 Actin filaments

Actin antagonists have previously been shown to alter responses of *Commelina communis* stomata to physiological stimuli, implicating actin

filaments in the control of guard cell volume changes (Kim et al., 1995). An actin filament depolymerizing agent, Cytochalasin D, activated K^+ inward channel and enhanced light induced stomatal opening (Hwang et al., 1997). Phalloidin, an actin filament stabilizer, in contrast inhibited K^+ inward channel currents and restricted light-induced stomatal opening (Hwang et al., 1997). These results imply the possible interaction of the actin cytoskeleton with guard cell plasma membrane K^+ inward channels in signal transduction.

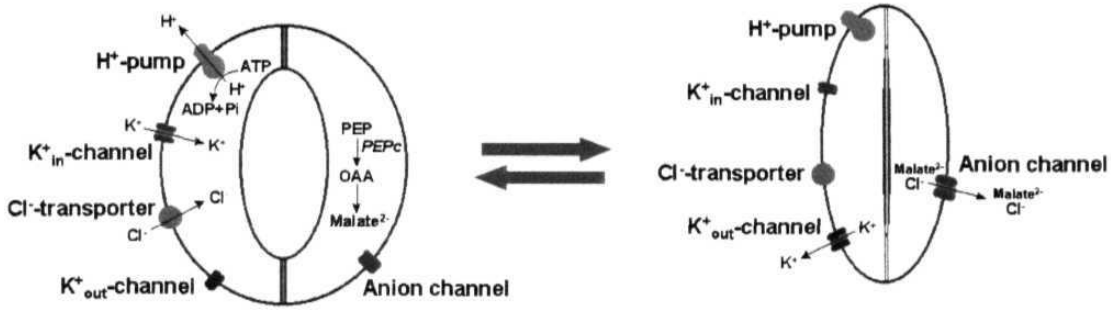

Figure 1.1. Schematic presentation of signaling in guard cells during stomatal opening and stomatal closure modulated by various ion movements.

1.6 Effects of ABA or MJ on stomatal signal transduction

The plant hormones like ABA, methyl jasmonate (MJ), salicylic acid induced marked stomatal closure (Raghavendra and Reddy, 1987, Mori et al., 2001). When plants are exposed to drought, they accumulate ABA in the leaves. ABA brings about reduction in stomatal aperture by promoting stomatal closure and inhibiting stomatal opening. Although the identity or location of the ABA receptor(s) are not known, a number of components acting downstream of ABA have been identified. These include protein kinases and phosphatases, an increase in cytosolic pH, slow anion channels, K^+ channels, activation of phospholipase D, and an increase in the concentration of free calcium ions in

the guard cell $[Ca^{2+}]_{cyt}$ via activation of several Ca^{2+} channel types. There is clear evidence that ABA activates release of Ca^{2+} from internal stores, but the source and trigger for ABA-induced increase in cytoplasmic Ca^{2+} are uncertain. The tonoplasts and another membrane, probably endoplasmic reticulum, have IP_3-sensitive Ca^{2+} release channels, and the tonoplasts has also cADPR-activated Ca^{2+} channels (MacRobbie, 1998). The increase in $[Ca^{2+}]_{cyt}$ then controls some of the ion channels whose activity regulates guard cell turgor changes. However, not all the channels are regulated by increases in $[Ca^{2+}]_{cyt}$, so it is likely that the overall control of guard cell turgor requires additional calcium independent components or pathways.

Several intermediates including reactive oxygen species (ROS) (Pei et al., 2000), nitric oxide (NO) (Neill et al., 2002), a putative heterotrimeric G protein α subunit (Wang et al., 2001), and the $[Ca^{2+}]_{cyt}$ mobilizing compound sphingosine-1-phosphate (Ng et al., 2001, Pandey and Assmann, 2004) have been added to the list of components involved in ABA signaling pathways. ABA enhanced the production of ROS in guard cell, activated Ca^{2+} channels in plasma membrane and caused stomatal closure (Bowler and Fluhr, 2000, Knight and Knight, 2001). It is possible that ROS also is a common signaling component during several cellular responses (Pei et al., 2000).

Apoplastic ABA and Ca^{2+} form a synergistic system to ensure stomatal closure. The apoplastic ABA inactivates the K^+ inward rectifying channel (Gepstein et al., 1982) and opens a plasma membrane Ca^{2+}-channel (Schroeder and Hagiwara, 1990) leading to increased cytoplasmic Ca^{2+}

(McAinsh et al., 1990). In contrast to inward channels, the K^+ and Cl^- outward rectifying channels are opened up by ABA (Schauf and Wilson, 1987, Blatt et al., 1998). As a result, the K^+ influx into guard cells is restricted and effluxes of K^+ or Cl^- out of guard cell are promoted by ABA. The action of ABA on Ca^{2+} channels appears to be mediated by a protein phosphatases type 2C (MacRobbie, 1998).

Similar to ABA, the accumulation of JA is induced by osmotic stress, wounding, drought, exposure to elicitors, such as chitins or oligosaccharides (Doares et al., 1995, Parchmann et al., 1997, Leon et al., 2001). Jasmonates are small lipid derivatives that affect senescence and abscission, stomatal closure, inhibition of root growth, and germination of non-dormant seeds (Creelman and Mullet, 1997). The derivative of jasmonic acid, particularly methyl jasmonate, is naturally found in high concentrations in flowers and reproductive tissues (Staswick, 1992, Creelman and Mullet, 1997). MJ enhances the expression of certain genes, such as vegetative storage proteins (VSP) in soybean (Staswick, 1992) and *A. thaliana* (Berger et al., 1995, 1996). Recently, Beardmore et al. (2000) reported MJ induced bark storage protein gene in leaves and stems of poplar and oil seed rape (Rossato et al., 2002).

Extensive studies have been carried out in ABA signaling cascade in response to development event, and environmental stress: such as seed maturation/germination inhibition and reduction of stomatal aperture (Schroeder et al., 2001a,b, Finkelstein et al., 2002, Fan et al., 2004). However, the studies on MJ response in guard cell signaling is very

limited (Raghavendra and Reddy 1987). Little is known about early steps in jasmonate signaling, but several important papers have highlighted rapidly executed jasmonate-triggered events in the plasma membrane. In one of these reports, patch-clamping of stomatal guard cells revealed that methyl jasmonate promoted K^+ efflux, a process necessary for stomatal closure (Evans, 2003). The present study is undertaken to compare the MJ signaling with the ABA signaling cascade in the guard cells, in terms of suppression of stomatal opening by MJ and ABA.

Readers interested in the effects of ABA or MJ on plant growth and development may consult the reviews of Leung and Giraudat (1998), Finkelstein et al. (2002), Turner et al. (2002), Devoto and Turner (2003), Borisjuk et al. (2004). There are excellent reviews on ABA effects on guard cells (Giraudat et al., 1994, Giraudat, 1995, Chrispeels et al., 1999, Comstock, 2002, Desikan et al., 2004, Ng et al., 2004, Wang et al., 2004, Tallman, 2004). However, the action of MJ on guard cells is not as frequently reviewed as that of ABA, as the work on this aspect has just picked up.

1.7 Use of *Arabidopsis* mutants for studies on stomatal function

Mutants of *Arabidopsis* have been employed to understand the plant function, including mechanisms of signaling cascades and various aspects of plant development. Several mutants are available which are either insensitive or hypersensitive to plant hormones. Similarly, there are mutants deficient in various signaling components and as a result exhibits altered responses to hormones or external stimuli, including stomatal movement. One of the most

popular among these *Arabidopsis* mutants, are those insensitive to ABA inhibition of seed germination: *abh1*, *abi1* and *abi2*. These mutants are also impaired in ABA induced stomatal closure. The *abi1* or *abi2* mutants have point mutations altering a conserved amino acid and encode type 2C protein phosphatases (Schroeder et al., 2001a, Merlot et al., 2001). It is shown that these mutants are impaired in several parameters: elevation of cytosolic Ca^{2+}, inward or outward K^+ channel regulations and anion channel activation that suggested to act upstream of cytosolic Ca^{2+} (Pei et al., 1997, Allen et al., 1999, Hugouvienx et al., 2001).

Using infrared thermography, two allelic *Arabidopsis* mutants *ost1* and *ost2*, were isolated which showed a reduction in ABA induced stomatal closure or impaired in transpiration upon drought (Mustilli et al., 2002). The mutations in OST1 gene, encoding a serine-threonine protein kinase, render *Arabidopsis* guard cells insensitive to ABA but not the tissues. The mutants were found to be impaired at a site in between ABA perception and ROS production (Merlot et al., 2002, Assmann, 2003). The signal from ABA to OST1 was modulated by ABI1 and then precedes via ROS to regulate cytosolic Ca^{2+} levels and ultimately leads to changes in stomatal aperture.

Wang et al. (2001) reported a T-DNA insertional mutant of heteromeric GTP-binding (G) protein α subunit GPA1, which lacks both ABA inhibition of guard cell inward K^+ channels and pH-dependent ABA activation of anion channels. Further, studies on the gpa1 mutants suggested that extracellular calmodulin involves a heteromeric G-protein, H_2O_2 generation and changes in cytosolic Ca^{2+} in the regulation of stomatal movements (Chen et al., 2004).

Another gcr1 mutant, which encode G-protein coupled receptors (GPCRs) exhibited hypersensitivity to ABA in root growth, gene regulation and stomatal reponse (Pandey and Assmann, 2004)

Mutation in NADPH oxidase catalytic subunit genes (*atrbohD* and *atrbohF*) impairs ABA signaling, ROS production and elevation of cytosolic Ca^{2+} including activation of plasma membrane Ca^{2+} channels in guard cells (Kwak et al., 2003). Studies on these mutants revealed that ROS, involving NAD(P)H oxidase are rate limiting secondary messengers in guard cell ABA signal transduction. Hosy et al. (2003) identified an insertional T-DNA disruption of outward rectifying K^+ channel gene (*gork1*) in the *Arabidopsis* guard cells. *GORK* showed no measurable outward K^+ channel activity. The gork1 mutant also showed slightly enhanced light induced stomatal opening, explaining the functions of K^+ balance with K^+ efflux in guard cells.

Recently, several mutants defective in jasmonate biosynthesis and signaling have been isolated and their phenotypes shed new light on the role of jasmonates and jasmonate signaling in plant responses to pathogens, insects and ozone (Berger et al., 2002). Mutation in three *Arabidopsis* loci (JAR1, COL1 and JIN1) reduced sensitivity to JA, suggesting that they might affect jasmonate signal transduction (Feys et al., 1994, Berger et al., 2002, Staswick et al., 2002). The JAR1 gene family is involved in multiple, and important plant signaling pathways like auxin-induced soybean GH3 gene, indole-3-acetic acid (IAA) and salicylic acid (Staswick et al., 2002)

Various aspects of guard cell signaling can be further dissected by employing suitable *Arabidopsis* mutants. In this study, we noticed an

interesting interplay between ABA and JA signaling pathways regulate stomatal movement. Mutant insensitive to ABA was also insensitive to MJ and the MJ-insensitive mutants did not respond very well to ABA. We further investigated the pattern of stomatal movement in mutants with disruption of NAD(P)H oxidase or K^+ out membrane channels. The results demonstrated NAD(P)H oxidase plays a significant role in ABA or MJ signaling pathways for stomatal regulation.

The approach and objectives of the present work are described in the next chapter.

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Chapter 2

Approaches and Objectives

Chapter 2

Approach and Objectives

Guard cells are very good model systems to study the signal transduction in plant tissues, both guard cells respond very quickly to hormones (e.g., ABA or MJ) and light. Most of the research, on the effects of ABA on plant tissues, was focused on the regulation of gene expression and to identify the secondary messengers involved in transduction of ABA signal. MJ is a plant hormone and powerful modulator of stomatal movement. In contrast to the extensive work on ABA effects, the work on guard cell responses with respect to MJ mediated signaling events is very limited.

Nicotiana glauca (tree tobacco), *Pisum sativum* (pea), *Arabidopsis thaliana* are popular choices for biochemical studies, and are also used for studies on stomatal movements and guard cell properties. It is very easy to prepare the epidermal strips from the leaves of these three plants. Further, the isolation of guard cell or mesophyll cell protoplasts from *Arabidopsis* or *Pisum sativum* leaves is easy and rapid (Pandey et al., 2002, Devi et al., 1992). The leaves of these species offer a very good experimental material in the form of intact leaves, leaf epidermal peels, isolated protoplasts and organelles, for the study of physiological features, in presence of added compounds and metabolites.

Most of the experiments in this thesis were done with tree tobacco and some with *Arabidopsis thaliana*. The plants of *Arabidopsis* were raised in controlled growth chambers under regulated temperature and light regimes. The mutant plants affected in ABA signalling (*ost1-2*), MJ signaling (*jar1-1*),

plasma membrane catalytic subunits of the plasma membrane NAD(P)H oxidase (*artbohD/F*) or guard cell outward K^+ channel (*gork1*) were used to assess the respective roles of these genes in ABA or MJ signaling pathways leading to stomatal closure. The results were with those with wild type plants.

We have used the system of either leaf epidermal strips or guard cell/mesophyll cell protoplasts according to the requirement of the experiment and convenience of manipulation. The externally added compounds are permeable through the plasma membrane of the epidermal guard cells and this has been reflected in stomatal responses to various treatments. Protoplasts are very close to *in vivo* situation compared to isolated organelles to that of the intact epidermal guard cells. However protoplasts had limited stability at room temperature, fragile nature and tendency to sediment.

Epidermal peels are not only easy to peel and handle but are also stable for a long time. However, the process of peeling epidermal tissue can create stress on epidermal strip and hence the guard cell epidermal strips have been kept in incubation buffer for some time, so that the effects of stress can subside. Moreover, it takes a few hours to collect sufficient epidermal strips for biochemical studies. Measurements of stomata on epidermal strips are very easy and are usually done by precalibrated ocular microscope.

Several responses of plants to jasmonate are similar to that of ABA, both these stress signals processed through signal transduction chain to result in stress responses (Creelman and Mullet, 1997). Jasmonate (JA)-dependent wound signaling in tobacco appears to involve kinase-dependent JA

accumulation the effects of which are, in turn transduced by Ca^{2+} (Kenton et al., 1999). Most of the research, on the effects of ABA and MJ on plant tissues was found on regulation of gene expression. Several aspects of these are yet to be studied in detail.

In view of the possible involvement of Ca^{2+}, we studied the role of Ca^{2+} in relation to ion channels or messenger elements like modulators or activators of calmodulin and protein kinases in MJ or ABA induced stomatal closure responses. The MJ- and ABA-signaling are similar e.g. calcium requirement and protein (de)phosphorylation. Further, alkalinization of the guard cell cytoplasm (Gehring et al., 1997), ROS production (Lee et al., 1999) and modulation of K^+ channels at the guard cell plasma membrane (Evans, 2003) and all known to occur in response to ABA or MJ. Several of these observations suggest the existence of a close similarity and a cross talk between MJ or ABA signaling cascades. However, it is not completely clear which molecular components are shared by MJ and ABA signal transduction and which are different.

In the first phase of work, the signaling components of MJ signal transduction pathway in guard cell were examined using pharmacological compounds known to inhibit various secondary messengers. The results with MJ were compared with those of ABA. The effects of inhibitors or modulators were assessed in relation to MJ or ABA-induced stomatal closure. These inhibitors are: lanthanum, ruthenium red, verapamil - inhibitors of Ca^{2+} channels; trifluoperazine (TFP), N-(6-aminohexyl)-5-chloro-1 naphathalene-sulphonamide (W7) - inhibitors of calmodulin (CaM); butanol and 1-[6-17β-3-

methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl-1H-pyrrole-2,5-dione (U73122) - phospholipase D (PLD); phospholipase C (PLC) and phorbol myristate acetate (PMA) - activator of protein kinase C (PKC); K252A - inhibitor of broad range protein kinase; ML7 - inhibitor of Ca^{2+}- calmodulin protein kinase; diphenylene iodonium chloride (DPI) - an inhibitor of NAD(P)H oxidase; N-nitro-L-arginine methylester (L-NAME) - inhibitor of nitric oxide synthase (NOS); nicotinamide - inhibitor of cADPR; wortmannin (WM) - inhibitor of phosphatidylinositol 3-phosphate (PI_3P); LY294002 - inhibitor of phosphatidylinositol 4-phosphate (PI_4P). The inhibitors were used at very low concentrations so as to inhibit the target function effectively, with minimal disturbance to other metabolic processes of the tissue.

There has been intense interest on signaling molecules like nitric oxide (NO) and reactive oxygen species (ROS) and their involvement on signal transduction. It is likely that NO does not act alone, but interacts with other signaling molecules such as H_2O_2 to affect stomatal movement (Neill et al., 2003). These components function as signaling molecules in plants that are synthesized during stress responses (Neill et al., 2002) alter the redox state of the guard cells. Compared to the extensive literature on the involvement of calcium, pH and ROS during the modulation of stomatal movement studies on the biochemical or molecular biological basis of stomatal response to MJ, in relation to NO are very few.

Recent work has demonstrated that NO and ROS are essential intermediated in ABA induced stomatal closure in *Pisum sativum* and *Vicia faba* (Garcia-Mata and Lamattina, 2002). The sequence of signaling events that

occur during stomatal closure by MJ or ABA with regard to NO or ROS and intervening of secondary messengers are unknown.

There are excellent probes for monitoring the pH, ROS or NO within plant cells. These have already been used to record novel results. For e.g. BCECF-AM (2',7'-bis(2-carboxy-ethyl)-5-(6)-carboxyfluorescein-acetoxymethyl ster) to monitor pH in epidermal strips of the orchid *Paphiopedilum tonsum* (Irving et al., 1992), H_2DCF-DA (2',7'-dichlorofluorescein diacetate) for studies on ROS in guard cells of *Arabidopsis* (Murata et al., 2001), DAF-DA (4,5-diaminofluorescein diacetate) to detect NO in tobacco epidermal cells (Fossiner et al., 2000).

The present work is undertaken to understand the mechanism and identify signaling components involved in the suppression of stomatal opening by MJ or ABA. Since the modulation of calcium appears to be a common effect of ABA or MJ, stomatal opening was monitored in presence of varying calcium or EGTA. Experiments were designed to assess the role of different secondary messengers by using pharmacological compounds, which are known to affect different steps during Ca^{2+} signaling processes. The results suggest that the stomatal closure in response to ABA or MJ may be mediated through different steps of signaling pathways. The sensitivity of modulation of calcium was much more pronounced in presence of MJ than that of ABA. With this background the role and pattern of their other secondary messengers in guard cells: pH, ROS, NO, were also studied.

The following are the main objectives of the present work:

1. To determine the role of signaling components that modulate intracellular Ca^{2+} during the stomatal closure caused by ABA or MJ in epidermal guard cells of *Nicotiana glauca*.
2. To study the role of cytosolic pH changes and ROS production in response to ABA or MJ in guard cells of *Arabidopsis*. This has been studied in the epidermal guard cells and guard cell protoplasts of wild type plants. Further, mutant plants affected in ABA signaling (*ost1*-2), MJ signaling (*jar1*-1), plasma membrane catalytic subunits of the plasma membrane NAD(P)H oxidases (*atrbohD/F*) or guard cell K^+ outward channel (*gork1*) were also used.
3. To examine the significance of NO during stomatal closure induced by ABA or MJ in epidermal strips of *Nicotiana glauca*.
4. To study secondary signals involved during ABA or MJ induced shrinkage of MCP (mesophyll cell protoplasts) and GCP (guard cell protoplasts) and to assess the suitability of protoplast system for studies on signal transduction.

The variation in the experimental plants, and particularly the use of *Arabidopsis* and mutants, is because some of the experiments were done in France (in the lab or Dr. Alain Vavasseur at CEA, Cedex). The study was a part of the Indo-French collaborative project of Prof. A.S. Raghavendra and Dr. Alain Vavasseur.

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Chapter 3

Materials and Methods

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Materials and Methods

3.1. Plant material

Plants of tree tobacco (*Nicotiana glauca* L. cv. Graham, seeds gifted by Prof. Gary Tallman) and pea (*Pisum sativum* L.) cv. Azad (purchased from Pocha Seeds Company Ltd, Pune, India). The *Arabidopsis thaliana* Landsberg *erecta*, *ost1-2* mutant plants (accession Landsberg, from Dr. Jerome Giraudat, France), *jar1-1* mutant plants (accession Columbia, from TAIR, California, USA), *atrboh(D) x atrboh(F)* mutant plants (accession Columbia, from Dr. Julian Schroeder, USA) and *gork1* mutant plants (accession Wassilewskija, from Dr. Herve Sentenac, France).

3.2. Plant growth conditions

Tree tobacco seeds were sown in the pots filled with soil along with farmyard manure (3:1, v/v). The plants were grown outdoors, under natural environment with average day/night temperature of about 30/20 ^{0}C and an approximate photoperiod of 12 h, and were watered daily. The second to fourth leaves were harvested from 2 to 3 week old plants (Fig. 3.1A).

The growth conditions of Arabidopsis grown in France were different from that of the plants grown in India for our experiments. Seeds of *Arabidopsis* grown in France were sown on agar medium in petri plates and were kept in darkness at 4 ^{0}C for 3 days. The petri plates were transferred into light (8 h photoperiod) at 22 ^{0}C kept for 3-4 days. The seeds then germinated and the seedlings were transplanted to in disposable plastic cups containing

sand soaked with Hoagland's solution. The plants were allowed to grow in growth chamber under the following conditions: light regime of 300 μmol m^{-2} s^{-1}, 8 h, 22°C and 70 % RH; dark regime of 16 h, 20°C and 75 % RH. Plants were watered three times a day with a half-strength Hoagland solution (Table 3.1). The second or third fully expanded leaves from 4-5 week old plants (Fig. 3.1) were used for epidermal bioassays.

The *A. thaliana* (ecotype Columbia) grown in India were sown in a 1:1:1 mixture of vermiculite, perlite and soil right in plastic disposable cups and kept at 4 ^{0}C in dark for 3 days before transfer to and germination at 20 ^{0}C. The plants were grown at a 16 h light (125-150 μmol m^{-2} s^{-1}) and 8 h dark regime. Nutrient solution (Somerville and Ogren, 1982, Table 3.2) was supplied once a week.

Table 3.1. The composition of nutrient solution used for watering *Arabidopsis* plants in France.

Macronutrients	(1 Litre)	Micronutrients	(1 litre)
KH_2PO_4	17.05 g	$FeSO_4$	14 mg
K_2HPO_4	21.8 g		(in 50 μM Na_2EDTA)
$Ca(NO_3)_2$. $4H_2O$	8.6 g	$ZnSO_4$. $4H_2O$	88 mg
KNO_3	20.20 g	$CuSO_4$. H_2O	20 mg
		H_3BO_4	58 mg
		$MnSO_4$. $4H_2O$	62 mg
		$MgSO_4$. $7H_2O$	27 g
		$(NH_4)_6MO_7O_{24}$. $4H_2O$	0.92 mg
		NaCl	1 g

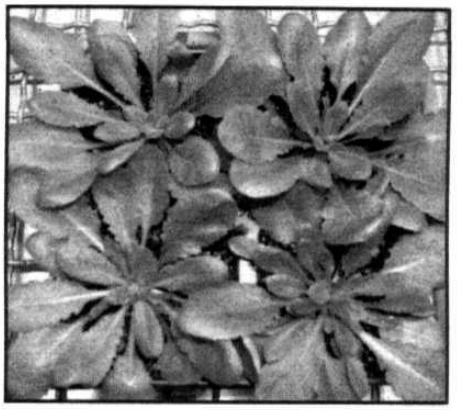
Table 3.2. The composition of nutrient solution used for watering *Arabidopsis* plants in India.

Macronutrients	(1 Litre)	Micronutrients	(1 Litre)
KNO_3	0.505 g	$FeSO_4$	$14x10^2$ mg
KH_2PO_4	0.34 g		(in 50 μM Na_2EDTA)
$MgSO_4$	0.492 g	$MnSO_4$	10 mg
$Ca(NO_3)_2$	0.47 g	$ZnSO_4$	2 mg
		$CuSO_4$	0.025 mg
		H_3BO_4	3 mg
		KI	0.75 mg
		Na_2MoO_4	0.25 mg
		$CoCl_2$	0.025 mg

The pea seeds were soaked in water overnight and then surface sterilized with 0.2% (v/v) sodium hypochlorite solution. The seeds were kept covered in a moist black cloth at 25 ^{0}C until they germinated, usually for 3 days. The germinating seeds were then sown in plastic trays filled with soil and farmyard manure (3:1, v/v) and were watered twice daily. The plants were grown out doors under natural photoperiod of approximately 12 h. The first and second fully expanded leaves were picked from 8-15 day old plants (Fig. 3.1D) and used for isolating protoplasts or epidermal bioassays.

A: *Nicotiana glauca* (Tree tobacco)

B: *Arabidopsis thaliana*



C: *Arabidopsis thaliana*

D: *Pisum sativum* (Pea)

Figure 3.1: A view of tree tobacco, *Arabidopsis* or pea plants, used in the experiments. (A) Tree tobacco (*Nicotiana glauca* L.) used for the epidermal bioassays; (B,C) *Arabidopsis* used for the epidermal bioassays as well as mesophyll and guard cell protoplasts; (D) Pea (*Pisum sativum* L. cv. Azad P1) used for the mesophyll and guard cell protoplasts.

3.3. Bioassays using epidermal strips

The abaxial (lower) epidermis was peeled off from the leaves (of tree tobacco or pea) and cut into strips of ca. 0.4 cm^2. The epidermal strips were transferred to 3-cm diameter petri dishes containing 3 ml of 25 mM MES-KOH, pH 7.0, 10 mM KCl and other test compounds (ABA or MJ or pharmacological

agents). The epidermal strips were left in darkness for 1 h and then exposed to white light. A bank of tungsten lamps, whose light was filtered through water jacket, provided the irradiation with white light (500 μmol m^{-2} s^{-1}). The epidermal strips were irradiated with white light for 3 h. Photon flux was measured with a Li-Cor quantum sensor (Li-Cor Instruments Ltd, Lincoln, NE, USA). The temperature was maintained at 25 ± 1^{0}C.

The leaves, from 4 to 5 week old plants *Arabidopsis* were harvested at about 8am and paradermal sections of abaxial epidermis were prepared. These epidermal sections were mounted on glass cover slips with the help of medical adhesive (Hollister, Libertyville, IL, USA) or Telesis V adhesive (Premiere Products Inc. Pacaima, California, USA) and incubated (10 mM MES-KOH, pH 6.5, 30 mM KCl) at 22°C for 2 h under light of (300 μmol $m^{-2}s^{-1}$). Then the test compounds (ABA or MJ or inhibitors) were added to the incubation medium and the epidermal strips were kept under the same conditions for another 2 h.

3.4. Measurements of stomatal aperture

Incase of tobacco and pea, the width of stomatal aperture was measured under a research microscope (DMIL, Leica, Germany) with the help of a precalibrated ocular micrometer. Randomly, ten stomata were examined in each of three different epidermal strips. Thus each observation was the result of at least 30 measurements. Incase of *Arabidopsis*, stomatal apertures were measured with an optical microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a camera lucida and a digitising table (Houston instrument TG 1017,

Austin, TX, USA) linked to a personal computer. For each treatment, at least 60 stomatal apertures were measured. It was ascertained that the 3 ecotypes studied have similar responses to ABA and MJ at the stomatal level.

Each experiment was at least repeated thrice on several days. The average values ± SE are reported.

3.5. Isolation of protoplasts

The guard cell and mesophyll cell protoplasts were isolated from *Arabidopsis* and *Pisum sativum* as described below.

3.5.1. Guard cell protoplasts (GCP) from Arabidopsis (Fig. 3.2 A)

GCP were prepared essentially as described by Pandey et al. (2002). The entire leaf was taken from 5 to 7 plants of *Arabidopsis*. The protoplasting media were as follows; Basic medium: 5 mM MES, pH 5.5, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.01 mM KH_2PO_4, 0.5 mM ascorbic acid, and 0.55 M mannitol; Enzyme medium 1: 0.7% Cellulysin, 0.1 % (w/v) polyvinyl pyrrolidone 40,000 (PVP-40), 0.25 % (w/v) bovine serum albumin (BSA) and 0.5 mM L-ascorbic acid, dissolved in 55 % (v/v) basic medium and 45 % (v/v) distilled water; Enzyme medium 2: 1.5 % (w/v) Onozuka RS Cellulase, 0.03 % (w/v) Pectolyase Y-23, 0.25 % (w/v) BSA, and 0.5 mM L-ascorbic acid in basic medium. Most commercially available wall-digesting enzymes are not highly purified and the low pH treatment by using HCl was used to inactivate contaminating proteases. The pH was therefore reduced to 3.5 for 5 min, and then raised to 5.5 using KOH.

After excision of the major veins with a razor blade, 60-80 young, fully expanded leaves were blended for 1 min in 100 ml cool distilled water, using a commercial Waring blender. The blended mixture was poured through a 200 μm nylon mesh to remove the broken mesophyll and epidermal cells. Large specks of mesophyll tissue, if any were removed with forceps. The epidermal peels retained were rinsed thoroughly with deionized water until the foam produced by blending had essentially been removed. The peels, which should be pale green, were then transferred into a 250 ml Erlenmeyer flask containing 50 ml of enzyme medium 1. The purpose of the first digestion is to release remaining epidermal cells, whose protoplasts then burst as a result of low osmolality. The flask was placed in a shaking water bath at 27°C for 30 min, with the shaking speed set at 160 excursions per min. As guard cells are responsive to light, digestion is performed in darkness (Dietrich et al., 2001). After 30 min, 100 ml of basic medium was added, and the mixture was shaken for an additional 10 min under the same conditions. This step provides an intermediate osmolality between enzyme medium 1 and enzyme medium 2; sudden plasmolysis would damage the guard cell membrane.

The digested peels were collected on 100 μm nylon mesh and rinsed gently with basic medium, then placed into 50 ml of enzyme medium 2 in 100 ml Erlenmeyer flask. Since this step releases guard cell protoplasts into medium, the osmolality is increased to maintain protoplasts integrity. The flask was shaken for 60 excursions per min in the dark at 17-20°C for 45-60 min, until most of the GCPs had rounded up. At the end of the digestion period the flask was swirled gently by hand for few seconds this would improve the

release of GCPs from the peels. The mixture was filtered through 10 μm nylon mesh. The peels remaining on the mesh were washed with 100-150 ml basic medium to release GCPs adhering to them. The filtrate, which contains GCPs, was collected into four 50 ml centrifuge for 5 min at 100 g. All but 1-2 ml of suspension was carefully removed from each loose pellet, and then the protoplasts pellet was gently resuspended. The tubes are refilled with basic medium and a second, identical centrifuge was performed.

After removal of the supernatant the GCP pellet is gently resuspended yielding a volume of 1-2 ml. GCPs (Fig. 3.2 C) were calculated on cell number basis. For kinetic studies, GCPs were incubated in the basic medium in the presence of ABA, MJ or methanol solvent control for the indicated time at room temperature (around 22°C).

3.5.2. Guard cell protoplasts (GCP) from Pisum sativum (Fig. 3.2 B)

GCP were isolated from pea leaves as described by Devi et al. (1992) with minor modifications. The lower epidermis of the leaf, was peeled off with forceps from the leaves of pea. Epidermal strips were kept in 10 ml of preplasmolysis buffer (1 mM $CaCl_2$, 0.3 M sorbitol, 10 mM MES-KOH buffer, pH 6) in 5 cm diameter petridishes for nearly 45 min. The strips were transferred to a 5 ml petri dish consisting of digestion medium (2 % (w/v) Cellulase Onozuka R-10, 0.2 % (w/v) Macerozyme R-10, 0.25 % (w/v) BSA, 10 mM sodium ascorbate, 1 mM $CaCl_2$, 0.4 M sorbitol and 10 mM MES-KOH). The pH of digestion medium was adjusted to 5.5 using 1 N KOH. The petri dish was subjected to orbital shaking (at 60 revolutions per min) for 20 min.

This step was done to remove the mesophyll tissue that was adhered to the epidermal strips.

The digestion medium was removed with a pasteur pipette and second digestion medium was added. The second digestion medium contained 3 % (w/v) Cellulase onozuka R-10, 0.3 % (w/v) Macerozyme R-10, and the remaining components and pH were similar to the first digestion medium. The petridishes were subjected to an orbital shaking. Both the digestions were under room light (light intensity of about 50 $\mu E\ m^{-2}s^{-1}$) in a water bath maintained at 30°C.

After incubation for 45 min, the digestion medium containing GCPs was removed with a pasteur pipette and fresh washing medium was added to the petri dishes. Protoplasts were collected by shaking gently the digested strips in the petridishes with washing medium. The pooled protoplast suspension in washing medium was passed successively through 100 and 30 μm nylon mesh. The filtrate containing guard cell protoplasts was centrifuged at 100 g for 5 min and the pellet resuspended in the suspension medium (10 mM HEPES-KOH pH 7, 0.4 M sorbitol, 1 mM $CaCl_2$, 10 mM KCl, and 0.5 mM $MgCl_2$).

3.5.3. Mesophyll cell protoplasts (MCP) from Arabidopsis (Fig. 3.2 C)

Fully expanded rosette leaves of *A. thaliana* were excised and kept in distilled water in the dark. The leaves were cut into 1 cm^2 pieces with a razor blade. The leaf pieces were then transferred to 10 ml of enzyme medium (5 mM MES-KOH, 0.5 M mannitol, 0.5 mM $CaCl_2$, 0.25 % (w/v) macerozyme R-10, 1.5 % (w/v) Cellulase R-10, 0.25 % (w/v) BSA and 0.1 % (w/v) PVP-40,

pH 5.5). The leaf pieces were vaccum infiltrated with enzyme medium for 2 min. Broken cells and debris were removed by decanting the medium and the leaf tissue was transferred to 15 ml of fresh enzyme medium. Digestion was performed at 24°C for 45 min with slow shaking (50 excursions per min) in a water bath. The enzyme medium developed a greenish hue, indicating the release of protoplasts. The medium was filtered through 60 μm nylon mesh into 50 ml centrifuge tube. The leaf pieces retained were rinsed with 25 ml of incubation medium (0.5 M mannitol, 1 mM $CaCl_2$) and the filtrate was collected. The suspension was centrifuged at 100 g for 5 min and the pellet obtained was resuspended in 50 ml of incubation medium. The medium was centrifuged, as before, and the final pellet protoplasts was gently resuspended in 1 ml of incubation medium and kept in ice in dark until use.

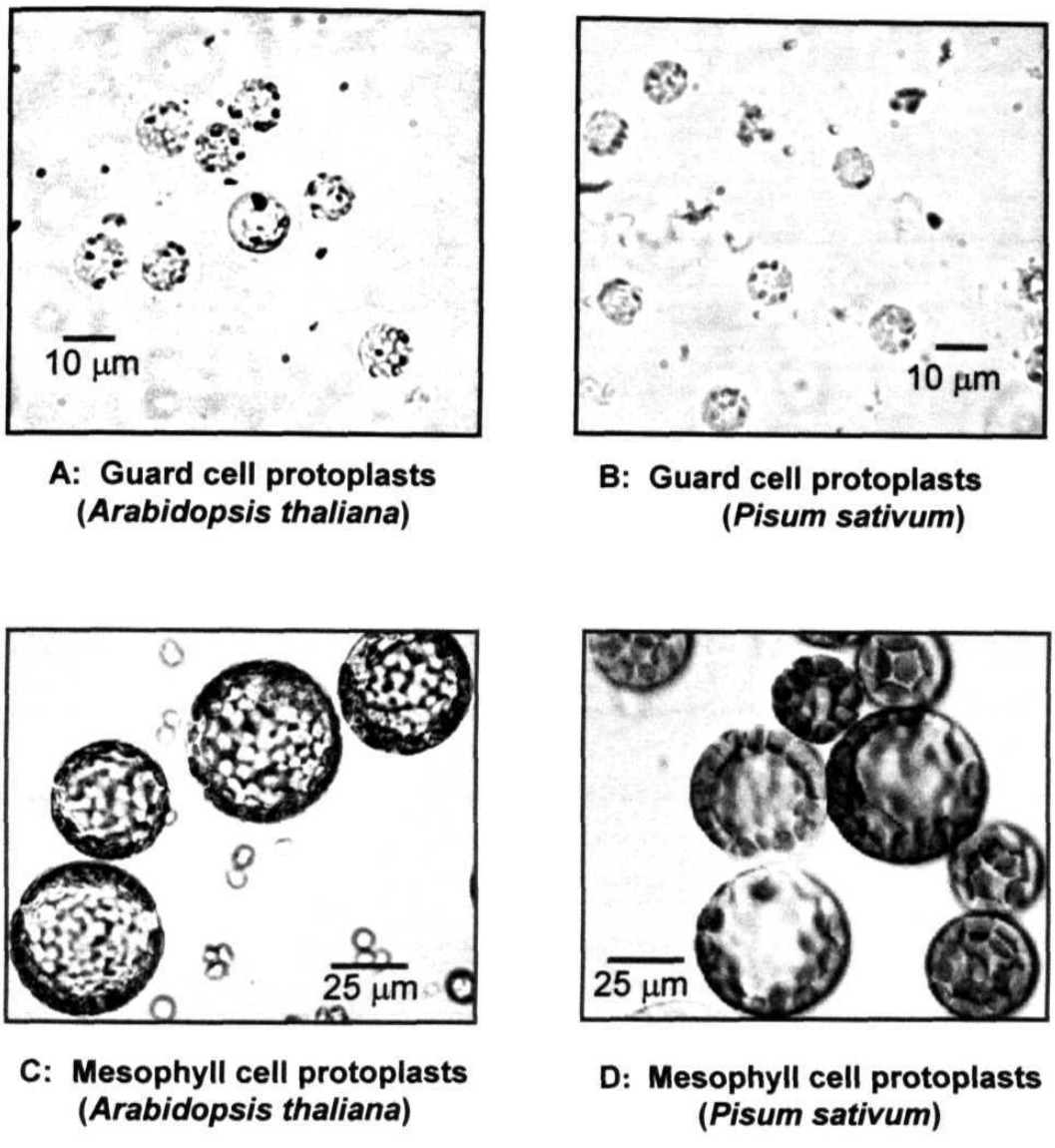
3.5.4. Mesophyll cell protoplasts (MCP) from Pisum sativum (Fig. 3.2 D)

Mesophyll protoplasts were isolated from pea leaves by minor modifications of the procedure of Devi et al. (1992). The abaxial epidermis of leaves was stripped off with forceps. The leaf was then cut into approximately 1 cm^2 pieces and floated on plasmolysis medium (0.3 M sorbitol, 1 mM $CaCl_2$ in 10 mM MES-KOH buffer pH 6.0) with the peeled lower surface touching the medium. After 30 min, the plasmolysis medium was removed and digestion medium (2% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10, 0.25% (w/v) BSA, 10 mM sodium ascorbate, 0.4 M sorbitol, 1 mM $CaCl_2$ in 10 mM MES-KOH buffer pH 5.5) was added. The enzymes cellulase, and macerozyme

macerozyme (pectinase), sodium ascorbate and BSA were added and the pH of the digestion medium adjusted just before use.

The leaf pieces were digested for 30 minutes, at 30^0 C under an illumination of 300 μmol m^{-2} s^{-1}. The digestion medium was then gently removed with a pasture pipette. Washing medium (0.4 M sorbitol, 1 mM $CaCl_2$ in 10 mM MES-KOH, pH 6.0) was added to the petri dish containing the digested leaf pieces. The petri dish was tapped and swirled gently to release the protoplasts into the medium. All further operations were done at 4°C. The suspension was filtered through a nylon filter of 60 μm pore size and filtrate was centrifuged at 50 g, for 5 minutes. The supernatant was discarded and the pellet was washed thrice with washing medium, to remove broken protoplasts. The washing was done by suspending the mesophyll protoplast pellet in 2-3 ml washing medium, centrifuging at 50 g, for 3 minutes, and then discarding the supernatant.

The pelleted protoplasts were washed once in suspension medium (0.4 M sorbitol, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ in 10 mM HEPES-KOH, pH 7.0). The pellet was finally suspended in 0.5 ml of suspension medium, mixed well by gently rocking and swirling the test tube containing protoplasts, and kept on ice. The protoplasts were kept on ice and handled gently through out the experiment.



A: Guard cell protoplasts (*Arabidopsis thaliana*)

B: Guard cell protoplasts (*Pisum sativum*)

C: Mesophyll cell protoplasts (*Arabidopsis thaliana*)

D: Mesophyll cell protoplasts (*Pisum sativum*)

Figure 3.2: Photomicrograph of mesophyll and guard cell protoplasts isolated from leaves of *Arabidopsis thaliana* (A, C) and *Pisum sativum* (B, D). The horizontal bar represents 25 µm for mesophyll protoplasts and 10 µm for guard cell protoplasts.

3.6. Characteristics of protoplasts

The number of protoplasts was counted using a haemocytometer. The size of the protoplasts was measured with a precalibrated ocular micrometer, under research microscope (DMIL, Leica, Germany).

3.6.1. Estimation of chlorophyll

Chlorophyll was estimated in mesophyll protoplast preparation by extracting into 80% (v/v) acetone (Arnon, 1949). An aliquot of 12.5 μl of protoplast suspension was added to 5 ml of 80 % (v/v) acetone and mixed on a cyclo-mixer. The absorbance of acetone extract was measured at 652 nm (A_{652} - to determine chlorophyll) and 710 nm (A_{710} - to correct for turbidity), using a spectrophotometer (Shimadzu UV-160A). The chlorophyll concentration was calculated using the following formula:

$$\text{Chl (mg ml}^{-1}\text{ of protoplast suspension)} = (A_{652} - A_{710}) \times 11.11$$

3.6.2. Measurement of protoplast volume changes

The diameters of protoplasts were measured with an optical microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a camera lucida and a digitizing table (Houston instrument TG 1017, Austin, TX, USA) linked to a personal computer. In some of the experiments, diameters were measured with a pre-calibrated ocular micrometer in a research microscope (DMIL, Leica, Germany). The volumes were computed by assuming spherical protoplasts, using the formula $4/3\ \pi r^3$ (r: radius of protoplast).

Thirty protoplasts were measured during each observation and experiment was repeated at least four times on different days. Each value of protoplasts volume of therefore represents an average of at least 120 measurements. Measurements were made at given time intervals for temporal kinetics (with or without the test compounds). Otherwise diameters were measured after 20 minutes of exposure to test compounds.

3.6.3. Photosynthetic oxygen evolution/ Respiratory oxygen uptake

Photosynthetic activity of protoplasts was measured in terms of O_2 evolution on illumination, while respiration was measured by monitoring O_2 uptake in darkness. The evolution or uptake of oxygen was measured at using Clark type oxygen electrode (Hansatech Instruments Ltd., King's lynn, UK). The reaction medium of 1 ml contained 0.4 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM bicarbonate in 10 mM HEPES-KOH, pH 7.5 and protoplasts equivalent to 20 μg Chl. Water at a constant temperature of 25°C was circulated through the outer jacket of reaction chamber. A 35 mm slide projector provided the illumination of 1250 $\mu E\ m^{-2}\ s^{-1}$. Calibration of the oxygen content in the electrode chamber was done with air saturated water, assumed to contain 252 nmoles of oxygen ml^{-1} at 25°C (Walker, 1988).

3.7. Determination of H_2O_2

Hydrogen peroxide production in guard cells was analysed using 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) as previously described (Murata et al., 2001). Epidermal peels were mounted on a microscope slide

with medical adhesive (Hollister, Libertyville, IL) or Telesis V (Premiere Products Inc. Pacaima, California, USA). Epidermal tissues were incubated for 2 h in 30 mM KCl and 10 mM MES-KOH, pH 6.5 for *Arabidopsis* were as for tobacco or pea 10 mM KCl, 25 mM MES-KOH, pH 7.0, was used. The dye, H_2DCF-DA, at 30 µM, was added to the incubation medium. After 20 min, the excess of dye was removed by three washes with distilled water. Epidermal tissues were then incubated for the indicated time with 20 µM ABA or MJ with an equal volume of methanol added to the control. When used, DPI (12.5 µM) was added 30 min before the dye to the epidermal strips.

Arabidopsis guard cells were observed with an epi-fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a charge-coupled device camera (AxioCam, Zeiss, Germany, Göttingen). The tobacco or pea guard cells were observed under an inverted fluorescence microscope (Optiphot-2, Nikon, Japan), fitted with monochrome high-resolution digital cooled CD camera (Coolsnap FX) that enabled to capture the images. The captured images and the relative fluorescence emission of guard cells was analysed using the NIH Image for Window, as previously described in Murata et al. (2001).

Confocal microscopy was also used to observe the fluorescence of ROS in epidermis of *Arabidopsis*.

3.8. Determination of NO

NO production in guard cells of pea and tobacco was analysed using the fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2DA) as previously

described (Neill et al., 2002). Epidermal peels were mounted on a microscope slide with an adhesive (Telesis V adhesive, Premiere Products Inc. Pacaima, California, USA). The coverslip along with epidermis was incubated for 2 h in 25 mM Mes-KOH, 10 mM KCl and pH 7 under light at 25°C. DAF-2DA at 10 µM was added to the incubation medium. After 20 min, the excess of dye was removed by three washes with distilled water. The inhibitors were added 30 min before the dye loading. Epidermal tissues were then incubated under the light for the indicated time with 20 µM ABA or MJ with an equal volume of methanol added to the control.

Guard cells were observed under an inverted fluorescence microscope (Optiphot-2, Nikon, Japan) fitted with with monochrome high-resolution digital cooled CD camera (Coolsnap FX) that enabled to capture the images.

Images were captured and the relative fluorescence emission of guard cells was analysed using the NIH Image for Windows, as previously described in Murata et al. (2001).

3.9. Determination of pH

Changes in pH were examined in epidermis of *N. gluaca* or *P. sativum* by incubation with 2',7'-bis(2carboxyethyl)5(6)carboxyfluorescein acetoxymethyl ester (BCECF-AM) as described earlier by Irving et al. (1992). The epidermal tissues were incubated for 3 h with 50 mM KCl and 10 mM MES-KOH, pH 6.5 in light (350-450 µmol m^{-2} s^{-1}) for *Pisum sativum*. While for *Nicotiana glauca* incubation medium was done in 10 mM KCl and 25 mM MES-KOH, pH 6.5. The strips were then treated with 20 µM BCECF-AM for 30 min in darkness.

The strips were rinsed several times in incubation buffer so as to remove the excess dye. The epidermal tissues were then treated with 20 μM ABA or MJ (or methanol in the control) and examined under the fluorescent microscope. The inhibitors were added to the incubation medium then after 30 min the dye was added to the medium under dark and then after 30 min MJ/ABA were added. Guard cells were then observed under an inverted fluorescence microscope (Optiphot-2, Nikon, Japan) fitted with monochrome high-resolution digital cooled CD camera (Coolsnap FX) that enabled to capture the images for the pH change with BCECF-AM fluorescence. The captured images and the relative fluorescence emission of guard cells was analysed using the NIH Image for Windows, as previously described in Murata et al. (2001).

3.10. Quantitative estimation of ROS, NO or pH

The levels of the fluorescence in the images acquired through either the epifluorescent microscopes or inverted fluorescent microscope was determined by using NIH Image for Windows. The images were imported to the NIH software and opened as TIFF files. A square box was drawn on the image window using the cursor and the intensity of fluorescence were calculated by analysing the pixels of the square box in the fluorescent image. The mean values of square area box were obtained by taking the pixels within the given fluorescence image window. After taking "n" different pixel intensities of the square box of the same size in the non-fluorescent area was taken as the control (background).

The pixel intensity value of fluorescent guard cells was recorded as (X) and the background of the fluorescence images as (Y). The difference of the background and area of interest was calculated and Y-X gives the actual intensity of the fluorescent image. The intensity of fluorescence was obtained, as intensity of pixels in the control/beginning of the experiment and taken as 100%. Based on the % of control the experimental analysis was done with various treatments.

3.11. Measurement of pH by 'Null-Point" Method

The method used for barley aleurone protoplasts, as described in van der Veen et al. (1992), was adapted for *A. thaliana* guard cell/mesophyll cell protoplasts. Disruption of the plasma membrane of cells in a weakly pH buffered solution will, in principle, lead to a change of external pH unless external pH is equal to internal pH. The "null-point method" for determination of internal pH is based on this principle. The internal pH can be determined by incubating protoplasts in weakly pH buffer solutions with external pH adjusted with KOH so as the internal pH is equal to the external pH, where no pH change could be observed. The experiments to know the change in the internal pH or the deflections can be determined by incubating protoplasts in weakly pH buffered solutions and the pH external was not adjusted to the changing internal pH.

The sample of 1 ml with 1 X 10^6 GCPs or 6 X 10^5 MCPs was placed in a weakly buffered medium (0.5 mM MES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 500 mM mannitol, pH 7). The suspension was continuously stirred with a

magnetic flea at low-speed magnetic stirring to avoid protoplasts damage in a Clark type oxygen electrode chamber (Hansatech Instruments Ltd. King's lynn, UK) (Fig. 3.3A). GCPs were incubated with 20 μM MJ or ABA, at room temperature. The external pH ($[pH]_{ext}$) was adjusted to the required value with diluted KOH. Subsequently, digitonin (0.01% w/v) was added to permeate the protoplast plasma membrane. The resulting pH changes in the external solution were recorded with a combined pH electrode (Ingold, Wilmington, MA, USA) coupled to a pH-meter (pHM85, Radiometer Copenhagen, Copenhagen, Denmark) and a pen recorder (Fig. 3.3A,B). Buffering capacity of the solution was determined by adding 10 nmoles of HCl at the end of each experiment. The values of the external pH at which permeablization induced no apparent shift in external pH were taken as an estimate internal pH.

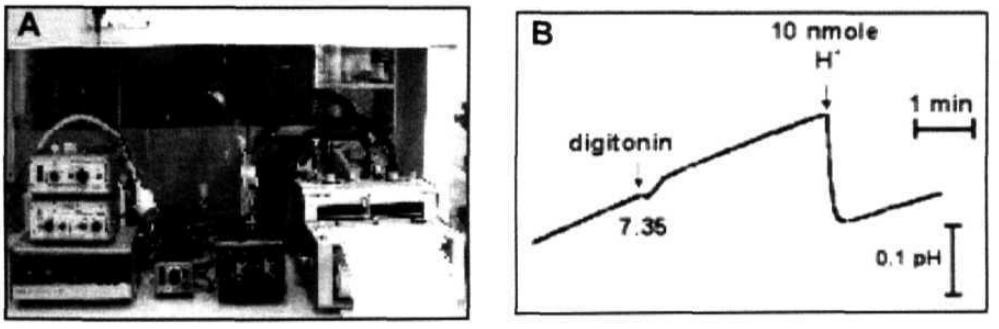


Figure 3.3: (A) Instrument used for null point method estimation of pH with very sensitive the recorded traces, indicating the titration at the end of the experiment are shown on (B) pen recorder attached to the pH meter.

3.12 Replication and Statistical Analysis

The data presented are the average values (± SE) of results from three to four experiments conducted on different days. Statistical analysis of the data was done using a computerized program written in a basic language.

3.13. Solvents, Chemicals and Materials

The solutions of MJ and ABA were prepared in the ethanol. While PMA; TFP, U73122, W7, K252A, ML-7, c-PTIO, H_2DCF-DA, BCECF-AM, DAF-2DA were dissolved in DMSO. The other components were water soluble. Most of the chemicals were from SIGMA (Sigma Chemical Company, St Louis, MO, USA). MJ was the gift from Prof. O. Miersch, Inst. für Pflanzenbiochemie, Halle (Saale), Germany. Cellulase R10, Cellulase RS and Pectolyase Y-23 were from Sheishin Corporation (Tokyo, Japan), W7 and protein kinase inhibitors were from BIOMOL (Plymouth, PA, USA). H_2DCF-DA, DAF-DA, cPTIO, L-NAME, BCECF-AM was from Calbiochem (La Jolla, CA, USA). Nylon filters were purchased from Sarayu Textiles, Mumbai. All other chemicals and materials were of analytical grade and were from following companies: Sisco Research Laboratories, E-Merk (India), Spectrochem, Loba Chemie, Himedia Laboratories and Qualigens: all from Mumbai.

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Chapter 4

Different Signaling Pathways Involved During the Suppression of Stomatal Opening by Methyl Jasmonate or Abscisic Acid

Chapter 4

Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid

4.1 Introduction

Stomata are model systems to study the responses of plant cells to not only by plant hormones such as abscisic acid (ABA) or auxins or blue light signal but also several secondary messengers including calcium, G-proteins or protein kinases/protein phosphatases (Parvathi and Raghavendra, 1995, Willmer and Fricker, 1996, Assmann and Shimazaki, 1999, McAinsh et al., 2000). Abscisic acid (ABA) and methyl jasmonate (MJ) suppress stomatal opening, while cytokinins and IAA promote stomatal opening (Incoll et and Whitelam, 1977, Mansfield et al., 1990, Gehring et al., 1990, Raghavendra and Reddy, 1987).

Compared to the extensive literature on ABA effects on guard cells, studies on MJ-induced stomatal closure are quite limited. Nevertheless the effects of MJ on other aspects of plant growth and development, particularly senescence or gene expression are examined in detail (Sembdner and Parthier, 1993). Jasmonates are involved in the regulation of a multitude of processes, such as promotion/inhibition of growth, wounding in response to pathogens, elicitation of secondary product formation, promotion of senescence, tuberization and bulb formation (Sembdner and Parthier, 1993, Creelman and Mullet, 1997).

Although the exact concentration of MJ in the vicinity of guard cells is not known, the level of MJ has been shown to increase rapidly after wounding

(Creelman et al., 1992, Albrecht et al., 1993). The effects of MJ on certain aspects of plant growth and development, particularly senescence or gene expression are examined in detail (Sembdner and Parthier, 1993, Kenton et al., 1999, Hung and Kao, 1998, Seo et al., 2001). A concentration range of 5-50 μM is frequently used for these studies. However, studies on MJ-induced stomatal closure are quite limited, compared to the extensive literature on ABA effects on guard cells.

Several responses of plants to jasmonate are similar to that of ABA (Wiedhase et al., 1987). It has been suggested that endogenous jasmonates represent an integral part of signal transduction chain between stress signals and stress responses (Creelman and Mullet, 1997). Thus, ABA and MJ are both stress related signals. Jasmonate (JA)-dependent wound signaling in tobacco appears to involve kinase-dependent JA accumulation the effects of which are, in turn transduced by Ca^{2+} (Kenton et al., 1999). The induction levels of NtCDPK1 mRNA, DAHPS mRNA's and ATPase activity of Ca^{2+}-ATPase went up after treatment with MJ (Yoon et al., 1999, Starling et al., 1994, Suzuki et al., 1995).

The involvement of Ca^{2+} during signal transduction pathway in guard cell responses has been of great interest. Elevated levels of extra cellular Ca^{2+} lead to stomatal closure even in presence of high K^+ or low CO_2 (DeSilva et al., 1985, Schwartz, 1985,1988). Similarly, stomatal closure in response to darkness, or elevated CO_2 or ABA is prevented by the presence of EGTA, a Ca^{2+}-chelator (DeSilva et al., 1985, Schwartz, 1985, 1988). Ca^{2+} may act on guard cells in several ways: inhibition of inward K^+ channels, stimulation of

Cl^- efflux leading to depolarization of plasma membrane, and interaction with calmodulin (CaM) (Schroeder et al., 2001, McAinsh et al., 1997, Blatt, 2000a). The present work is undertaken to examine the mechanisms involved in the suppression of stomatal opening by MJ or ABA. Since the modulation of Ca^{2+} appears to be a common effect of ABA and MJ, experiments were designed to assess the role of different secondary messengers, which could lead to the modulation of intracellular Ca^{2+}. Stomatal opening was monitored in presence of varying Ca^{2+} or EGTA or in the presence of pharmacological compounds, which are known to affect different steps during the Ca^{2+} signaling process. The results suggest that the stomatal closure in response to ABA or MJ may be mediated through different steps of signaling pathways. The sensitivity to modulation of Ca^{2+} was much more pronounced in presence of MJ than that of ABA.

4.2 Results

MJ or ABA reduced the extent of stomatal opening in epidermal strips under white light compared to the untreated epidermal strips (control). The concentration of 10 μM of MJ or ABA showed similar responses of stomatal closing of nearly 50 % response compared to the control. The presence of Ca^{2+}, even in range of 5-20 μM levels, promoted stomatal closure induced by both ABA and MJ (Fig. 4.1). In contrast, the presence of EGTA in the incubation medium stimulated stomatal opening and relieved the stomatal closure caused by ABA or MJ (Fig. 4.2). EGTA at 5 mM was effectively reversed the closure response of MJ. The effect of EGTA was more pronounced in presence of MJ than that of ABA.

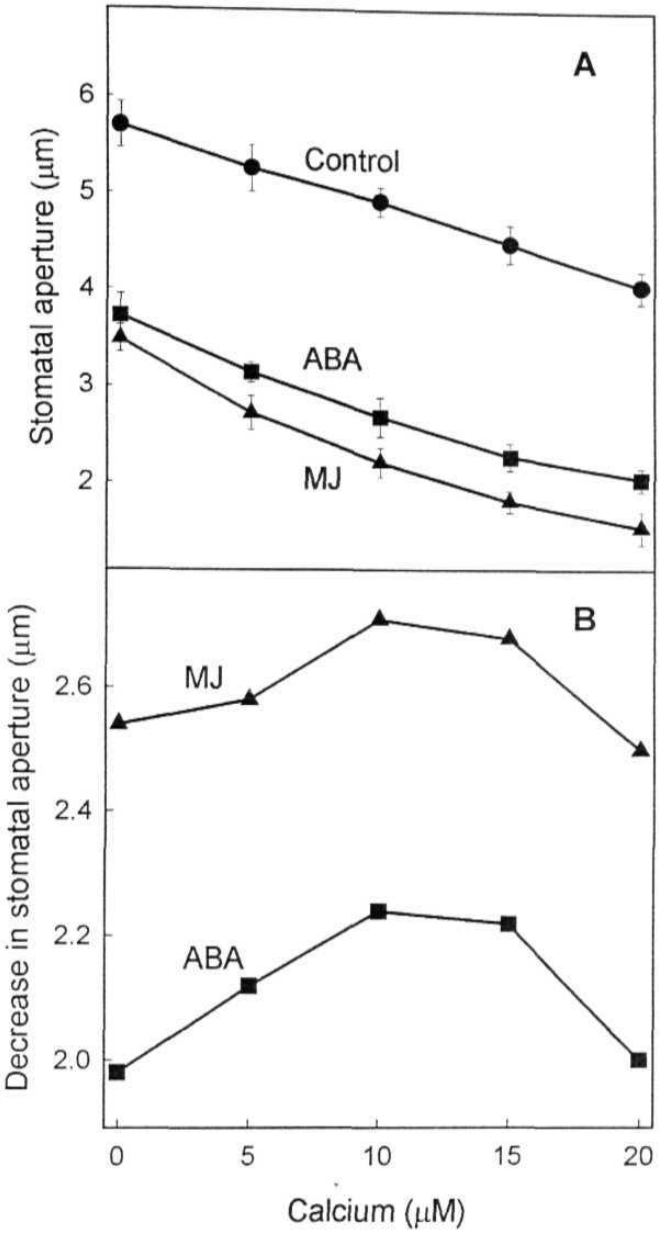

Figure 4.1: The effect of stomatal opening in epidermal strips of *Nicotiana glauca* by calcium in the absence or presence of 10 μM ABA or MJ. (A) The sensitivity of stomatal opening to range of calcium in presence of ABA or MJ under light. (B) The extent of decrease in stomatal opening by ABA or MJ responses in relation to concentration of calcium. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The pattern of inhibition was similar in presence of both ABA and MJ.

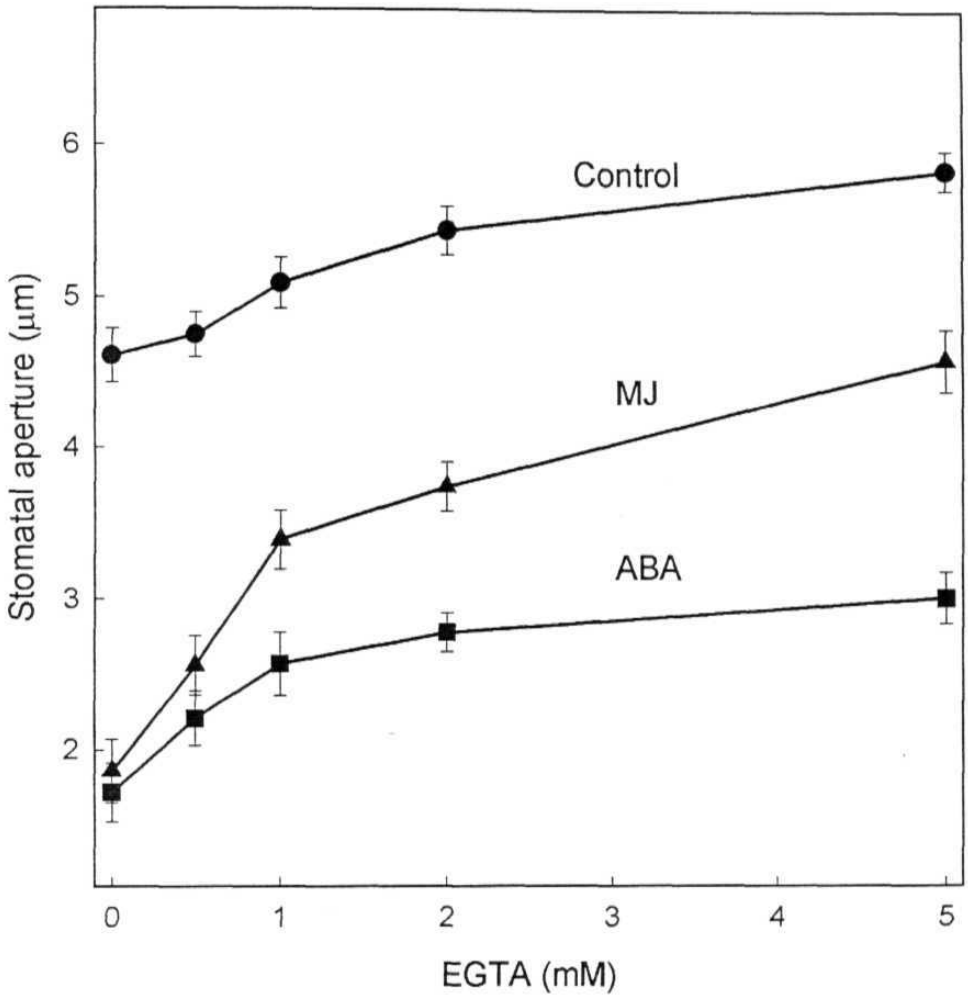

Figure 4.2: Promotion of stomatal opening in epidermal strips of *Nicotiana glauca* by EGTA (calcium chelator) in the absence or presence of 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The stimulation was more pronounced in the presence of MJ than that of ABA.

The increase in intracellular Ca^{2+} can occur due to either Ca^{2+} influx through calcium channels or release of Ca^{2+} from internal compartments, through again Ca^{2+} channels (Ca^{2+} induced calcium increase). Experiments were therefore designed to study the sensitivity of stomatal closure induced by ABA or MJ to pharmacological compounds, that are known to affect different secondary messengers. These pharmacological agents are: lanthanum/ ruthenium red/ verapamil (calcium channel blockers), TFP/ W7 (CaM antagonists), butanol/U73122 (inhibitors of phospholipase C/ phospholipase D, PLC/PLD) and PMA (stimulator of protein kinase C, PKC).

Lanthanum, a Ca^{2+} channel blocker, totally reversed the stomatal closure caused by MJ but had only limited effect in presence of ABA (Fig. 4.3). Ruthenium red and Verapamil two more Ca^{2+} channel blockers, also had similar effect: reversal of stomatal closure caused by MJ, but not that of ABA (Fig. 4.4 and 4.5). Two CaM antagonists, TFP and W7, reversed almost completely stomatal closure caused by MJ, with only marginal effect on ABA action (Fig. 4.6 and 4.7).

In a strong contrast to the effect of Ca^{2+} channel blockers, U73122 (an inhibitor of PI-PLC) reversed the stomatal closure caused by ABA, but not that of MJ (Fig. 4.8). The stomatal closure by U73122 is interesting and needs to be examined further. Similarly, 1-butanol (an inhibitor of PLD) prevented stomatal closure induced by ABA but could relieve only 50% of the stomatal closure caused by MJ (Fig. 4.9). The presence of PMA an activator of PKC almost completely relieved the stomatal closure caused by ABA. But showed much less effect on closure induced by MJ (Fig. 4.10).

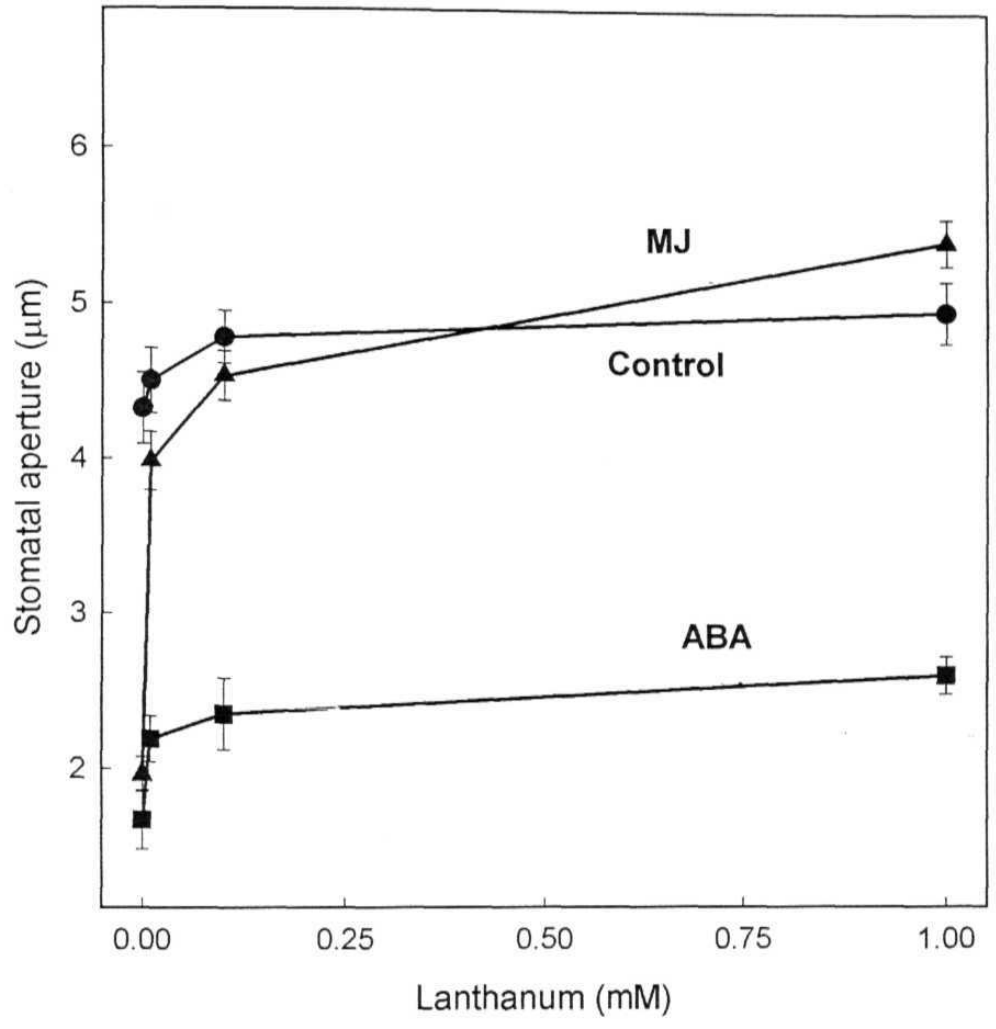

Figure 4.3: The effect of lanthanum (calcium channel blocker) on stomatal opening in the epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal by lanthanum of stomatal closure was pronounced in case of MJ but not that of ABA.

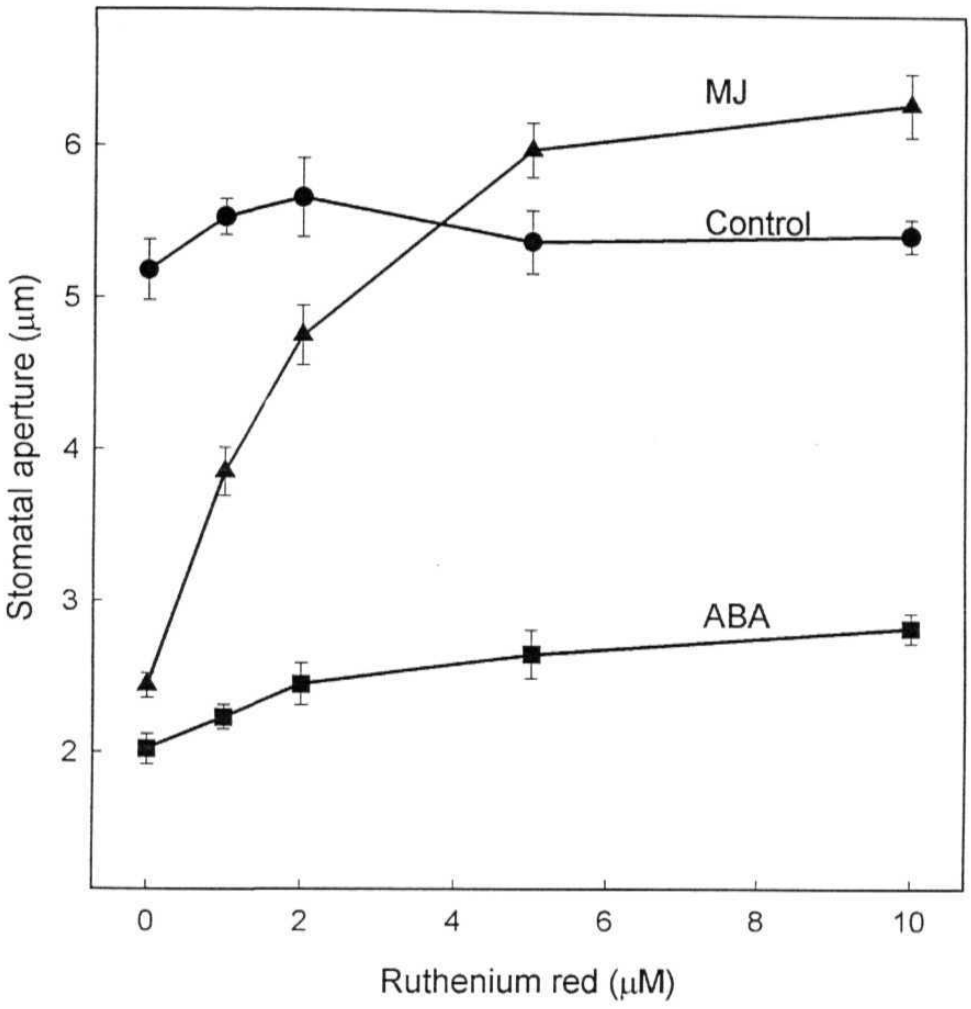

Figure 4.4: The effect of ruthenium red (calcium channel blocker) on stomatal opening in epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal by ruthenium of stomatal closure was pronounced in case of MJ but not that of ABA.

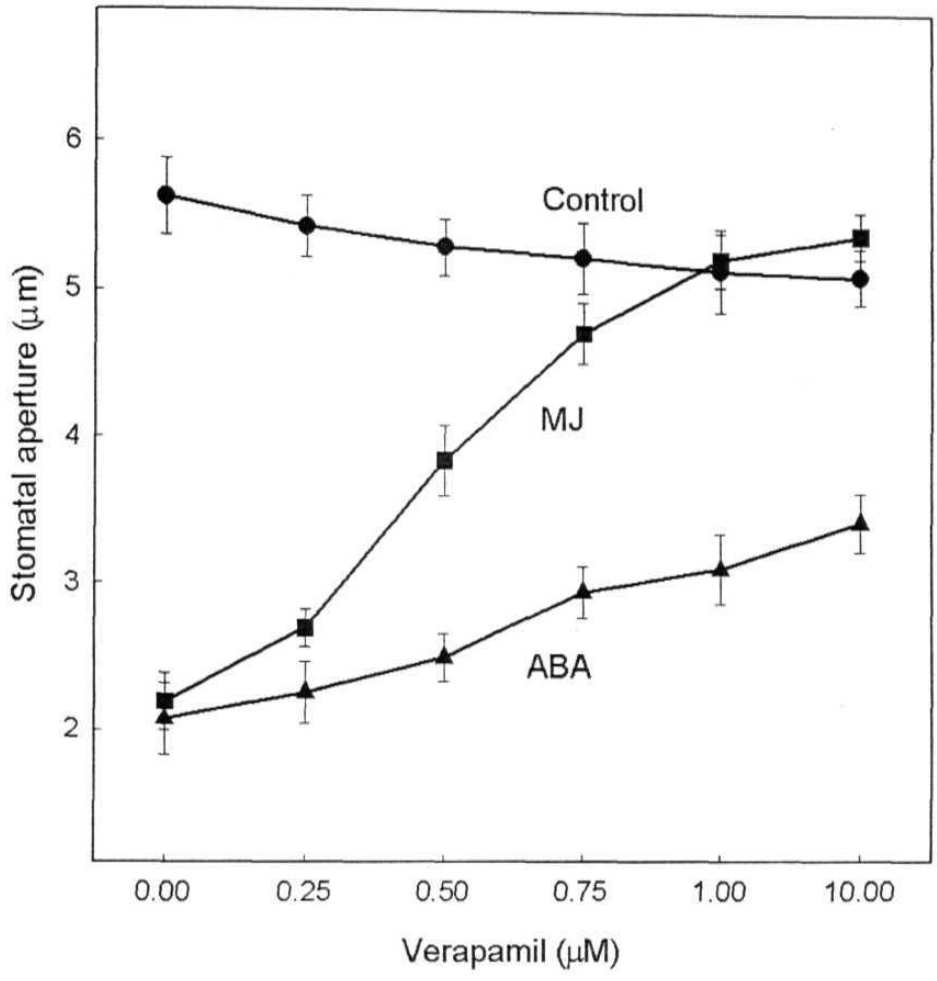

Figure 4.5: The effect of Verapamil (calcium channel blocker) on stomatal opening in epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal of stomatal closure was more pronounced in presence of MJ than that of ABA.

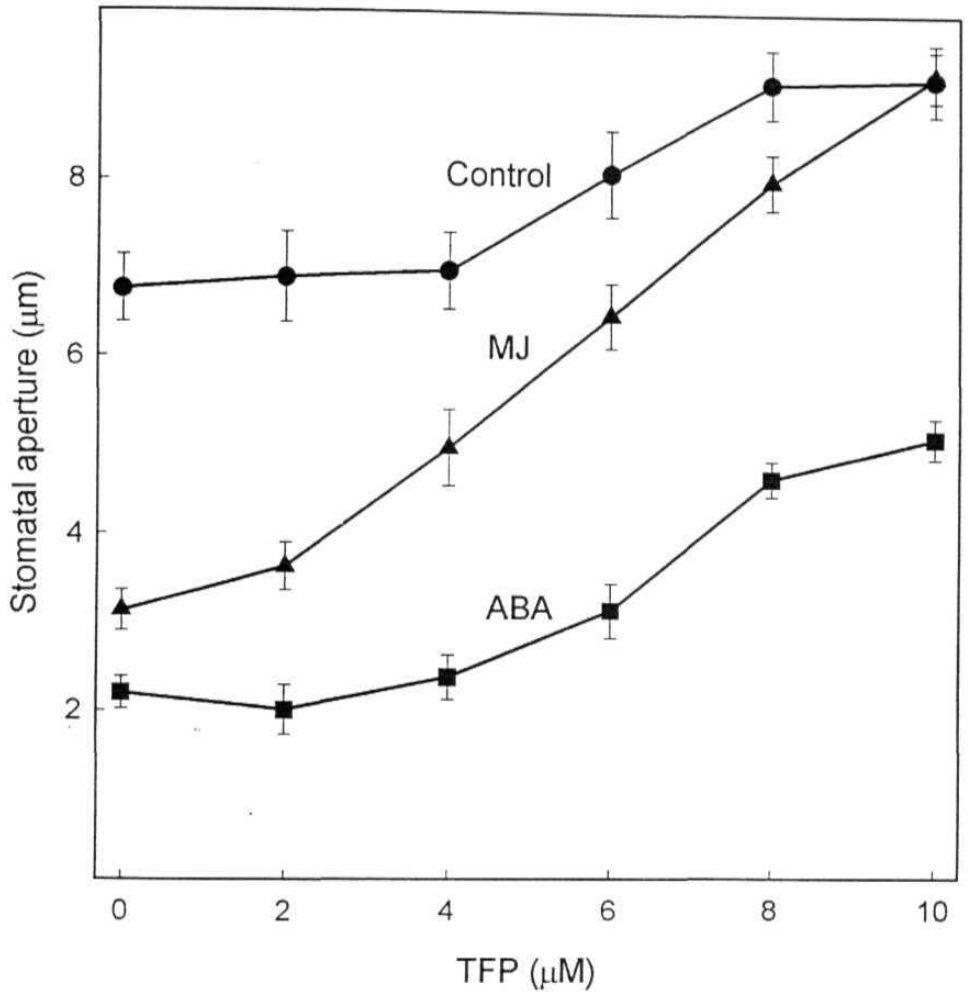

Figure 4.6: The effect of TFP (calmodulin antagonist) on stomatal opening in epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal of stomatal closure was more pronounced in presence of MJ than that of ABA.

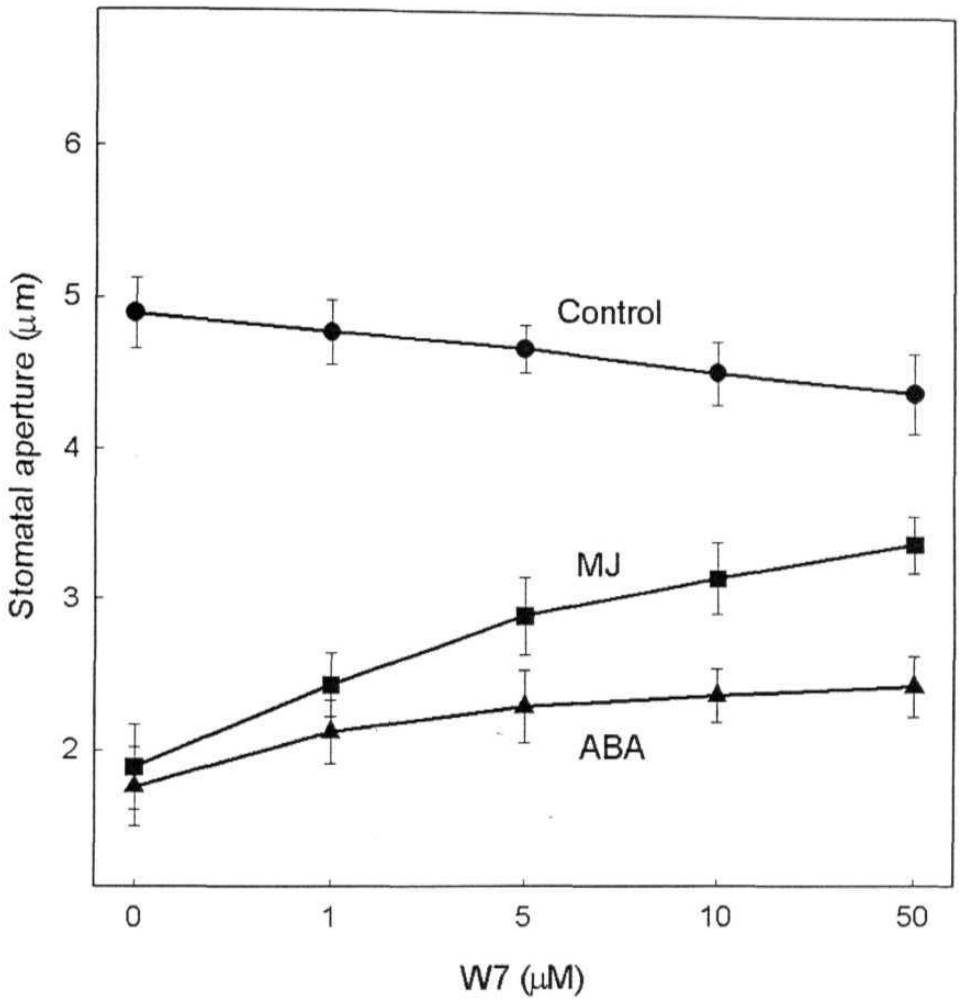

Figure 4.7: The effect of W7 (calmodulin antagonist) on stomatal opening in epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal of stomatal closure was more pronounced in presence of MJ than that of ABA.

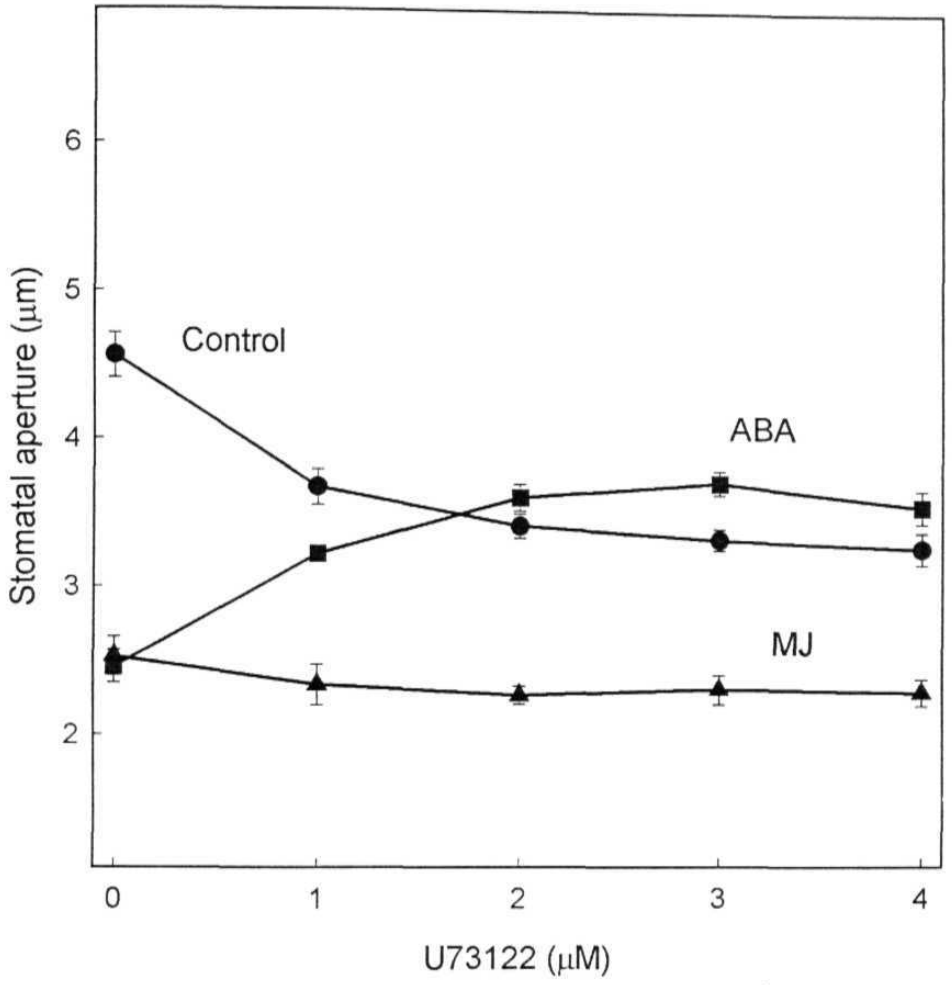

Figure 4.8: The effect of U73122 (inhibitor of phospholipase C) on stomatal opening in the epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal by U73122 of stomatal closure was more pronounced in case of ABA but not that of MJ.

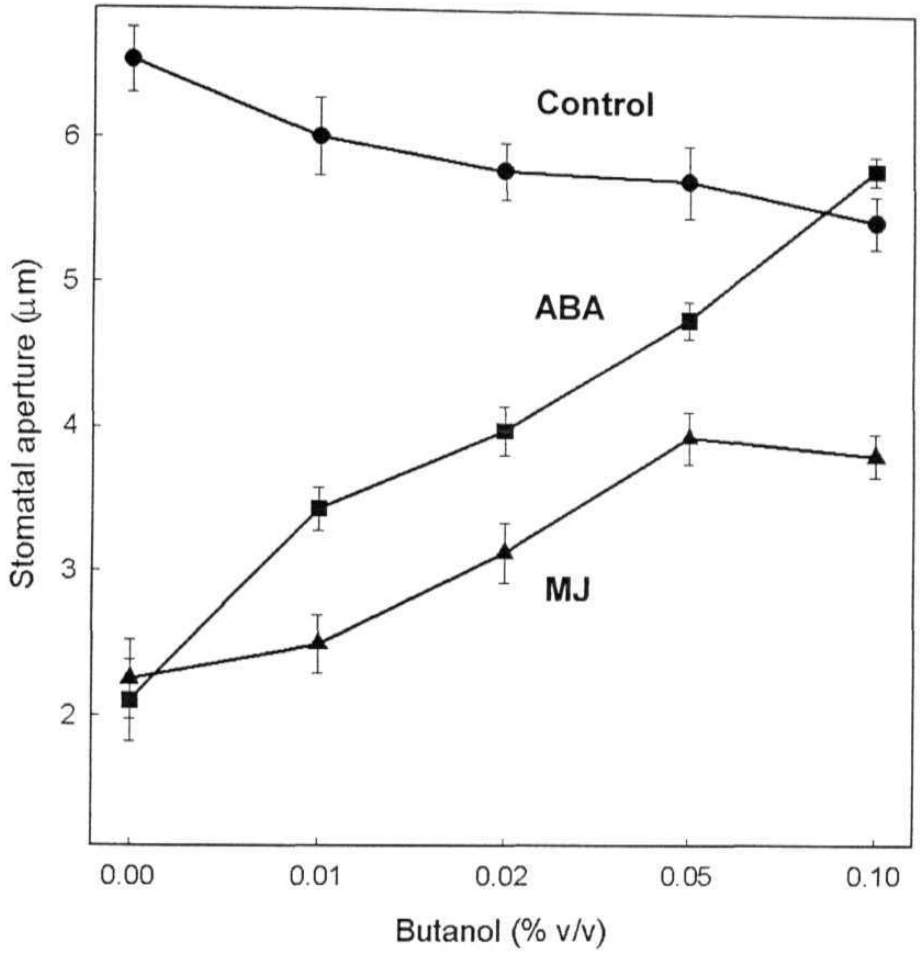

Figure 4.9: The effect of 1-butanol (inhibitor of phospholipase D) on stomatal opening in the epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal by 1-butanol of stomatal closure was more pronounced in case of ABA but not that of MJ.

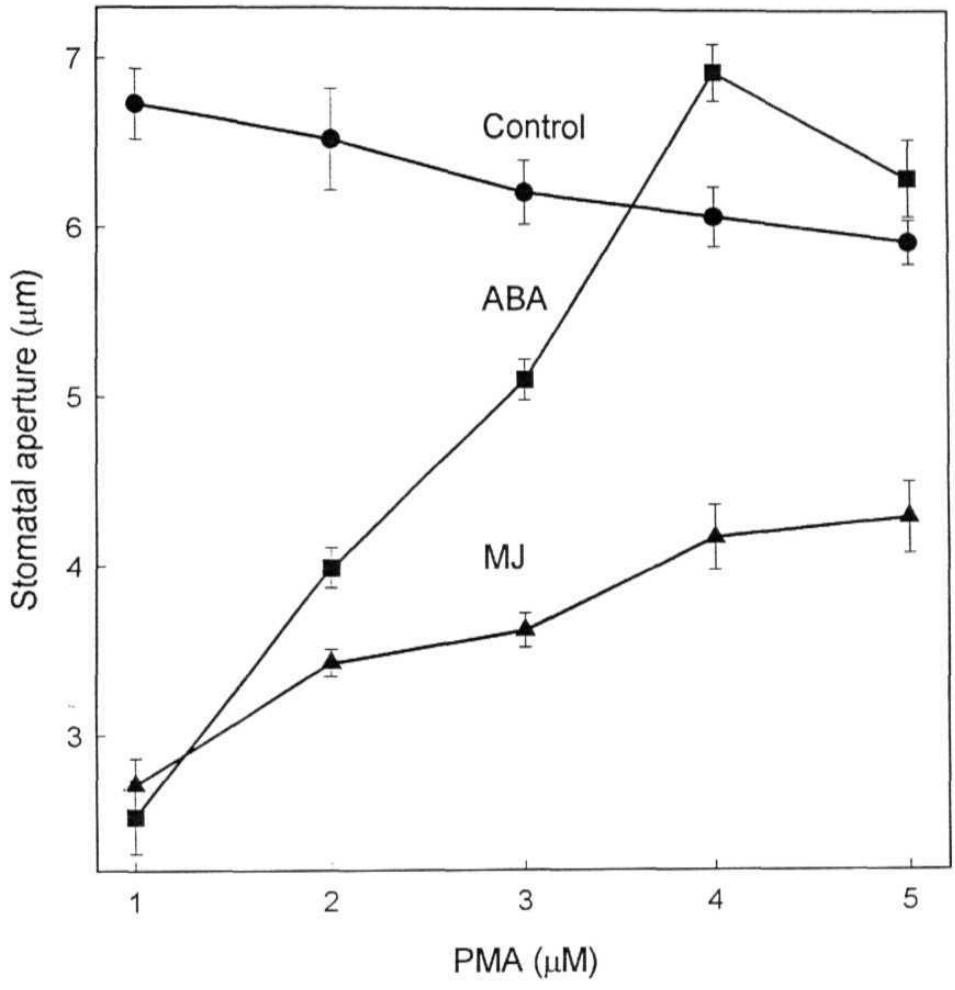

Figure 4.10: The effect of phorbol myristate acetate (PMA, activator of protein kinase C) on stomatal opening in the epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM ABA or MJ. The strips were exposed to white light (500 μmol m^{-2} s^{-1}) for 3 h. The reversal by PMA of stomatal closure was more pronounced in case of ABA but not that of MJ.

Fig. 4.11 summarizes the effects of several pharmacological reagents on stomatal aperture in presence of ABA and MJ. The concentrations in most of the cases are chosen in such a way that they have no significant effect on stomatal opening in the absence of ABA or MJ. The small decrease in stomatal opening caused by U73122 was taken into consideration, while calculating the effects in presence of ABA or MJ. Lanthanum/ verapamil/ ruthenium red (Ca^{2+} channel blockers) and TFP/ W7 (CaM antagonists) reversed the stomatal closure induced by MJ but not that of ABA. These results showed that anti-calcium or calmodulin was more efficiently playing role in MJ induced stomatal closure responses when compared to ABA. A regulation of free calcium levels in the cytosol, possibly involving CaM, is playing a role in stomatal opening under light.

Similarly, butanol/ U73122 (inhibitors of PLD/ PLC) and PMA (stimulator of PKC) relieved the stomatal closure by ABA but not that of MJ. The anti-phospholipases (PLD/ PLC) worked efficiently in ABA induced stomatal closure when considered in comparison to MJ effects on stomatal movements. However, it should be noted that MJ induced promotion of stomatal closure requires calcium as the calcium chelator, EGTA efficiently responded. The partial interferes with PLD on MJ effect showed modulated responses of phospholipases at stomatal level signaling.

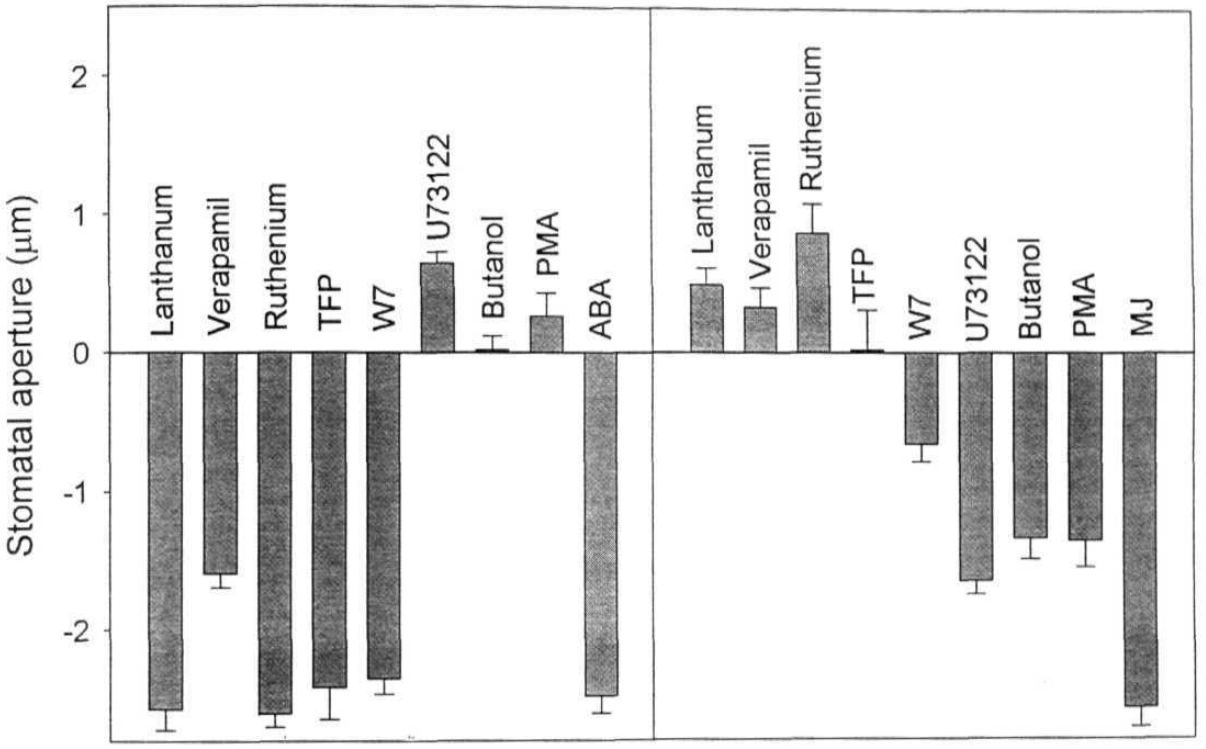

Figure 4.11: ABA (A) or MJ (B) induced stomatal closure as influenced by Ca^{2+} flux modulators, calmodulin antagonists, PLC/PLD inhibitors, and protein kinase activator. Lanthanum (1 mM), Verapamil (0.01 mM), Ruthenium (0.01 mM), TFP (0.01 mM), W7 (50 μM), U73122 (3 μM), 1-Butanol (0.1% v/v) or PMA (1.5 μM), were added to the incubation medium, containing either 10 μM ABA or MJ. The stomatal apertures were measured 3 h after illumination with white light (500 μmol m^{-2} s^{-1}). The extent of decrease (or increase) over respective control (i.e. without ABA or MJ) is represented.

4.3 Discussion

The results from our study establish that calcium is an important messenger during the stomatal closure induced by not only ABA but also MJ. However, there seems to be a difference in the mode of action by ABA or MJ. The closure induced by ABA was dependent on the functioning of PLC/PLD and PKC. In contrast, the action by MJ was strongly dependent on Ca^{2+} channels and CaM.

The importance of intracellular Ca^{2+} of guard cells in mediating stomatal closure by ABA or CO_2 or H_2O_2 has been demonstrated by several workers (McAinsh et al., 1997, Blatt 2000b, Webb and Hetherington, 1997, Assmann, 1999). Our observations of promotion by external Ca^{2+} (Fig. 4.1) and reversal by EGTA (Fig. 4.2) of stomatal closure imply that the intracellular Ca^{2+} in guard cells may increase on exposure to MJ. Strong modulation by Ca^{2+} of response to MJ has been observed in case of plant pathogen interaction and leaf senescence (Kenton et al., 1999, Hung and Kao, 1998, Leon et al., 1998, Leon et al., 2001).

The differential responses to Ca^{2+} in relation to stomatal closure by ABA or MJ could be due to differences in Ca^{2+} mobilization through Ca^{2+} channels or other processes. For example, several secondary messengers are known to be involved while responding to ABA e.g. phosphoinositides, G-proteins, PLC, Ca^{2+} channels, protein kinases including PKC or myosin light chain kinases (Assmann and Shimazaki, 1999, Schroeder et al., 2001a, Cousson and Vavasseur, 1998, Staxen et al., 1999, Lee et al., 1996, Jacob et al., 1999). The action of MJ appears to be strongly dependent on the Ca^{2+} channels as

indicated by the complete reversal of ABA effect by three Ca^{2+} channel blockers: lanthanum, ruthenium red or verapamil (Figs. 4.3, 4.4 and 4.9).

The effects of Ca^{2+} are often mediated through CaM. The importance of CaM can be assessed by using CaM antagonists such as TFP or W7 (Shimazaki et al., 1992). The reversal of stomatal closure by TFP or W7 suggests that the effects of MJ are mediated by CaM, while such dependence on CaM appears to be low in case of ABA. Further, the dependence on calcium appears to be much stronger in case of MJ than that of ABA as the presence of EGTA reversed completely the stomatal closure caused by MJ while being only partial in case of ABA (Fig. 4.2). Thus, the involvement of calcium channels and CaM was more pronounced during stomatal closure induced by MJ that that incase of ABA.

The function of PLC is to hydrolyze the membrane bound PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 then releases Ca^{2+} from intracellular sources (e.g. endoplasmic reticulum, vacuole) while DAG activates PKC. The presence of PMA, stimulator of PKC, reversed ABA-induced stomatal closure, but not that of MJ (Fig. 4.10). These observations indicate that the effects of ABA involve the modulation of PLC/PLD and PKC. In contrast, the stomatal closure caused by MJ does not seem to depend much in PLC or PKC. While the Ca^{2+} dependence of ABA or MJ action on stomata is quite logical, the strong involvement of PLC/PLD only in case of ABA action (as indicated by the effects of U73122/ Butanol and PMA) is quite interesting. The involment has been of ABA with these phospholipases has been reported (Schroeder et al., 2001a, Jacob et al., 1999).

ABA regulation of stomatal aperture is proposed to involve both Ca^{2+}-dependent and Ca^{2+} independent signal transduction pathways (Cousson and Vavasseur, 1998, Li and Assmann, 1996, Allan et al., 1994, Romano et al., 2000). Elevation of cytosolic Ca^{2+} in guard cell possibly by the increase in IP_3 and subsequent Ca^{2+} release from intracellular organelles is the initial mechanism of ABA induced stomatal closure (McAinsh et al., 1992, Lee et al., 1996, McAinsh et al., 1990, Schroeder and Hagiwara, 1990). However, Gilroy et al. 1991 found that ABA induced elevation of cytosolic Ca^{2+} occurred in only some guard cells, but not all.

Although the changes in cytosolic Ca^{2+} levels were small or undetectable, ABA induced stomatal closure could still occur. Further, the low temperature induced stomatal closure in the epidermis of *Commelina communis* is due to an increase in apoplastic calcium and does not involve ABA (Wilkinson et al., 2001). Thus, the regulation by ABA of stomatal aperture may involve both Ca^{2+} -dependent and -independent signal transduction pathways. Some of the Ca^{2+} -independent signaling elements that could mediate the ABA responses are cytosolic pH, PLC/PLD, PKC and protein phosphatases (Jacob et al., 1999, Li and Assmann, 1996, Grabov and Blatt, 1997, Leung and Giraudat, 1998).

The results are summarized and integrated into a schematic representation of signaling components, which respond to ABA or MJ (Fig 4.12). As per the present results, Ca^{2+} plays a vital role that mediates the effects of both ABA/MJ during stomatal closure. However, the regulation by ABA of stomatal movement appears to involve both calcium-dependent and

calcium-independent pathways. The Ca^{2+}-dependence of MJ action appears to be much greater than that in case of ABA, as indicated by the effects of EGTA. Moreover MJ-induced stomatal closure requires the presence of external calcium and involves a calmodulin-like domain. The PLC / PLD / PKC are key elements in the ABA signalling pathway involving towards increasing cytoplasmic calcium levels. In summary ABA and MJ signaling pathways converge at Ca^{2+}, there was difference in perception sites that mediate the cytoplasmic calcium waves. These results show that both ABA and MJ signaling pathways with differences in their actions converge at the accumulation of calcium with in the guard cells.

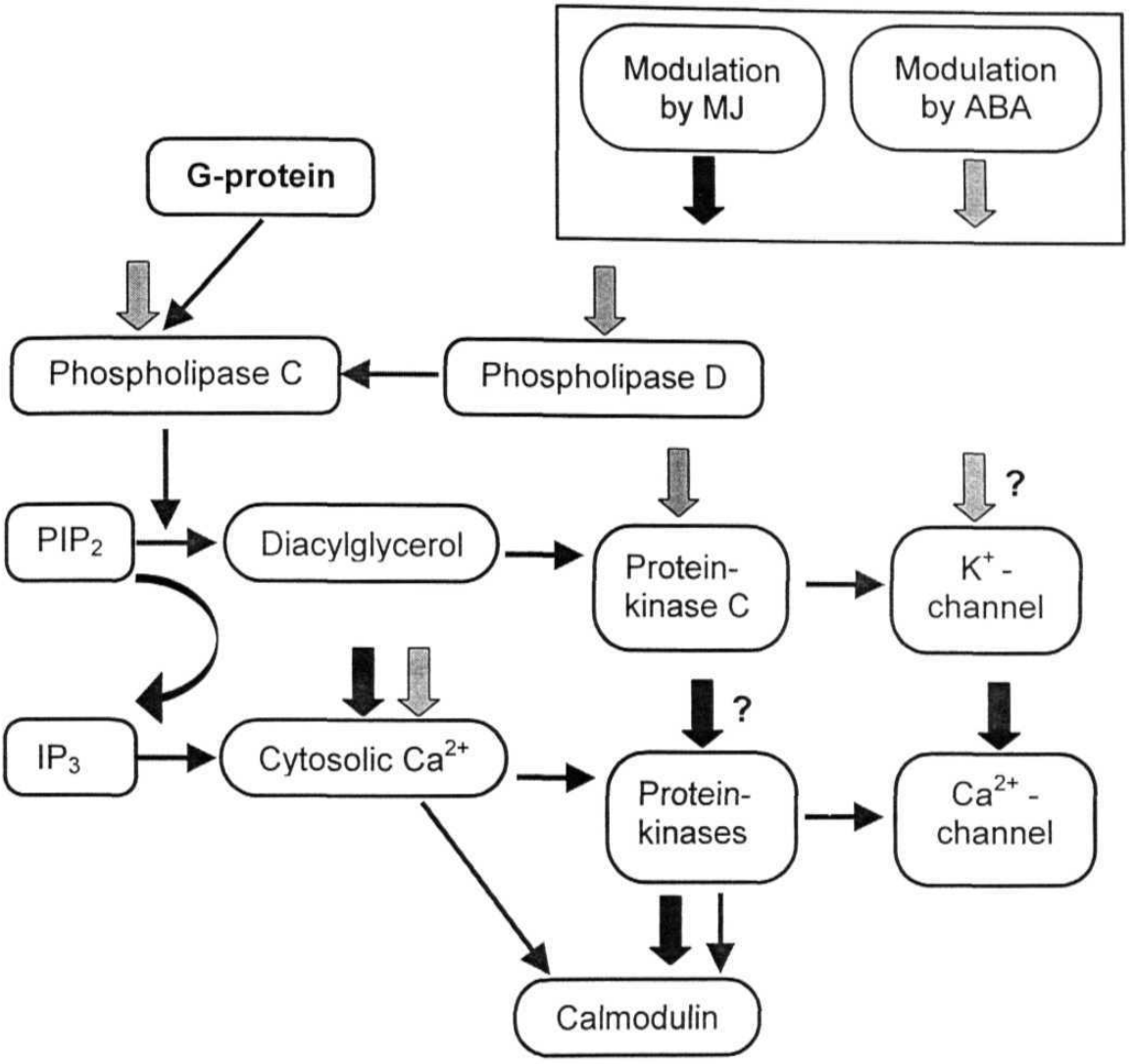

Figure 4.12: A hypothetic diagram of secondary messengers involved in the regulation of stomatal movement and their modulation by ABA or MJ. The calcium independent effects of ABA could be on potassium channels, while the calcium channels appear to be sensitive to MJ. PIP_2: Phosphatidylinositol 4,5-bisphosphate; IP_3: Inositol 1,4,5-triphosphate.

4.4 Conclusion

1. Ca^{2+} is a common signalling component during MJ/ABA mediated stomatal closure. External calcium promoted, while EGTA prevented the process of stomatal closure, in case of both ABA and MJ.
2. The Ca^{2+} dependence of MJ action appears to be much greater than that in case of ABA as indicated by the effects of EGTA.
3. Lanthanum, verapamil, ruthenium red (Ca^{2+} channel blockers), trifluoperazine and W7 (calmodulin antagonists) prevented MJ induced stomatal closure but had no such effect on ABA mediated stomatal closure. Although the primary actions of ABA and MJ appear to be different: the targets being Ca^{2+} channels in case of MJ, and PLC/ PLD/ PKC in case of ABA, the signal transduction pathways are converging at levels of Ca^{2+}.
4. ABA and MJ could be useful tools to analyze the sequence and pattern of secondary messengers during signal transduction in stomatal guard cells.

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Chapter 5

Cytoplasmic Alkalization Precedes Reactive Oxygen Species Production During Methyl Jasmonate- and Abscisic Acid-Induced Stomatal Closure

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5.1 Introduction

Methyl jasmonate, a linolenic acid derivative, is involved in plant development and defence and is overproduced during wounding, fruit ripening and drought stress (Creelman and Mullet, 1997). MJ affects plant transpiration (Lee et al., 1996, Wang, 1999) by promoting stomatal closure (Raghavendra and Reddy, 1987, Gehring et al., 1997, Suhita et al., 2003). MJ-induced stomatal closure is accompanied by an alkalization of the guard cell cytoplasm in *Paphiopedilum* spp. (Gehring et al., 1997). A recent study has shown that this response to MJ requires external calcium and involves a calmodulin-like domain (Suhita et al., 2003). Interestingly, Evans (2003) demonstrated that MJ activates the outward potassium channel from guard cell protoplast of *Vicia faba*, the main conductance allowing K^+ efflux and loss of turgor. All these steps, involved in stomatal closure, are also key elements in the ABA signaling pathways, such as cytoplasmic calcium waves (Allen et al., 2000), protein (de)phosphorylation (Leung et al., 1994, 1997, Meyer et al., 1994, Merlot et al., 2001, Li et al., 2000, Kwak et al., 2002, Mustilli et al., 2002), cytoplasmic pH modification (Irving et al., 1992) and modulation of potassium channels at the guard cell plasma membrane (Armstrong et al., 1995).

In addition, reactive oxygen species (ROS) have been recently identified as essential second messengers in ABA signaling (Guan et al., 2000, Pei et al., 2000, Zhang et al., 2001a). In guard cells, ABA induces ROS production,

which in turn activates Ca^{2+} channels at the plasma membrane (Pei et al., 2000, Murata et al., 2001). Cytosolic Ca^{2+} elevation activates slow anion channels triggering cell depolarisation and inactivates inward rectifying K^+ channels, leading to ion efflux and turgor reduction. Interestingly, MJ together with various elicitors also induces an accumulation of H_2O_2 in leaves, (Orozco-Cardenas and Ryan, 1999). Thus, it is likely that ABA and MJ transduction pathways leading to stomatal closure involve overlapping signaling elements. Such interaction has already been suggested by Herde et al., (1997) who observed that ABA deficient mutants were insensitive to jasmonic acid in reducing the transpiration stream. Additionally, the jasmonate-insensitive mutant *jar1*-1 shows increased sensitivity to ABA inhibition of germination (Staswick et al., 1992). All together, these observations suggest the existence of a crosstalk between MJ and ABA signaling cascades. However, it remains largely unknown which molecular components are shared by ABA and MJ signal transduction.

In the present study, cytoplasmic pH changes and ROS production in response to ABA or MJ were studied in guard cells of *A. thaliana*. Additionally, mutant plants affected in ABA signaling (*ost1*-2, Mustilli et al., 2002), MJ signaling (*jar1*-1, Staswick et al., 1992, 2002), plasma membrane catalytic subunits of the plasma membrane NAD(P)H oxidases (*atrbohD/F*; Kwak et al., 2003) or guard cell outward K^+ channel (*gork1*, Hosy et al., 2003) were used to assess the respective roles of these genes in ABA or MJ signaling pathways leading to stomatal closure.

5.2 Results

5.2.1 Jar1-*1 mutants are insensitive to MJ but not to ABA while* ost1-*2 mutants are insensitive to ABA but not to MJ*

The *jar1*-1 MJ insensitive mutant has been isolated on the basis of a diminished sensitivity of root growth to MJ (Staswick et al., 1992). The *jar1*-1 mutation affects the biochemical capability of JAR1 in the adenylation of jasmonic acid (Staswick et al., 2002). The *ost1*-2 mutant has been isolated using infrared thermography and characterised as ABA insensitive at the stomatal level (Mustilli et al., 2002). OST1 is an ABA-activated protein kinase, an ortholog of the *Vicia faba* Ca^{2+}-independent ABA-activated protein kinase (AAPK, Li et al., 2000). The *ost1*-2 mutation (Gly-33 to Arg) affects an invariant residue required for ATP-binding, and is thus predicted to abolish OST1 kinase activity (Mustilli et al., 2002).

Figure 5.1 presents the stomatal sensitivity to ABA and MJ in wild type plants, *jar1*-1 and *ost1*-2 mutant plants. Dose-response curves for MJ and ABA were quite similar in wild type plants (Fig. 5.1A), with a 50% effect observed at around 5 µM. In the case of the *jar1*-1 mutant (Fig. 5.1B), stomata did not respond to MJ while a residual response to ABA was still observed, 28% of stomatal closure observed in wild type plant at 20 µM ABA. As previously described (Mustilli et al., 2002), stomata from *ost1*-2 mutant plants were insensitive to ABA (Fig. 5.1C).

However, they were still able to close in response to MJ, with a diminished sensitivity compared to wild type plants, 60% of stomatal closure observed in wild type plant at 20 µM MJ. These results demonstrate that JAR1

and OST1 are not absolutely required in a common ABA and MJ signaling pathway leading to stomatal closure. However the diminished response of *ost1*-2 to MJ and *jar1*-1 to ABA suggests a cross talk between two signaling pathways through interacting elements.

Further it was ascertained that the 3 ecotypes of Arabidopsis used in this study have similar responses to ABA and MJ at the stomatal level (Table 5.1).

5.2.2 Protein kinases are essential elements in stomatal closure by ABA and MJ

Protein (de)phosphorylation events play important roles in ABA signaling in guard cell (Li et al., 2000, Merlot et al., 2001, Kwak et al., 2002, Mustilli et al., 2002). Three compounds, K252A (broad range protein kinase inhibitor; Kase et al., 1987), ML7 (Ca^{2+}-calmodulin protein kinase inhibitor; Saitoh et al., 1987, Hidaka and Kobayashi, 1999) and W7 (calmodulin I nhibitor; Yorio et al., 1985), were compared in their abilities to inhibit ABA- or MJ-induced stomatal closure (Fig. 5.2). K252A was found to abolish the ABA and MJ-induced stomatal closure (Fig. 5.2B). In contrast, ML7 and W7 were able to suppress the response to MJ but only partly effective against ABA (Fig. 5.2 C, D).

These results strongly suggest that at least one protein kinase, regulated by calcium and a calmodulin-like domain is involved in stomatal response to MJ. The results also suggest that the ABA signaling cascade involved Ca^{2+}-dependent and Ca^{2+}-independent protein kinases as previously suggested (Allan et al., 1994, MacRobbie, 1998).

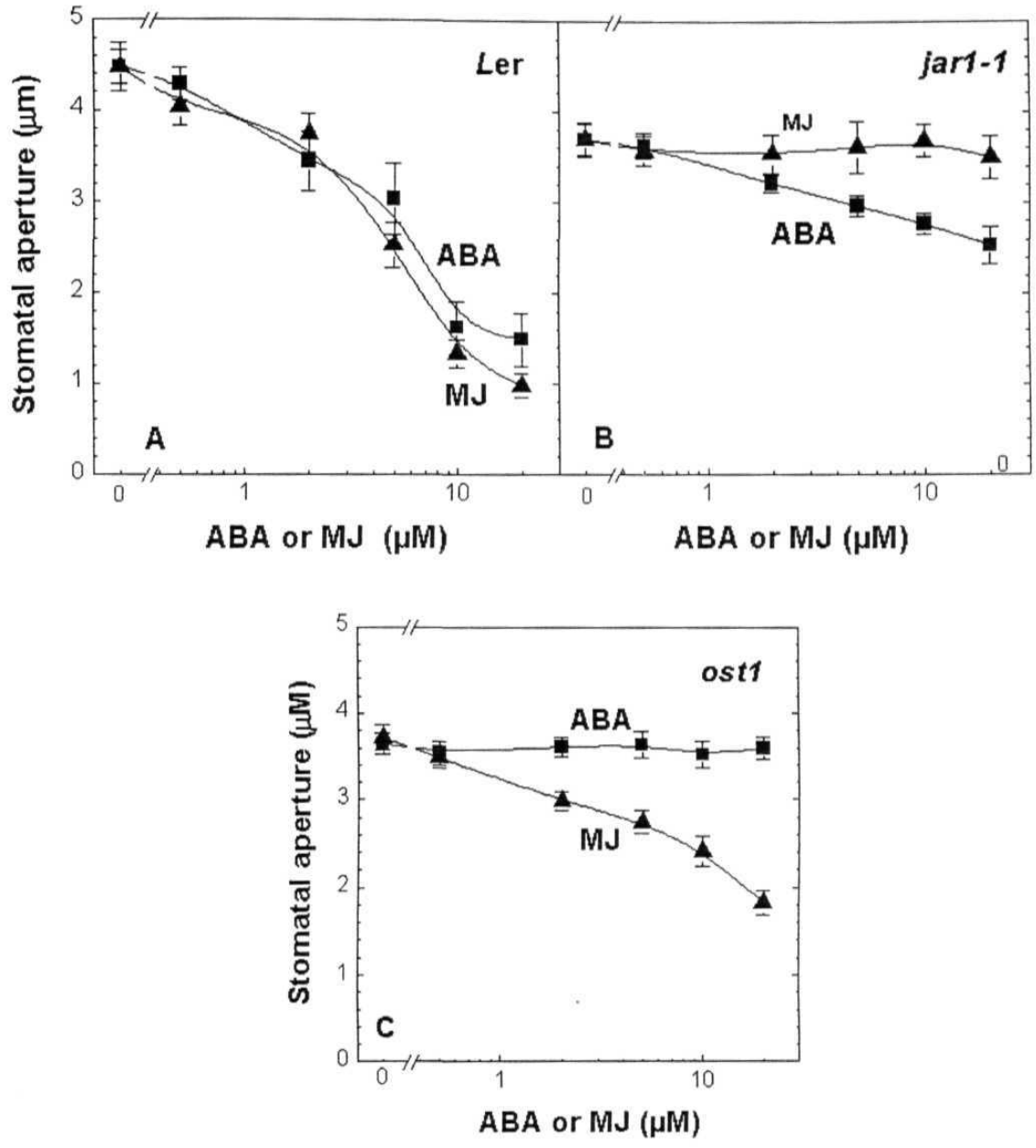

Figure 5.1: Dose-response curves of ABA- (squares) and MJ- (circles) induced stomatal closure in Landsberg *erecta* (A), *jar1*-1 mutant (B) and *ost1*-2 (C) mutant plants of *Arabidopsis*, respectively. The stomata of abaxial epidermis from leaves were allowed to open in light for 2 h, then ABA or MJ was applied for 2 h. Results are the averages ± SEM (n=60) from at least 3 independent experiments.

Table 5.1: The stomatal opening was measured as described in the Materials and Methods, in the absence or presence of 20 μM ABA or MJ. Results are averages ± SE of 3 to 4 independent experiments.

Ecotype	Hormone (20 μM)		
	Control	ABA	MJ
	Stomatal aperture (μm)		
Landsberg	3.9 ± 0.24	1.1 ± 0.21	1.2 ± 0.23
Columbia	3.8 ± 0.17	0.8 ± 0.25	1.0 ± 0.19
Wassilevskija	4.3 ± 0.21	1.2 ± 0.29	0.9 ± 0.13

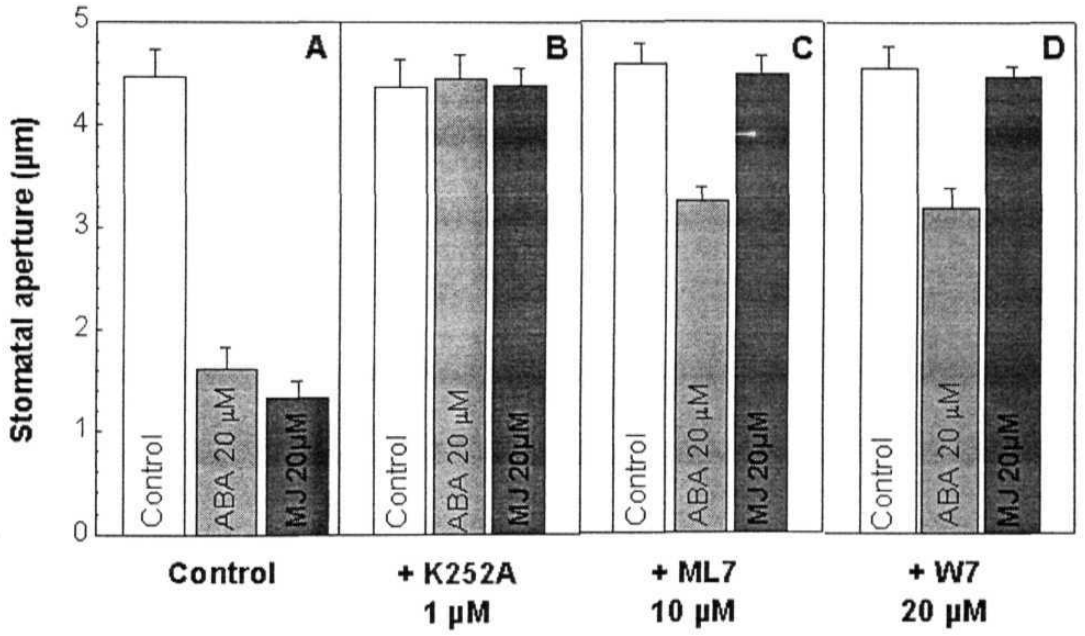

Figure 5.2: ABA or MJ induced stomatal closing (A) in the presence of the protein kinases inhibitors K252a (B), ML7 (C) and the calmodulin antagonist W7 (D). Stomata of leaf epidermis were allowed to open in light for 2 h, and then incubated for 2 h in ABA or MJ. K252a, ML7, W7, were added 30 min before the addition of ABA or MJ.

5.2.3 JAR1 and OST1 are located upstream of ABA and MJ-induced H_2O_2 production through an NAD(P)H oxidase

ROS production in guard cells is induced by not only ABA (Pei et al., 2000, Murata et al., 2001) but also by chitosan, an elicitor of defence reactions (Lee et al., 1999) or in leaves by an exposure to MJ vapours (Orozco-Cardenas and Ryan, 1999). Analysis of ROS levels in guard cells using the fluorescent dye 2',7'-dichlorofluorescein diacetate (H_2DCF-DA)confirmed that ROS production was significantly stimulated after a treatment with ABA and unravelled an even greater stimulation by MJ in wild type plants (Table 5.2).

In the presence of ABA or MJ, ROS production developed in guard cell during the first 30 min of treatment and then plateaued for at least 30 min (Fig. 5.3B). Interestingly, MJ did not increase ROS levels in *jar1*-1 guard cells while ABA had no effect on ROS level in *ost1*-2 guard cells (Table 5.2). Additionally, ROS production and stomatal closure triggered by ABA were slightly impaired in *jar1*-1, identical results were obtained when *ost1*-2 mutants were submitted to MJ.

However, externally applied H_2O_2 elicited the same degree of stomatal closure in *jar1*-1 (55% decrease over control, with out H_2O_2) and *ost1*-2 (60% decrease over control) as in wild type plants (53% over control), suggesting that OST1 and JAR1 are placed upstream of ROS production in ABA or MJ signaling.

Pei et al. (2000) reported that diphenyl iodonium (DPI) could partially prevented ABA-induced stomatal closure and proposed that DPI could limit H_2O_2 production in guard cells. To test that the fluorescence observed in guard

cells was the result of the product of an NAD(P)H oxidase, DPI was supplied to the incubation medium for 30 min before treating the epidermal strips with ABA or MJ. In the presence of 12.5 µM DPI, ROS production was indeed restricted even after a treatment with 20 µM ABA or MJ (Table 5.3).

In parallel stomatal closure by ABA and MJ was also prevented. These results indicate that ROS production by an NAD(P)H oxidase (Auh and Murphy, 1995) or another flavoenzyme (O'Donnell et al., 1993) is a key elelment in the MJ signaling pathway as already shown for ABA (Pei et al., 2000).

A recent work has shown that a double mutant of two isoforms of catalytic subunits of plasma membrane NAD(P)H oxidases (*atrbohD/F*) led to a diminished ABA sensitivity at the stomatal level (Kwak et al., 2003). Interestingly, stomatal response to MJ was almost completely abolished in this double mutant (Fig. 5.3A), underlining the importance of NADPH oxidases not only in ABA- but also in MJ-induced stomatal closure.

5.2.4 Alkalization of pH_{Cyt} is necessary and precedes ROS production in response to ABA and MJ

A modification of guard cell cytoplasmic pH is essential in ABA-induced stomatal closure (Irving et al., 1992, Blatt and Armstrong, 1993). We have therefore studied the response of the cytoplasmic pH to ABA and MJ. The null point method, first developed to measure cytoplasmic pH ($[pH]_{Cyt}$) in animal cells, has been shown to be valuable in measuring $[pH]_{Cyt}$ changes induced by MJ in barley aleurone protoplast (van der Veen et al., 1992). The basic principle of this method is that when extracellular solution pH ($[pH]_{Ext}$) is equal

to $[pH]_{Cyt}$ and buffering capacity is low, a selective disruption of the plasma membrane by digitonin will not affect $[pH]_{Ext}$. We used this method to evaluate the $[pH]_{Cyt}$ change of guard cell protoplasts (GCPs) in response to MJ or ABA. In preliminary experiments, very little change of $[pH]_{Ext}$ was observed when GCPs were treated with digitonin at $[pH]_{Ext}$ 7.35 ± 0.03 (Fig. 5.4A). Then, two types of experiments were performed. In a first set $[pH]_{Ext}$ was fixed to 7.35 ± 0.03 using KOH, then changes in pH of external solution, after addition of digitonn to MJ- or ABA- treated GCPs were recorded (Fig. 5.4).

In a second batch of experiments, the precise values of $[pH]_{Cyt}$ in presence of ABA or MJ were estimated by changing the external pH by 0.1 unit to determine the null-point value. Based on triplicate measurements, the estimated $[pH]_{Cyt}$ for untreated guard cell protoplasts was 7.33 ± 0.04 ($[pH]_{Cyt}$ ± SE), and 7.47 ± 0.02 or 7.68 ± 0.02 after a 30 min treatment with 20 μM ABA or MJ, respectively. In the presence of MJ or ABA an alteration of the cytoplasmic pH took place in 15 min and then slowly decreased (Fig. 5.5A). During the first 30 min of treatment ABA or MJ induced a ROS production increase in guard cells, which then plateaued for at least 30 min (Fig. 5.5B).

Technically it is difficult to use the null-point method for observing pH changes within 15 min. The pattern was therefore cross-checked with another species, *Pisum sativum*, using short durations of incubations and monitoring pH with an fluorescent dye, BCECF-AM. These results again indicate that the rise in pH of guard cells started within 5 min and peaked by 15 min, while the ROS production started only after 10 min and reached maximum by 30 min (Fig. 5.6). Interestingly MJ triggered a stronger response

than ABA despite kinetics were similar. These results are in accordance with the results obtained by Gehring et al. (1997) using *Paphiopedilum* spp. guard cells and the fluorescent dye BCECF. The controls without ABA or MJ showed no significant change in pH (Fig. 5.6A)

Addition of the weak acid butyrate (0.5 mM), which causes an acidification of cytoplasm (Blatt and Thiel, 1994), limited stomatal closure caused by ABA or MJ in wild-type plants as well as in *jar*-1 and *ost*-1 mutants of *Arabidopsis* (Table 5.4). Butyrate also diminished ABA and MJ-induction of ROS production (Table 5.5, Fig. 5.7). Butyrate at 0.5 mM did not significantly affect the rates of either photosynthesis (122 μmol mg chl^{-1} h^{-1} in the absence and 120 μmol mg chl^{-1} h^{-1} in the presence of butyrate) or respiration (9 μmol mg chl^{-1} h^{-1} and 10 μmol mg chl^{-1} h^{-1}, ± butyrate, respectively). Thus, butyrate at the concentration used in our experiments did not influence the metabolism of guard cells. These results together with the kinetics of ROS production and pH changes (Fig. 5.5 and 5.6), gave strong indications that pH changes preceded ROS production during stomatal closure induced by MJ and ABA. Moreover, preincubation of GCPs with 1 μM K252A also led to the suppression of the pH change (Table 5.5), suggesting that a protein kinase is involved upstream of pH change in ABA and MJ signaling cascades.

Confocal microscopy was also used to analyse the reactive oxygen species fluorescence in *Arabidopsis* (Fig. 5.8).

Table 5.2: Influence of *jar1*-1, *ost1*-2 and *gork1* mutations on H_2O_2 production and stomatal closure in response to 20 µM MJ or ABA. Changes in ROS levels were analysed by measuring H_2DCF-DA fluorescence levels in guard cells in response to a 30-min treatment with ABA, MJ or solvent control addition. To determine the consequence of mutations on stomatal closure, leaf epidermis were allowed to open in light for 2 h, then ABA or MJ was applied for 2 h. Results are the averages ± SE (n=60) of 3 to 4 independent experiments. The extents of H_2O_2 production in the guard cells of wild-type plants, without MJ or ABA, are taken as 100%.

Hormone	Ler		*jar1*-1		*ost1*-2		*gork1*	
	H_2O_2 (%)	SA* (*µm*)	H_2O_2 (%	SA (*µm*)	H_2O_2 (%)	SA (*µm*)	H_2O_2 (%)	SA (*µm*)
Control	100 ± 3.1	4.5 ± 0.27	94 ± 3.8	3.7± 0.17	99 ± 2.7	3.7 ± 0.12	101 ± 3.5	4.5 ± 0.2
MJ	128 ± 2.5	1.3 ± 0.16	98 ± 3.0	3.5 ± 0.26	113 ± 3.5	2.4 ± 0.17	127 ± 3.5	4.2 ± 0.1
ABA	122 ± 3.1	1.6 ± 0.29	116 ± 2.8	2.5 ± 0.20	99.3 ± 3.7	3.6 ± 0.13	121 ± 3.7	2.7 ± 0.2

*SA, stomatal apertures.

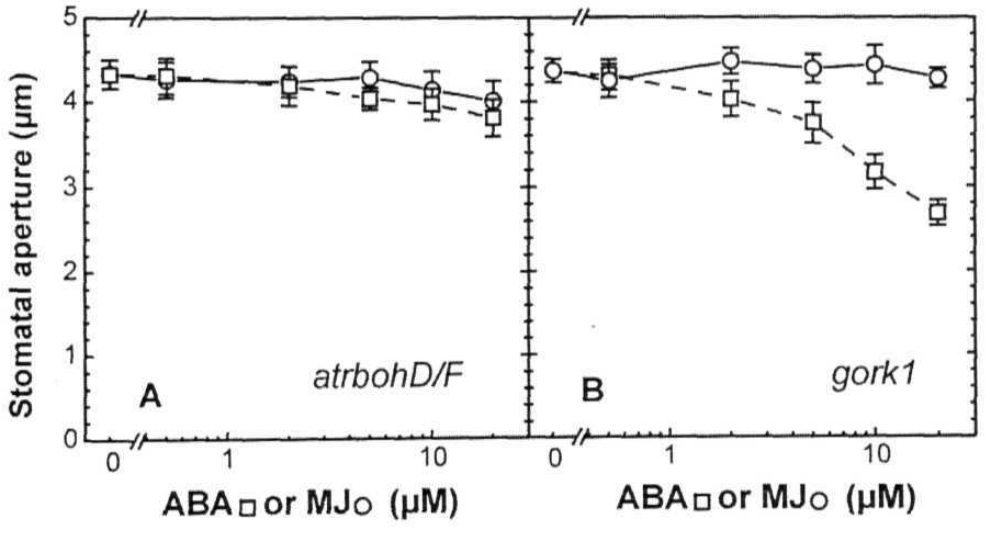

Figure 5.3: Dose-response curves of ABA-induced (squares) and MJ-induced (circles) stomatal closure in (A) *atrbohD/F* double mutant plants and (B) *gork1* mutant plants. Stomata of leaf epidermis were allowed to open for 2h under light, then ABA or MJ was applied for 2 h. Results are the average ± SE (n=60) of 3 to 4 independent experiments.

Table 5.3: The ROS production was monitored by using fluorescence dye, H_2DCF-DA as described in materials and methods. The extent of ROS production in guard cells, without 20 μM MJ or 20 μM ABA or 12.5 μM DPI in wild type was taken as control and 100%. Results are averages of (± SE) from at least 3 experiments.

	None		ABA		MJ	
Plant	No DPI	DPI	No DPI	DPI	No DPI	DPI
Wild type	100 ± 1.9	103 ± 2.5	121 ± 2.2	104 ± 2.1	126 ± 3.5	102 ± 3.2
Jar1-1	96 ± 2.9	105 ± 3.2	112 ± 3.5	106 ± 3.2	97 ± 3.4	103 ± 3.1
ost1-2	99 ± 2.8	101 ± 3.0	99 ± 3.1	98 ± 3.0	113 ± 2.8	97 ± 3.6
gork1	101 ± 3.1	102 ± 2.9	120 ± 2.7	103 ± 3.3	127 ± 3.7	106 ± 2.9

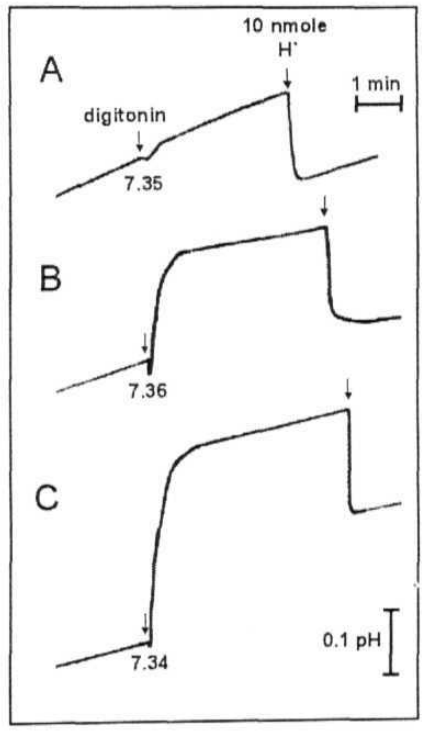

Figure 5.4: Change in the pH of the external solution after permeation of the guard cell plasma membrane by digitonin in control protoplasts (A), ABA-treated protoplasts (B) or MJ-treated protoplasts (C). Guard cell protoplasts were incubated in the presence of 20 μM ABA or MJ for 30 min before application of digitonin. Values indicate the external solution pH at time of application of digitonin, 10 nmoles of HCl were injected into the medium at the end of each experiment for calibration.

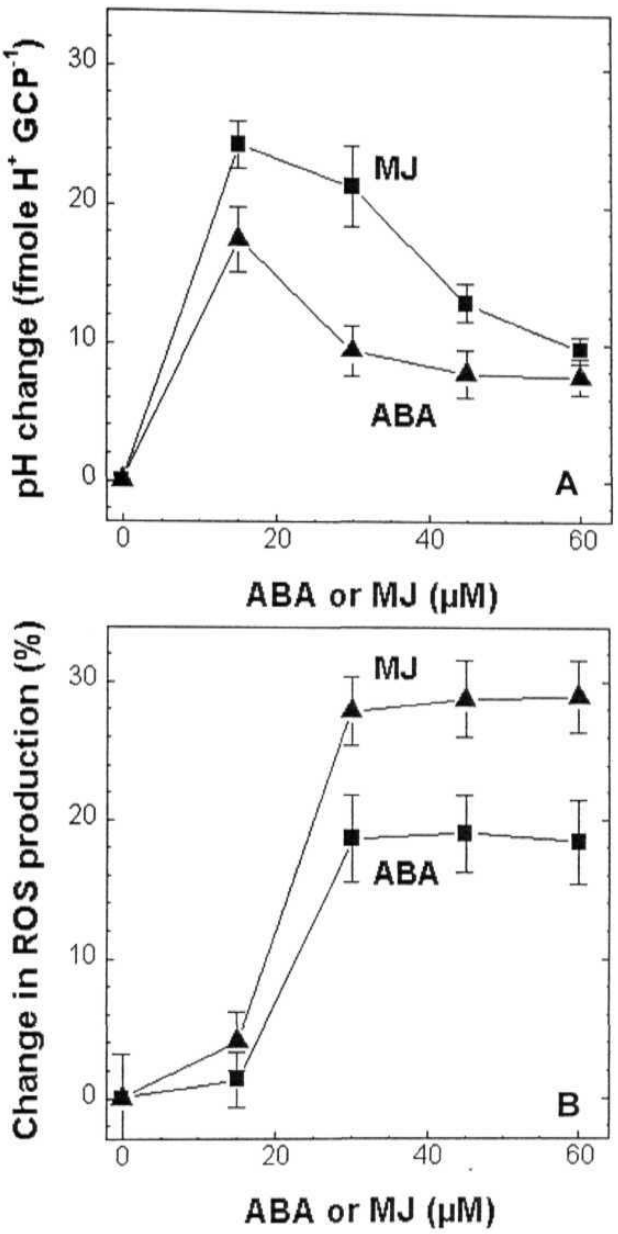

Figure 5.5: Kinetics of pH change (A) and ROS production (B) in guard cells in response to 20 mM ABA or 20 mM MJ; pH changes (A) were determined using the null-point method and H_2O_2 production (B) using the fluorescent dye H_2DCF-DA as described in "Materials and Methods".

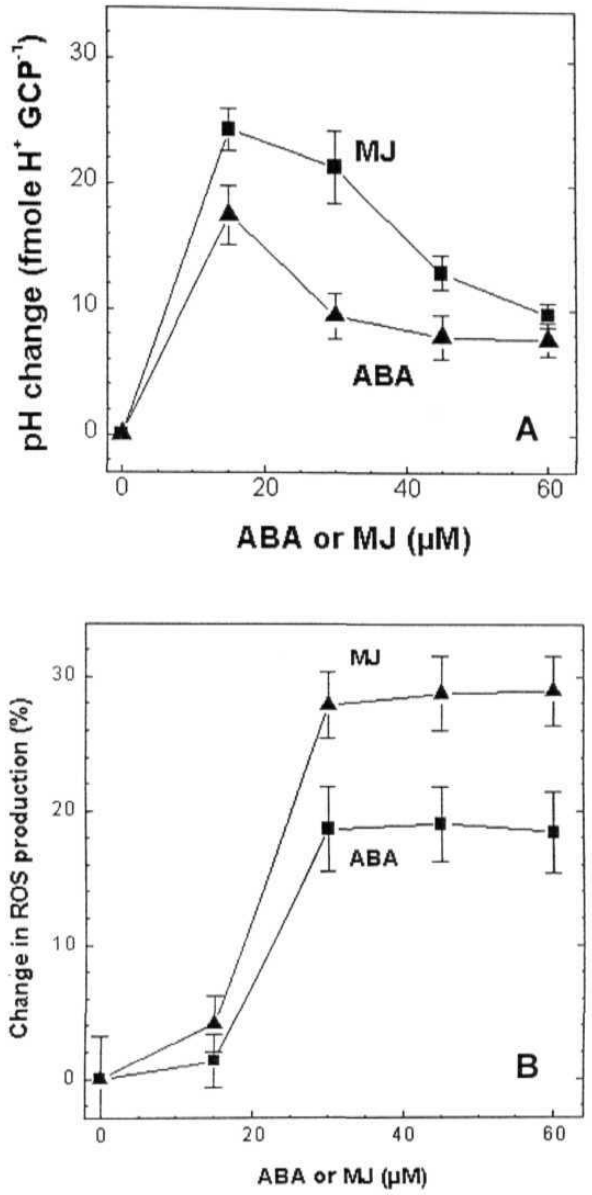

Figure 5.6: The pH changes and ROS production in guard cells of *Pisum sativum*. Changes in pH (A) or ROS (B) were monitored by using BCECF-AM or H_2DCF-DA after the addition of ABA (squares) and MJ (circles). The pixel intensities of fluorescence at each given point were determined and the relative changes in pH or ROS production.

Table 5.4: Modulation of pH change in presence of butyrate on wild type, *jar1*-1, *ost1*-2 and *gork1* with respect to stomatal closure in response to 20 μM ABA or MJ. The leaf epidermis were allowed to open in light for 2 h, then ABA or MJ or 0.5 mM butyrate was applied for 2 h. Results are the averages ± SE (n=60) of 3 to 4 independent experiments.

Plant	Control		ABA (20 μM)		MJ (20 μM)	
	No butyrate	0.5 mM butyrate	No butyrate	0.5 mM butyrate	No butyrate	0.5 mM butyrate
	Stomatal aperture (μm)					
Wild type	4.12 ± 0.2	4.03 ± 0.5	1.09 ± 0.3	2.72 ± 0.2	0.98 ± 0.1	2.96 ± 0.2
jar1-1	3.41 ± 0.2	3.24 ± 0.7	2.54 ± 0.2	2.75 ± 0.2	3.5 ± 0.3	3.29 ± 0.1
ost1-2	3.87 ± 0.2	3.56 ± 0.4	3.59 ± 0.5	3.61 ± 0.1	1.83 ± 0.1	2.62 ± 0.3
gork1	4.19 ± 0.3	4.07 ± 0.2	2.76 ± 0.2	2.89 ± 0.3	4.22 ± 0.2	4.01± 0.4

Table 5.5: Changes in pH of external solution were recorded after permeation of guard cell plasma membrane by digitonin in control or hormone treated protoplasts. The ROS production was monitored by using fluorescence dye, H_2DCF-DA as described in materials and methods. The extent of ROS production in guard cells, without MJ or ABA or butyrate or K252A (control) is taken as 100%. Results are averages (± SE) from at least three experiments.

Hormone	Rise in pH (fmol H^+ GCP^{-1})			ROSproduction (% of control)		
	Control	+ 0.5 mM butyrate	+ 1 μM K252a	Control	+ 0.5 mM butyrate	+ 1μM K252a
None	0.34 ± 0.01	0.26 ± 0.0	10.87± 1.2	100 ± 3.1	101 ± 3.5	101 ± 2.7
ABA	20.8 ± 1.21	8.6 ± 1.3	0.82 ± 2.1	122 ± 3.1	110 ± 3.5	105 ± 3.1
MJ	26.5 ± 1.15	10.1 ± 1.0	0.74 ± 1.4	128 ± 2.5	114 ± 3.7	103 ± 2.9

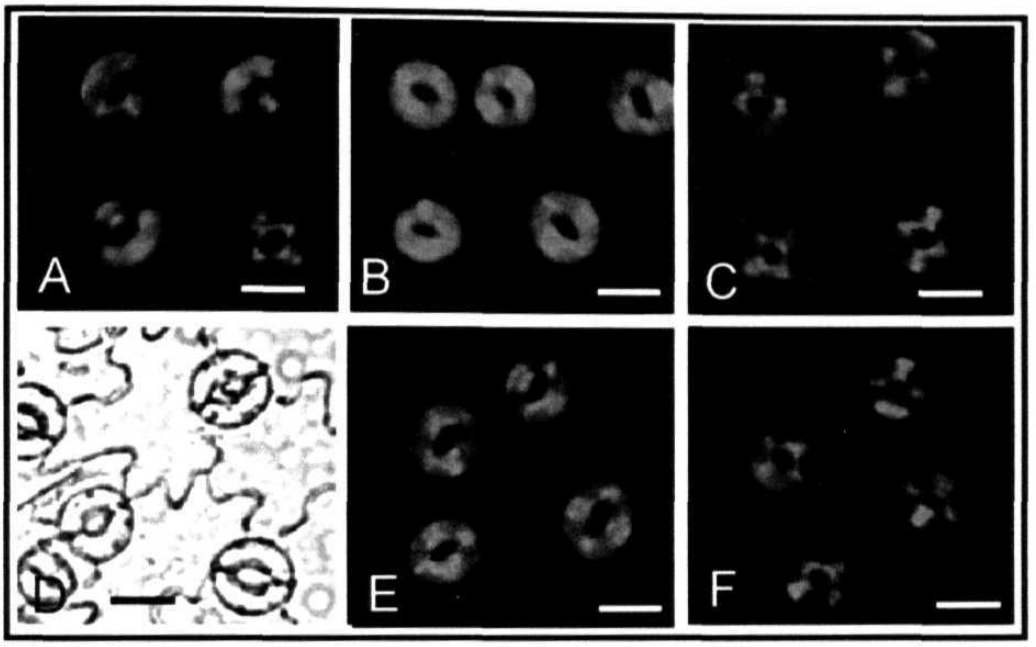

Figure 5.7: MJ- or ABA-induced H_2O_2 production in guard cells is inhibited by 0.5 mM butyrate. Photographs were taken from a representative lot of guard cells from epidermal strips loaded with H_2DCFDA, untreated (A) or submitted to a 30 min pre-treatment with 20 µM MJ (B), or 20 µM ABA (E). (C, F) Effects of 0.5 mM butyrate on MJ - or ABA -induced H_2O_2 production, respectively. Photographs were taken using fluorescence (A-C, E, F) or light microscopy (D), bars represent 10 µm.

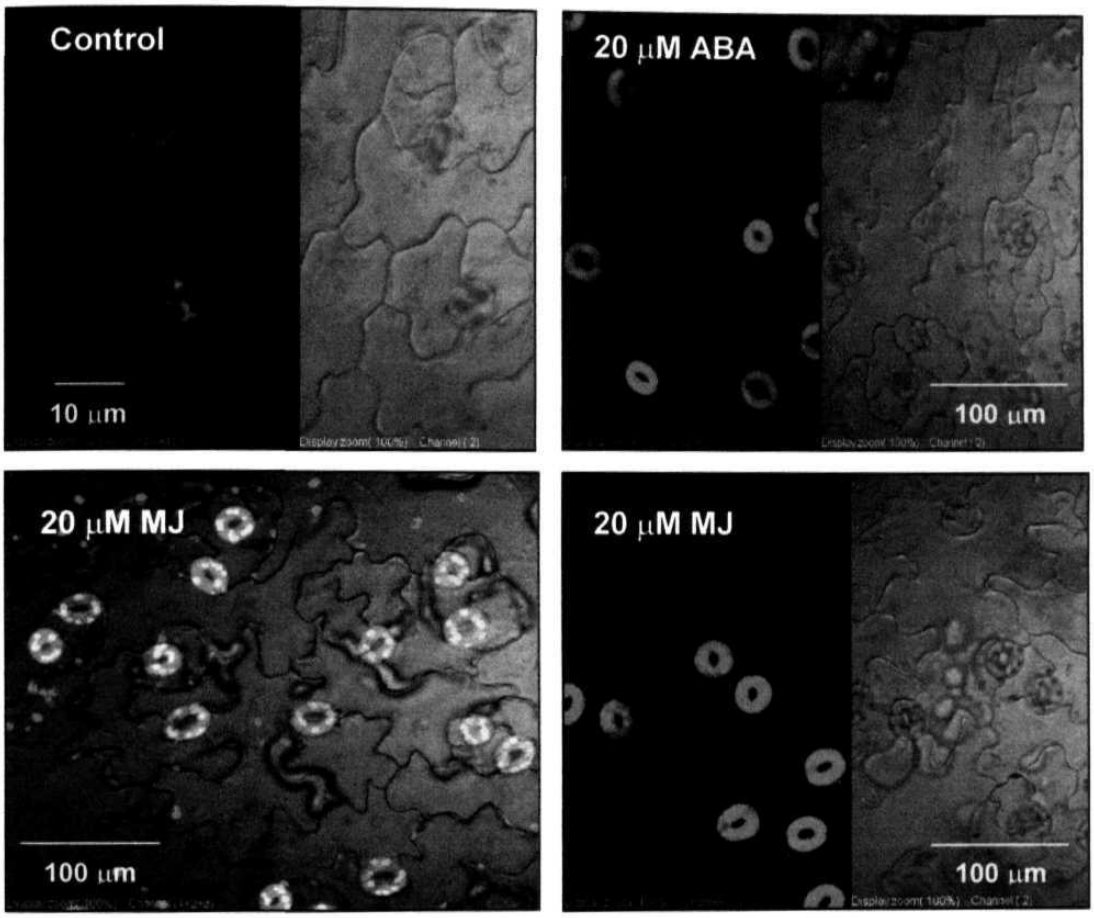

Figure 5.8: Confocal views: ROS production in guard cells of *Arabidopsis*, visualized by 2,7-dichlorofluorescein diacetate (H_2DCF-DA).

5.2.5 Role of calcium or calmodulin in pH or ROS production

Although changes in calcium, pH, and ROS are observed in response to hormonal signals, their interrelationship and the exact sequence of these events have not been clear. It may be argued that changes in pH or ROS in guard cells are brought about by external/internal calcium. The pattern of pH change and ROS production were therefore assessed after modulating calcium, by the addition of either external calcium or EGTA. Added external calcium or EGTA did not affect ROS production (Table 5.6), confirming the involvement of calcium down stream of ROS production. We noticed that ML-7, a Ca^{2+}-calmodulin (CaM) protein kinase inhibitor, was quite effective in reversing the stomatal closure caused by MJ but not that of ABA. Therefore, the effect of W7 (CaM antagonist) was checked. Again, W7 was effective in reversing the effect of MJ but not of ABA. Thus, a CaM-like domain appears to play more active role in the case of MJ than that of ABA (Fig. 5.2, C and D).

5.2.6 gork1 *mutant is insensitive to MJ*

The guard cell outward K^+ channel GORK was for a long time suspected to be the main K^+ conductance supporting ion efflux during stomatal closure. The molecular nature of this ion channel has been recently identified (Ache et al., 2000) and a GORK knockout mutant, *gork1*, characterised at the stomatal level (Hosy et al., 2003). In this mutant, the outward K^+ currents, generally observed upon membrane depolarisation, are absent in guard cell protoplasts, and the mutant displays a limited stomatal closure in response to the stress hormone ABA compared to wild type plants.

Table 5.6: Change in pH or ROS levels were analysed by measuring BCECF-AM or H_2DCF-DA fluorescence in guard cells in response to ABA or MJ or solvent control. Stomata were allowed to open in light for 2 h, then ABA or MJ was applied. The cellular pH and ROS production were examined after 15 and 30 min, respectively. The fluorescence intensity of the dye in the control sets (without ABA or MJ or calcium or EGTA) is taken as 100 and other values were expressed in relation to control. Results are the averages ± SE of 3 to 4 independent experiments.

Phenomenon & Calcium modulator	Hormone (20 μM)		
	None	ABA	MJ
Stomatal opening (μm)			
None	4.0 ± 0.5	1.9 ± 1.0	1.8 ± 0.7
10 μM $Ca(NO_3)_2$	2.8 ± 0.5	2.5 ± 1.1	2.0 ± 0.9
2 mM EGTA	4.8 ± 0.8	3.4 ± 1.2	4.4 ± 0.8
Change in pH (% Control)			
None	100 ± 2.7	120 ± 2.1	125 ± 3.1
10 μM $Ca(NO_3)_2$	99 ± 3.2	121 ± 2.8	124 ± 2.9
2 mM EGTA	100 ± 2.9	120 ± 3.2	124 ± 3.7
Change in ROS (% Control)			
None	100 ± 3.3	122 ± 2.8	130 ± 3.2
10 μM $Ca(NO_3)_2$	105 ± 2.8	126 ± 3.1	135 ± 3.0
2 mM EGTA	96 ± 3.4	118 ± 3.4	123 ± 3.7

We have therefore examined stomatal responses to ABA and MJ in *gork1*. As previously observed, the *gork1* mutation led to a diminished response to ABA (Fig. 5.3B). Interestingly, stomatal closure in response to MJ was completely suppressed in the *gork1* mutant. A recent work from Evans (2003) has already described a MJ dose-dependent modulation of inward and outward rectifier K^+ channels at the guard cell plasma membrane. The results from the present study suggest that GORK contributes to one of the conductance's involved in K^+ efflux during ABA-induced stomatal closure while it is an essential element in MJ-induced ion efflux and stomatal closure. MJ did not induce stomatal closure but caused significant ROS production in guard cells of *gork1* (Table 5.3).

5.3 Discussion

ABA and MJ play a crucial role in plant adaptation to stress conditions. These two phytohormones inhibit root growth, limit transpiration, interfere with seed germination and cell cycle and induce stomatal closure (Raghavendra and Reddy, 1987, Staswick et al., 1992, Wang, 1999, Swiatek et al., 2002). Considerable efforts have been devoted to identify signaling elements in the guard cell response to ABA, a fundamental process in drought resistance (Schroeder et al., 2001a).

In comparison, very few studies have focused on MJ signaling cascade leading to stomatal closure. It has been shown that MJ is produced during water stress (Creelman and Mullet, 1997) and that stomatal closure contributes to diminish the entry of certain pathogens in the leaf tissues (Agrios, 1997).

Additionally, stomatal closure could limit plant growth and help redirect plant metabolism towards defence reactions. Previous studies have shown that some events in MJ- and ABA-signaling are similar e.g. calcium requirement and protein (de)phosphorylation (Suhita et al., 2003), alkalization of the guard cell cytoplasm (Gehring et al., 1997), ROS production (Lee et al., 1999) and modulation of K^+ channels at the guard cell plasma membrane (Evans, 2003).

In the present study we observed that the response to MJ was more sensitive to Ca^{2+}-calmodulin (CaM) protein kinase inhibitors than the stomatal response to ABA (Fig. 5.2). These inhibitors were able to reverse the response to MJ, while the response to ABA was only partially affected. These findings suggest that at least one protein kinase with a Ca^{2+}-CaM like regulatory domain plays an essential role in MJ response, while such activity appears to participate to a limited extent in the ABA cascade. In contrast, a broad range inhibitor of protein kinase (K252A) was able to suppress both responses, suggesting that Ca^{2+}-dependent and Ca^{2+} independent protein kinases are involved parallely during the ABA signaling.

Interestingly, K252A was able to suppress MJ- or ABA-induced pH changes and ROS production (Table 5.4), suggesting that a protein phosphorylation event is essential and located upstream of these responses. While the kinetics of ROS production and pH change in response to MJ or ABA were almost similar, the amplitude of responses were always higher with MJ than that with ABA. These results confirm the previous observations from Gehring et al. (1997), who used different techniques and species. Additionally, the limited responses of *jar1*-1 and *ost1*-2 allow placing JAR1 and OST1

upstream of cytoplasmic alkalization in the MJ and ABA-signaling pathways, respectively.

The outward-rectifying K^+ channels appear to play an important role in stomatal closure. However, the reports on the regulation of these outward-rectifying K^+ channels are ambiguous. These channels were found to be down-regulated by H_2O_2 (Köhler et al., 2003). However, a rise in cytoplasmic pH (which is expected to raise H_2O_2 levels) led to the up-regulation of these outward K^+ channels (Miedema and Assmann, 1996).

In a recent study, Evans (2003) found that MJ down-regulated the outward K^+ channels. As per our observations, the *gork1* mutant, whose outward K^+ channels are impaired, was insensitive to MJ (Fig. 5.3B). Further, external addition of H_2O_2 caused marked stomatal closure in all the three mutants, including GORK. We suggest that GORK is one of the limiting elements during stomatal response to MJ and possible mechanisms could also be involved, for example modulation of influx of sucrose or K^+.

An important point from the present study is that the sequence of events signaling stomatal closure can be traced, which appear broadly similar for MJ and ABA. At least one protein phosphorylation event is necessary for the cytoplasmic alkalization, which leads to ROS production by the NAD(P)H oxidase. In turn, ROS would activate hyperpolarization-activated Ca^{2+} channels (Pei et al., 2000, Murata et al., 2001) and the resulting elevation of free cytoplasmic Ca^{2+} triggers plasma membrane anion channels leading to cell depolarisation.

Hedrich et al. (1990) showed R type anion channel activation by extracelllular $CaCl_2$. Schroeder and Hagiwara (1989) showed S type anion channel activation by cytoplasmic Ca^{2+} elevation.

ROS would also inhibit inward-rectifying K^+ channels (Köhler et al., 2003, Torsethaugen et al., 1999). Among the last steps in the cascade leading to stomatal closure is the activation of the outward K^+ rectifier from guard cell (Armstrong et al., 1995), allowing potassium efflux and loss of turgor. Thus GORK appears to be an essential component in the MJ signaling cascade, leading to stomatal closure (Fig. 5.9).

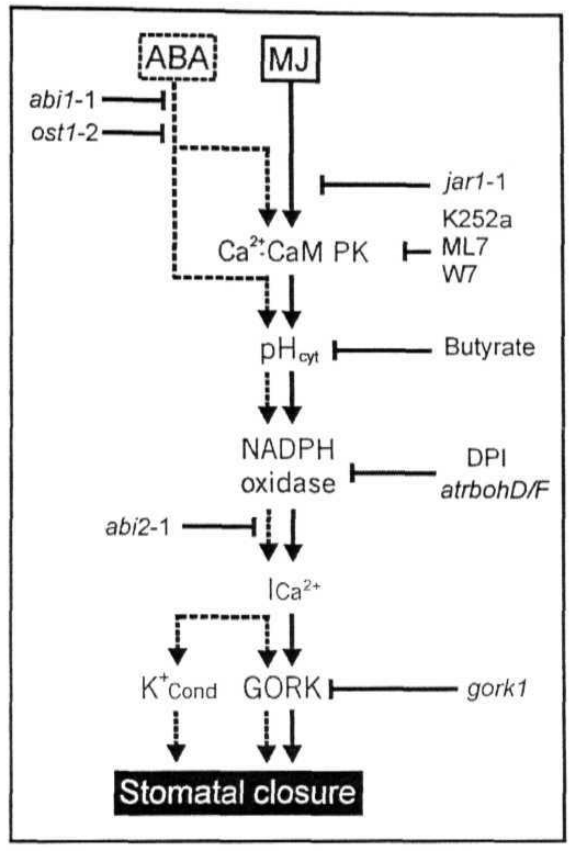

Figure 5.9: Model for the sequence of events in the MJ signaling cascade leading to stomatal closure. This linear model integrates the present results from the different mutants and the use of inhibitors. Ca^{2+}-CaM PK (calcium-calmodulin activated protein kinase). ICa^{2+}, calcium influx at the plasma membrane, *abi1* and *abi2* have been placed according to Murata et al. (2001) and Mustilli et al. (2002).

5.4 Conclusion

1. Dose response curves of MJ and ABA in *jar1*-1 mutant plants are insensitive to MJ but are able to close stomata in response to ABA, their senstivity to ABA is less than that of wild type plants.
2. Reciprocally, the stomata of *ost1*-2 are insensitive to ABA but are able to induce stomatal closure in response to MJ to a lesser extent compared to wild type plants.
3. MJ and ABA promotes H_2O_2 production in wild type guard cells while exogenously application of diphenylene iodonium (DPI) chloride, an inhibitor of NAD(P)H oxidases, results in the suppression of ABA- and MJ-induced stomatal closure.
4. Comparative analysis of responses to MJ and ABA points out some specificity in the ABA cascade, which appears to involve parallel Ca^{2+}-dependent and Ca^{2+}-independent pathways and K^+-conductance(s) other than GORK.
5. From the present study, a Ca^{2+}-CaM protein kinase seems to be involved in a very early MJ signaling, whereas H_2O_2 production occurs downstream of phosphorylation events that activate Ca^{2+}-channels at the plasma membrane leading to Ca^{2+} elevation in the cytoplasm.
5. ABA elevates H_2O_2 production in wild type and *jar1*-1 guard cells but not in *ost1*-2, where as MJ induces H_2O_2 production in both wild type and *ost1*-2 guard cells but not in *jar1*-1.
6. MJ induced stomatal closure is suppressed in NAD(P)H oxidase double mutant *atrbohD/F* and in the outward potassium channel mutant *gork1*.

7. MJ induces alkalization in guard cell cytosol, and MJ induced stomatal closure was inhibited by weak acid (butyrate).
8. Analysis of the kinetic of cytosolic pH changes and reactive oxygen species (ROS) production showed that the alkalization of cytoplasm precedes ROS production during the stomatal responses to ABA and MJ.

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Chapter 6

Nitric oxide Production is a Common Event During the Signal Transduction by Guard Cell of Both MJ and ABA

Chapter 6

Nitric oxide production is a common event during the signal transduction by guard cell of both MJ and ABA

6.1 Introduction

Nitric oxide (NO) functions as a signal in hormonal and defense responses (Dangl and Jones, 2001, Lamattina et al., 2003, Neill et al., 2003). Plant hormones like methyl jasmonate (MJ) or abscisic acid (ABA) are known to restrict stomatal opening (Raghavendra and Reddy, 1987, Mansfield et al., 1990). Recent evidence suggests the existence of cross talk between NO and some plant hormones (auxins, ethylene, ABA) during adaptive responses to adverse conditions, including abiotic stress (Durner and Klessig, 2001, Correa-Aragunde et al., 2004).

The NO molecule can interact with ROS-induced cell death in soybean suspension cells. The NO synthase (NOS) inhibitor of tobacco blocked hypersensitive resistance responses (HR) in *Arabidopsis* (Delledonne et al., 1998, 2001). NO may combine with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$), which damages lipids, proteins and nucleic acids (Lipton et al., 1993, Yamasaki et al., 1999). NO and H_2O_2 appear to operate in tandem during plant defense responses as well as during stomatal closure by ABA (Neill et al., 2002). Consequently, it is expected that NO can be a signal to which guard cells may respond and interact with other signals to fine tune signal transduction and stomatal movement.

NO is generated from L-arginine (Arg) by NOS, the activity of which can be inhibited by Arg analogues such as L-NAME (N-nitro-L-arginine

methylester). Biochemical and immunological evidence prove the existence of NOS in plants (Neill et al., 2002). The NOS like enzyme activity, inhibited by L-NAME, has been identified and partially purified from pea leaves (Barroso et al., 1999). Recently a pathogen-inducible plant enzyme catalyzing NOS like activity has been identified as variant of the P- protein of glycine decarboxylase complex (GDC) (Chandok et al., 2003). NO production in plants is not restricted to NOS like activity and NO can be generated also through either a light mediated conversion of carotenoids (Cooney et al., 1994) or from nitrate reductase (NR) (Yamasaki and Sakihama, 2000, Rockel et al., 2002). The mechanisms, by which ABA induces an increase in guard cell NO synthesis, are not all clear and may differ between the species.

There have been extensive studies on the mechanism and components of ABA action on guard cells. ABA activates (or elevates) a complex web of signaling components, including G proteins, protein kinases, protein phosphatases, cytosolic pH, free radicals, cytosolic calcium and ion channels (Schroeder et al., 2001a, Hetherington, 2001, Wang et al., 2001). Further additions to ABA signaling components include phospholipid sphingosine-1-phosphate (SIP), phospholipase C and reactive oxygen species (ROS), reactive species nitrogen species, i.e. nitric oxide (NO) (Hetherington, 2001, Neill et al., 2003). The participation of NO as a key signal in ABA-induced stomatal closure was observed in *P. sativum*, *V. faba*, *Arabidopsis* (Neill et al., 2002, Garcia-Mata and Lamattina, 2002; Desikan et al., 2002).

Compared to the vast literature on ABA effects on guard cells, the studies on stomatal responses to MJ are very few and have started picking up. Some of the recent reports indicated changes in guard cells on exposure to MJ in properties of K^+(out) channel, cytosolic pH, and ROS levels (Evans, 2003, Suhita et al., 2004). However, the role of NO during MJ-induced stomatal closure has not been examined so far.

The present work is an attempt to check the role of NO in stomatal responses of *Nicotiana glauca*, particularly after the treatment with MJ or ABA. In plants as in animals NO interacts with other signaling elements such as cADPR, lipids, cGMP, ion channels, Ca^{2+} (Garcia-Mata and Lamattina, 2003). The present study examines the responses of events related to NO pathway in response to MJ or ABA and the up-stream or down-stream interaction of NO with pH and ROS. Our results indicate that the level of NO in guard cells increases in response to MJ as well as ABA. This process seems to involve protein kinases, cGMP, PI-PLC and cADPR.

6.2 Results

6.2.1 Effect of NO modulation on stomatal closure caused by MJ or ABA

The presence of MJ or ABA at 10 μM decreased stomatal opening. In presence of sodium nitroprusside (SNP), an NO releaser, the inhibitory effect on stomatal closure by MJ or ABA was further enhanced (Fig. 6.1A). Such promotion by SNP of stomatal closure (by MJ or ABA) was pronounced at 0.01 or 0.1 mM SNP (Fig. 6.1B).

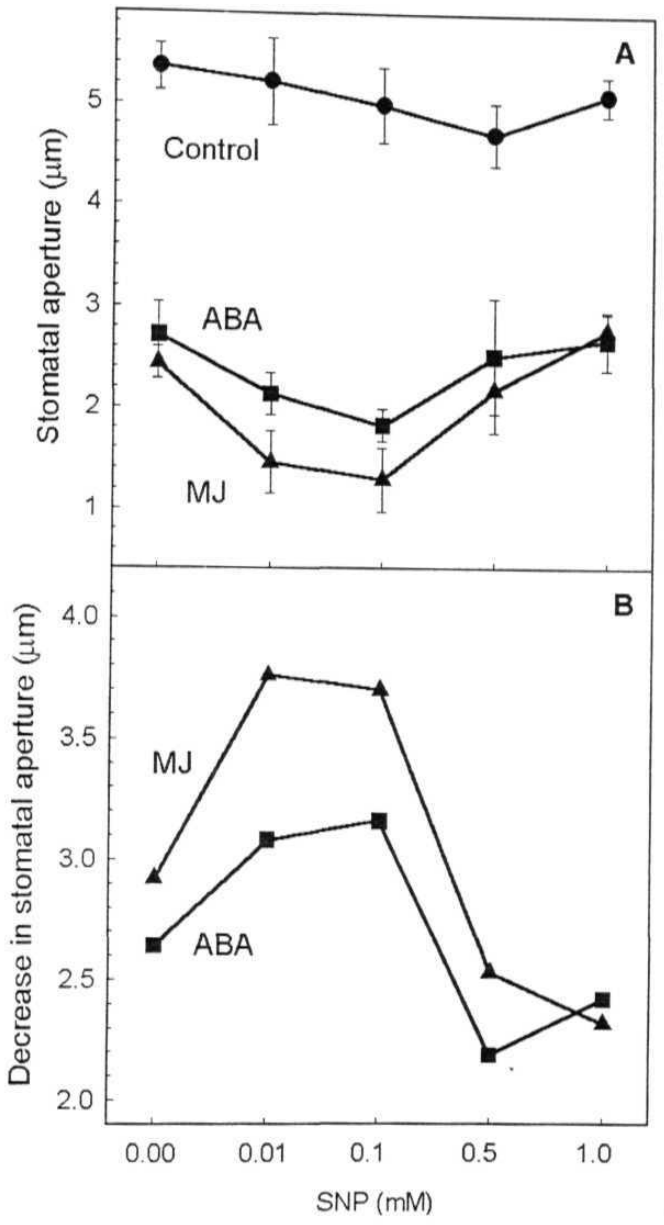

Figure 6.1: Dose response curves of stomatal opening to SNP (nitric oxide releaser) in epidermal strips of *Nicotiana glauca* in the absence or presence of 10 μM MJ or ABA. (A) Stomatal opening in the absence or presence of MJ or ABA; (B) The extent of decrease in stomatal aperture due to MJ or ABA, in relation to the presence of SNP. Stomata were allowed to open under light (500 μmol m^{-2} s^{-1}) for 3 h. Results are the averages ± SE from at least 3 independent experiments.

The next sets of experiments were with compounds which decrease endogenous NO levels: c-PTIO (NO scavenger) and L-NAME (an inhibitor of NOS). When added to the medium, c-PTIO did not affect the stomatal opening but reversed the stomatal closure caused by MJ or ABA (Fig. 6.2A). The reversal of MJ-effect was more than that of ABA (Fig. 6.2B). Similarly, NOS inhibitor L-NAME also reversed the effects of MJ or ABA (Fig. 6.3). However, the reversal by L-NAME (Fig. 6.3A) was not as complete as that with c-PTIO (Fig. 6.2A).

The levels of NO in guard cells were monitored by using a cell permeable NO sensitive fluorophore 4,5-diamino fluorescein diacetate (DAF-2DA). DAF-DA has been used for detecting of NO presence in not only animal and plant cells (Kojima et al., 1998, Foissner et al., 2000), but also in guard cells (Desikan et al., 2002, Mata and Lamattina, 2002). When epidermal guard cells were exposed to SNP, the NO production increased markedly (Fig. 6.4).

Use of DAF-DA gave beautiful fluorescent images of guard cells and demonstrated that both MJ and ABA elevated the NO levels (Fig. 6.5). The fluorescence tended to be high in nucleus of guard cells treated with MJ (Fig. 6.5C and 6.5E). In contrast the dispersion of NO fluorescence in the ABA treatment appeared to be high in cytosol of guard cells (Fig. 6.5D and 6.5F). The intensity of NO-fluorescence was markedly decreased in presence of c-PTIO or L-NAME (Fig. 6.6).

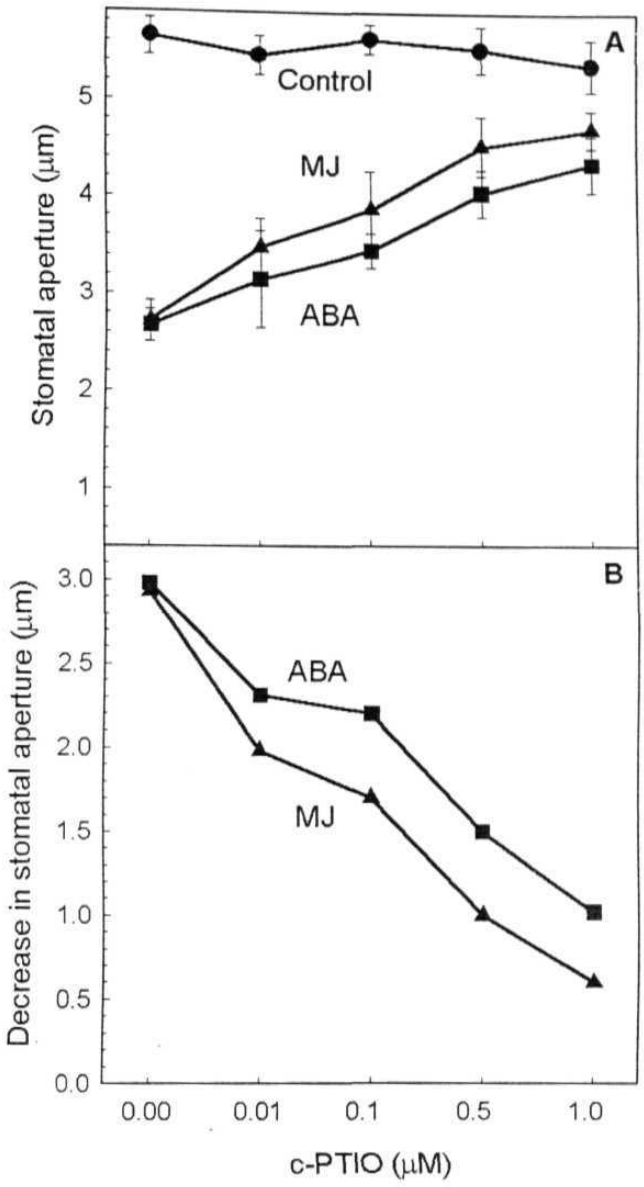

Figure 6.2: Relief by c-PTIO (NO scavenger) of stomatal closure induced by 10 μM MJ or ABA. (A) Stomatal opening in the absence or presence of MJ or ABA; (B) The extent of decrease in stomatal aperture due to MJ or ABA, in relation to the presence of c-PTIO. Further details are as in Fig. 6.1.

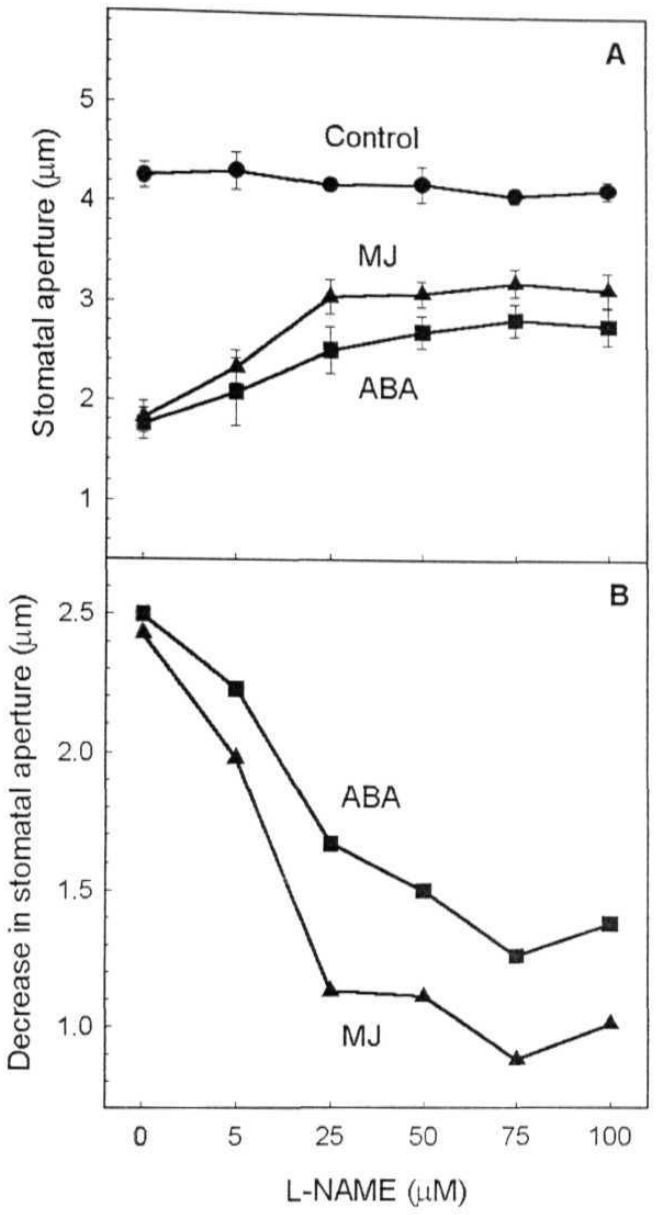

Figure 6.3: The effect of L-NAME (NOS inhibitor) on stomatal opening in the epidermal strips of *Nicotiana glauca* in the absence or presence of either 10 μM MJ or ABA. The reversal by L-NAME of stomatal closure was partial. (B) The extent of decrease in stomatal aperture due to MJ or ABA, in relation to the presence of L-NAME. Further details are as in Fig. 6.1.

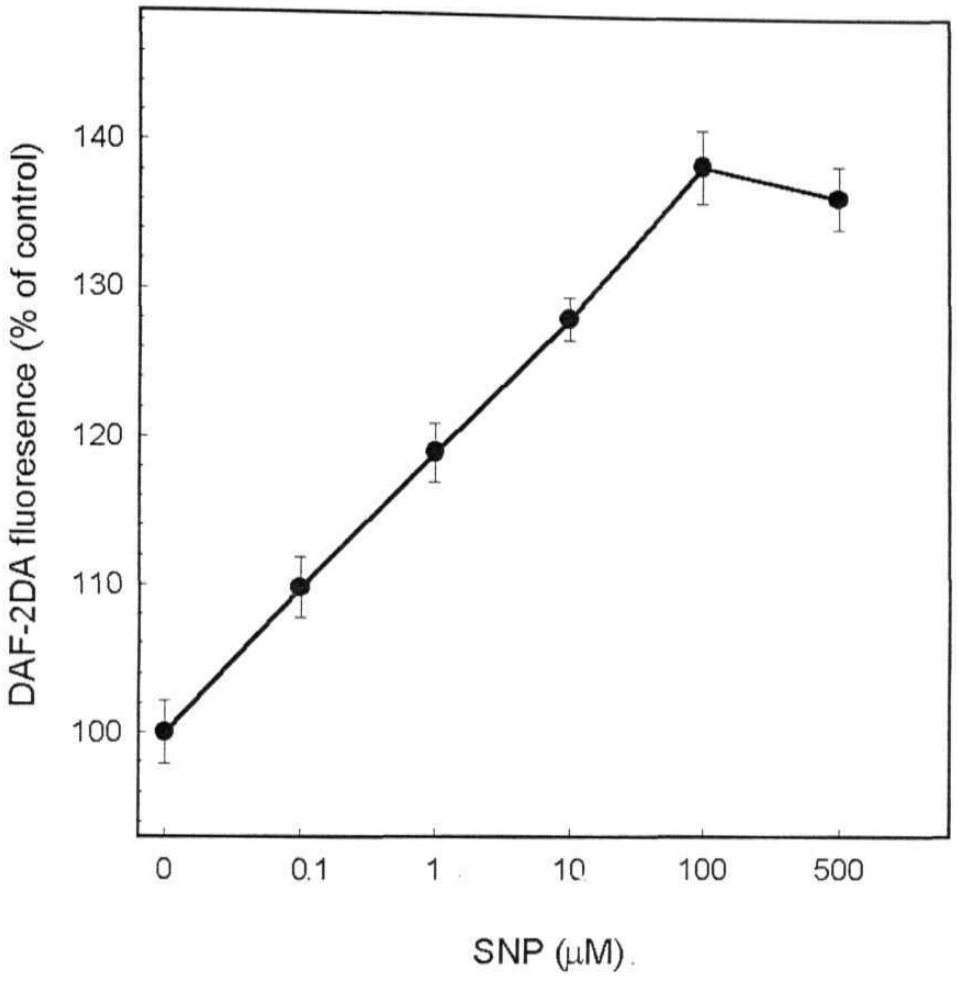

Figure 6.4: Increase in NO production in epidermal guard cells in presence of SNP. The epidermal strips were incubated with varying concentrations SNP in light. The levels of NO were monitored by exposing the strips with DAF-2DA, as described in Materials and Methods. The increase in fluorescence reflects an increase in NO.

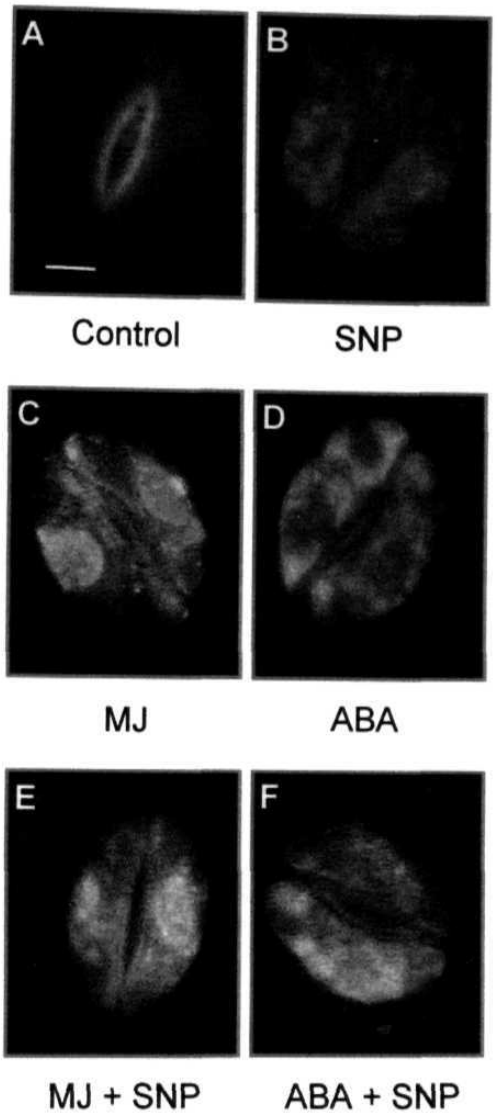

Figure 6.5: Increase in NO induced by 20 μM ABA or MJ in *Nicotiana glauca* guard cells visualized under epifluorescent microscope (C, D). The NO fluorescence appeared to be intense around the nucleus in MJ treatment. After ABA treatment, the dispersion of the fluorescence was intense in cytoplasm. The fluorescence was further enhanced by SNP, even in presence of ABA or MJ. Details of NO-visualization and ABA or MJ treatment are described in Materials and Methods. Bar represents 25 μM.

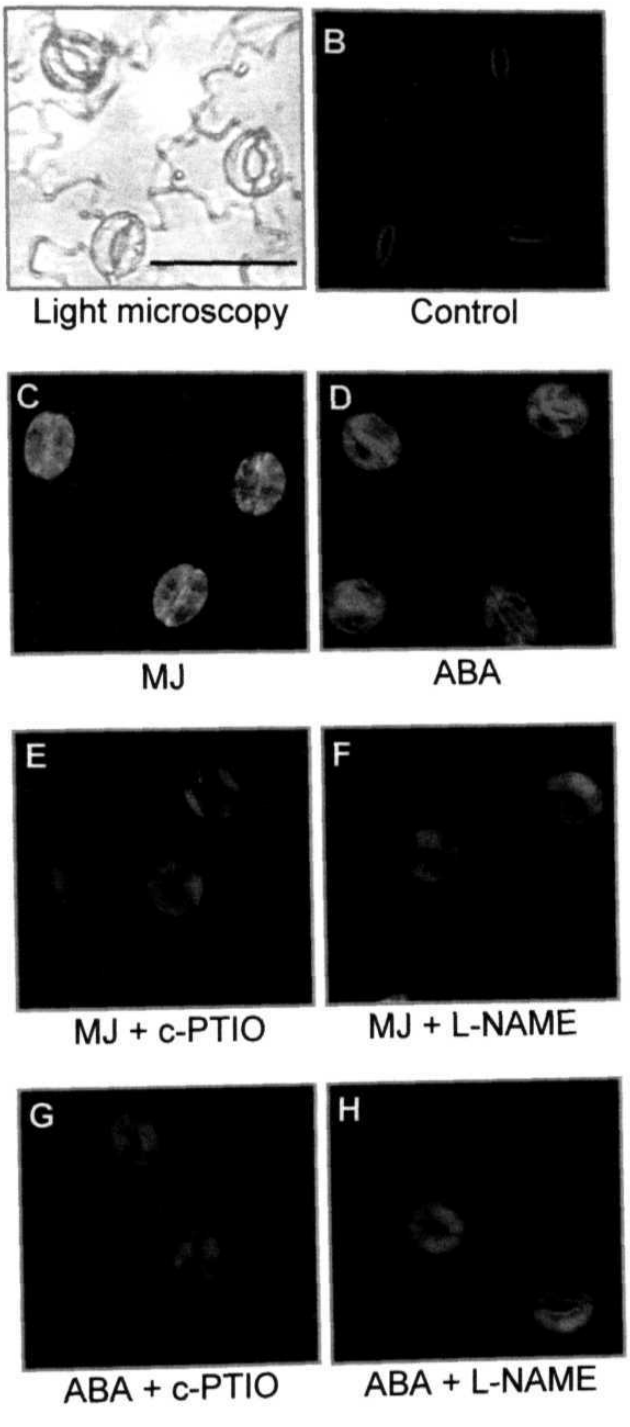

Figure 6.6: The modulation of NO-levels by c-PTIO and L-NAME in relation to MJ or ABA in *Nicotiana glauca* guard cells. The NO - levels were raised by treatment with MJ or ABA (C, D). The presence of c-PTIO and L-NAME reduced markedly the NO-levels, in presence of MJ or ABA of guard cells visibly fluorescing (E to H). Details of NO-visualization and ABA or MJ treatment are described in Materials and Methods. Bar represents 100 μM.

6.2.2 Comparative kinetics of increase in NO or ROS or pH in guard cells

For an interesting comparison, the levels of NO, ROS and pH were monitored by using three fluorescent probes. These were DAF-DA (for NO), H_2DCF-DA (for ROS), BCECF-AM (for pH). The NO production in guard cells induced by MJ or ABA peaked at 18 min and stayed high until 30 min (Fig. 6.7). In contrast, MJ or ABA induced ROS production in guard cells continued to increase up to 30 min of treatment and then plateaued (Fig. 6.8). The increase in pH due to MJ or ABA also peaked at 15 min (Fig. 6.9), as in the case of NO. However pH fell sharply soon after attaining the peak, unlike the case of NO.

6.2.3 pH change in guard cells associated with NO production

The data in Fig. 6.9 demonstrated that MJ or ABA caused marked alkalinization in guard cells. The alkalinization of pH can be restricted by exposing the cells, with weak acids, such as butyrate. Not surprisingly, the presence of butyrate at 1 mM restricted stomatal closure by MJ or ABA and also decreased the NO levels (Table 6.1).

6.2.4 Role of protein kinases in NO production

Protein kinases are involved at different levels of signal transduction in guard cells. Four protein kinase inhibitors were chosen: ML-7 (myosin light chain kinase (MLCK) inhibitor), staurosporine (broad spectrum of protein kinase inhibitor), wortmannin (PI_3K inhibitor) and LY294002 (PI_4K inhibitor). Experiments using these four pharmacological compounds did not yield any specific result. All the four inhibitors, restricted the effect of MJ or ABA on

stomatal opening and decreased NO-production (Table 6.2). Among the four inhibitors, ML-7 was the most effective in preventing MJ or ABA promoted stomatal closure and NO production.

6.2.5 Importance of calcium mobilizing components in MJ or ABA mediated NO production

Stomatal closure in response to ABA typically requires elevated cytosolic Ca^{2+}. Such elevation of calcium often requires mobilization involving components such as phospholipases, guanyl cyclase and cADPR. Incubation with 2 mM EGTA (a Ca^{2+}-chelator) reversed the stomatal closure, but had no effect on NO-levels, increased by MJ or ABA (Table 6.3). The presence of U73122, (inhibitor of PLC) or ODQ, (inhibitor of guanyl cyclase) or nicotinamide, (an antagonist of cADPR synthesis) all restricted the increase in NO production by ABA or MJ (Table 6.3). However, stomatal closure due to ABA or MJ was prevented by these three compounds. Similar effect was noticed with W7 (a CaM antagonists); decrease in NO-levels and relief in stomatal closure by ABA or MJ.

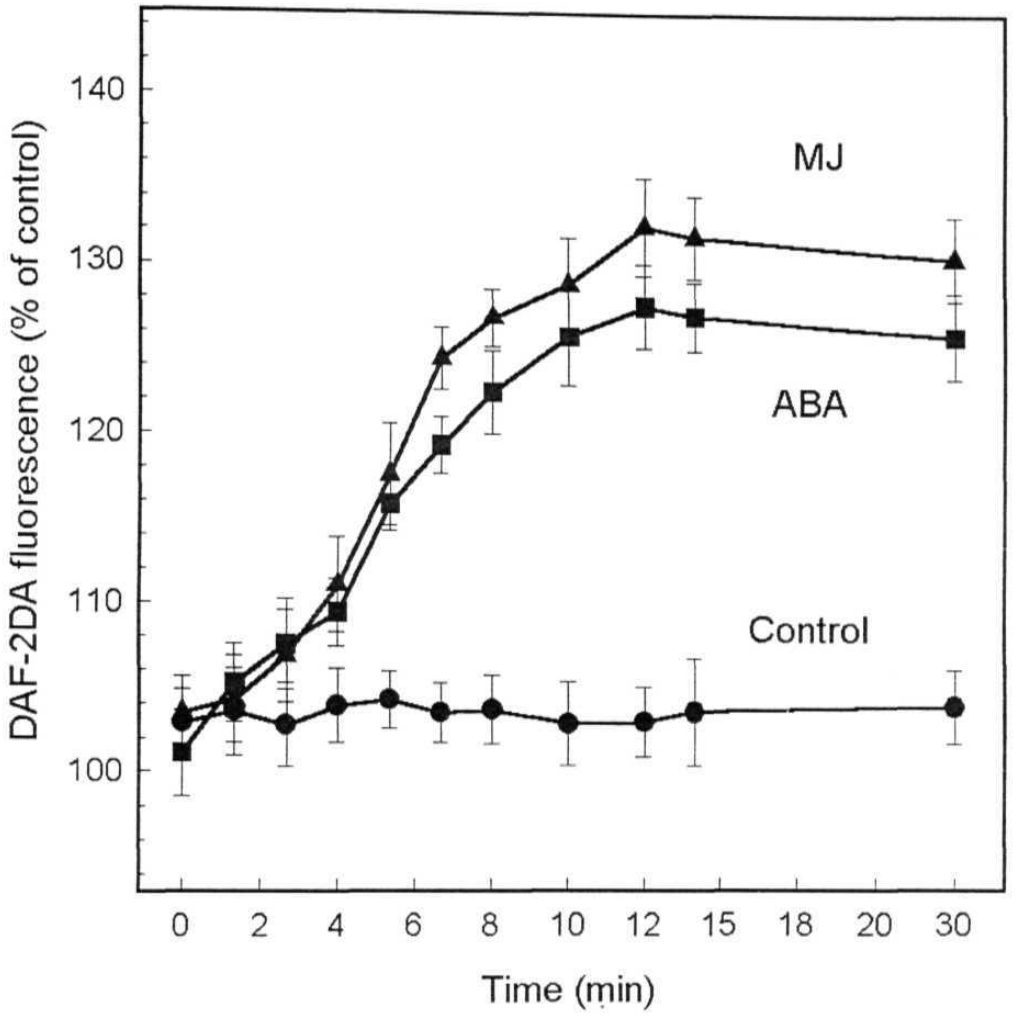

Figure 6.7: Kinetics of NO production in *Nicotiana glauca* guard cells in response 20 μM MJ or ABA. Epidermal strips were loaded with DAF-2 DA, washed and then incubated with ABA or MJ. Further details are described in Materials and Methods.

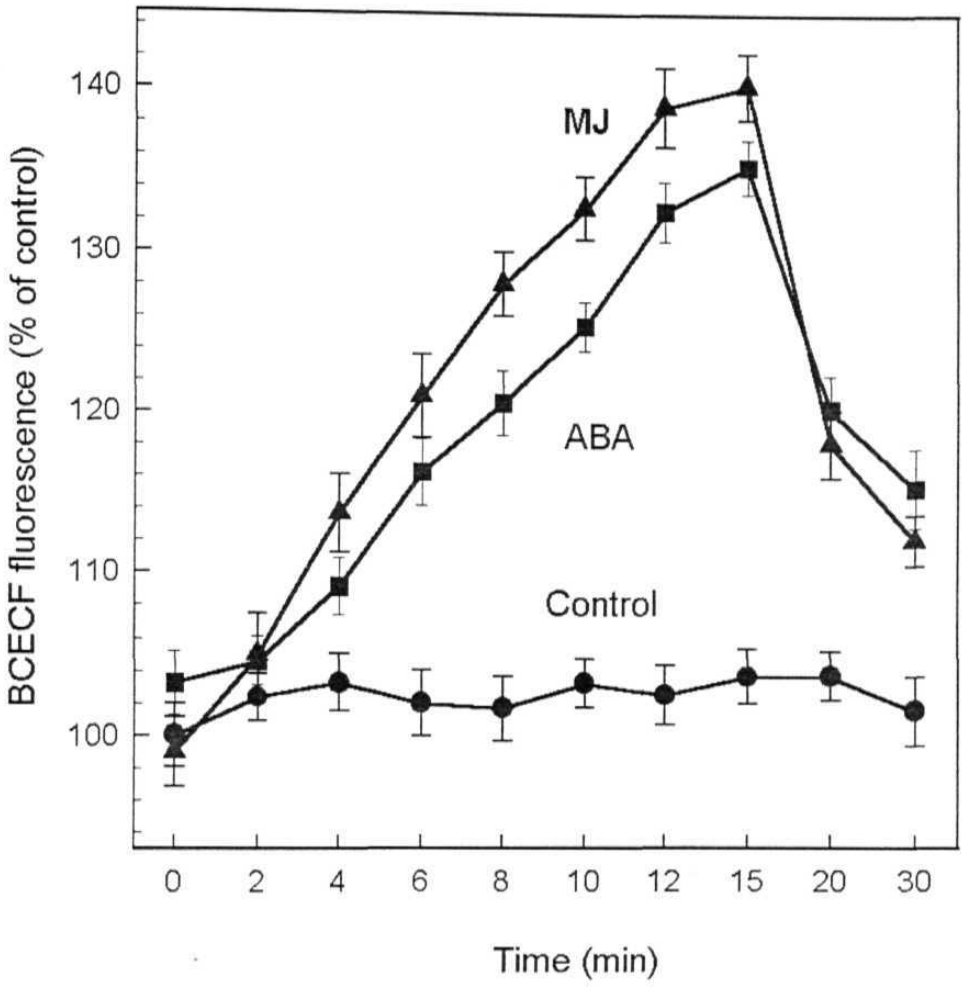

Figure 6.8: The time course of cytosolic alkalinization in guard cells of *Nicotiana glauca* in presence of MJ or ABA. The pH was monitored using BCECF-2AM as described in Materials and Methods.

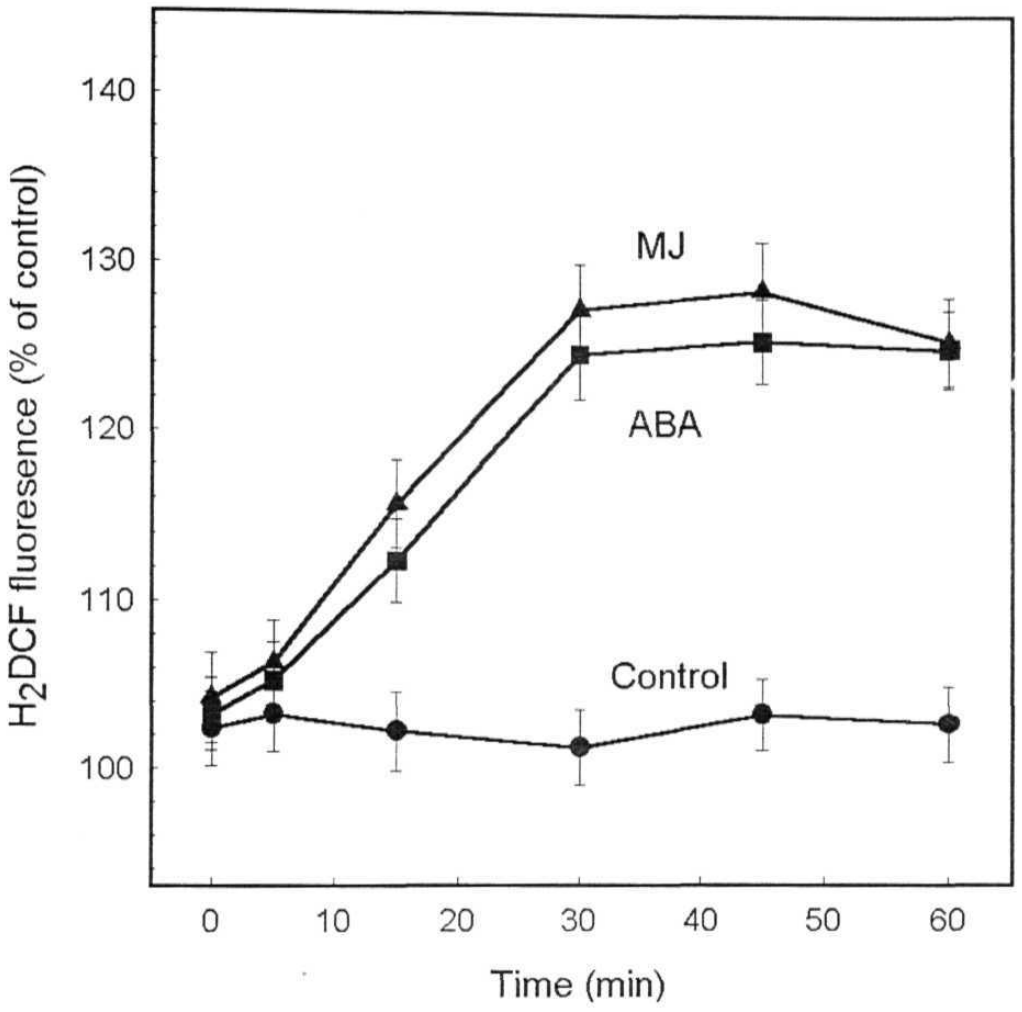

Figure 6.9: Kinetics of ROS production in *Nicotiana glauca* guard cells in response 20 μM MJ or ABA. Epidermal strips were loaded with H_2DCF, washed and then incubated with MJ or ABA respectively. Further details are described in Materials and Methods.

Table 6.1: Change in NO levels as indicated by DAF-2DA fluorescence in guard cells of *Nicotiana glauca* with 20 μM MJ or ABA, in presence of 1mM butyrate (a weak acid). The epidermis was incubated in light for 3 h with MJ or ABA in presence or absence of 1 mM butyrate. Results are the averages ± SE of 3 to 4 independent experiments. The extents of NO production in the guard cells without ABA or MJ are taken as 100%.

Hormone	No butyrate		1 mM butyrate	
	NO (%)	SA* (μm)	NO (%)	SA* (μm)
Control	100 ± 2.1	5.9 ± 0.4	100 ± 2.1	5.7 ± 1.0
MJ	129 ± 2.4	2.4 ± 0.8	107 ± 2.5	4.4 ± 0.8
ABA	124 ± 2.2	2.4 ± 0.7	113 ± 2.2	4.1 ± 1.0

*SA : Stomatal aperture.

Table 6.2: Changes NO levels by DAF-2DA fluorescence and stomatal opening in response to 20 μM ABA or MJ in presence of protein kinase inhibitors. The protein kinase inhibitors were ML-7 (MLCK inhibitor), staurosporine (broad spectrum inhibitor of protein kinases), wortmannin (PI_3K inhibitor) and LY294002 (PI_4K inhibitor). Further details are as in Table 1.

Effectors	Control		ABA		MJ	
	NO (%)	SA* (μm)	NO (%)	SA* (μm)	NO (%)	SA* (μm)
None	100 ± 2.9	4.7 ± 0.2	123 ± 2.1	1.8 ± 0.5	125 ± 3.1	1.7 ± 0.4
ML-7 (20 μM)	104 ± 2.2	4.7 ± 0.3	113 ± 3.4	4.2 ± 0.5	110 ± 2.7	4.5 ± 0.3
Staurosporine (2 μM)	108 ± 3.2	4.9 ± 0.2	107 ± 2.8	4.4 ± 0.3	108 ± 2.9	4.5 ± 0.4
Wortmannin (10 μM)	103 ± 2.6	4.1 ± 0.6	110 ± 2.7	4.0 ± 0.4	116 ± 2.3	3.6 ± 0.6
LY294002 (10 μM)	102 ± 3.0	4.2 ± 0.5	114 ± 2.9	3.6 ± 0.6	111 ± 2.6	4.1 ± 0.3

*SA: Stomatal aperture.

Table 6.3: The MJ or ABA mediated NO production as a function of stomatal aperture. The calcium chelator (EGTA), calmodulin antagonists (cADPR), PLC inhibitor (U73122), guanyl cyclase inhibitor (ODQ) and cADPR inhibitor (nicotinamide) secondary messengers were assessed in presence of 20 µM MJ or ABA. Changes in NO levels were analysed by measuring DAF-2DA fluorescence in guard cells. Further details are as in Table 1.

	Control		MJ		ABA	
Effectors	NO (%)	SA* (µm)	NO (%)	SA* (µm)	NO* (%)	SA* (µm)
None	100 ± 1.9	4.8 ± 0.3	127 ± 3.1	1.7 ± 0.3	124 ± 2.9	1.9 ± 0.2
EGTA (2 mM)	100 ± 2.1	6.5 ± 0.8	127 ± 2.3	5.8 ± 0.3	124 ± 2.7	4.5 ± 0.6
W7 (20 µM)	103 ± 2.6	4.9 ± 0.4	110 ± 2.8	4.2 ± 0.9	118 ± 2.9	3.9 ± 0.5
U73122 (1 µM)	105 ± 2.3	3.7 ± 0.4	125 ± 2.5	2.8 ± 0.4	113 ± 2.2	3.8 ± 0.7
ODQ (2 µM)	104 ± 2.4	4.4 ± 0.3	115 ± 2.4	3.7 ± 0.6	112 ± 2.8	3.5 ± 0.5
Nicotinamide (5 mM)	103 ± 1.9	5.5 ± 0.6	113 ± 3.0	5.5 ± 0.7	110 ± 2.5	5.3 ± 0.9

*SA: Stomatal aperture.

6.3 Discussion

It has been already established that ROS and NO are essential signaling components in mediating ABA induced stomatal closure (Neill et al., 2002). The presence of SNP (a NO donor) promoted stomatal closure while c-PTIO and L-NAME, which are expected to decrease NO, reversed stomatal closure by ABA or MJ (Fig. 6.2 and 6.3). Thus, the present study demonstrates, for the first time, the importance of NO mediated signaling in MJ dependent stomatal closure. The synergism of MJ or ABA with SNP suggests that MJ or ABA interacts with NO and augment each other.

We have been studying the pattern of stomatal response to MJ in relation to the signal transduction (Suhita et al., 2003). In our continuing effort to compare the signaling compounds during stomatal closure induced by MJ or ABA, cytosolic pH and ROS were shown to be among signaling components during the effects of ABA or MJ (Suhita et al., 2004). The present results add that NO is also involved during the transduction of ABA or MJ-signals. At least three enzymes could mediate NO production in plants: nitrate reductase (NR) (Desikan et al., 2002, Kaiser et al., 2002) inducible NOS (iNOS) (Chandok et al., 2003) and constitutive NOS that are activated by pathogen-derived elicitors (Fossiner et al., 2000). The partial reversal by L-NAME of MJ or ABA induced stomatal closure suggests that NOS is important but is possibly not the sole source of NO during MJ or ABA effects on guard cells.

The release of NO in cells can be monitored real time imaging with confocal microscopy and NO sensitive fluorophore DAF-2DA (Foissner et al., 2000, Kojima et al., 1998). Kinetic studies using DAF-2DA revealed that ABA

or MJ induced increase in NO reached maximum by 20 minutes (Fig. 6.7). This is similar to the time observed for the rise in NO, along with accumulation of large amounts of H_2O_2 in soybean suspension cells (Delledonne et al., 2001). Compared to the increase in NO levels, the ROS accumulation in guard cells continued until 30 min (Fig. 6.9). The time of 30 min for ROS accumulation is quite similar to the pattern observed in *Arabidopsis* and *Pisum sativum* by us earlier (Suhita et al., 2004).

Changes in cytosolic pH are involved in stomatal regulation. Substances that raise cytosolic pH (ABA or MJ) result in stomatal closure (Blatt and Armstrong, 1993, Suhita et al., 2004) and substances lowering cytosolic pH (auxin, fusicoccin) open stomata (Irving et al., 1992). Butyrate is a weak acid and can decrease cytoplasmic alkalinization and modulate related events, for e.g. K^+ channel (Blatt and Theil, 1994). The presence of butyrate not only decreased the extent of stomatal closure, but also decreased NO production caused by ABA or MJ (Table 6.1). The changes in NO are almost as fast as that in the pH (Fig. 6.8). We therefore feel that cytoplasmic pH would be an important feature in promoting or maintaining NO synthesis. There are reports that the NO-molecule could be very active at alkaline pH (Beckman et al., 1990) while at neutral pH being unreactive (Reiter et al., 2000). Further experiments are needed to study in detail the interrelationship of cytosolic pH changes and NO production in guard cells.

Signals such as ABA or high CO_2 cause stomatal closure, by elevating cytosolic free Ca^{2+} (Allen et al., 1999, Webb et al., 1996). It is therefore proposed that the signaling components during these events converge at the

level of cytosolic Ca^{2+}. The marked reversal of stomatal closure by EGTA without affecting the rise in NO-levels (Table 6.3) indicates that rise in cytosolic Ca^{2+} possibly occurs after the changes in NO -levels, due to either ABA or MJ. A similar observation changes in Ca^{2+} occurs after the cytosolic alkalinization and ROS-production by MJ has recently been reported (Suhita et al., 2004). It has been suggested by protein phosphorylation involving a MLCK could be a step before pH changes during MJ-effects (Suhita et al., 2004).

The increase in cytosolic Ca^{2+} due to ABA occurs due to the involvement of several secondary messengers and signaling components. Some of these are protein kinases, PLC, cytosolic pH, IP_3, cGMP, cADPR. These components can be modulated by suitable pharmacological compounds. However experiments with pharmacological compounds affecting protein kinases and calcium channels were inconclusive. However experiments using selected compounds did not give any specific result. The reversal of stomatal closure along with restriction in ABA induced NO-production by ML-7, staurosporine, wortmannin, LY294002, W7, U73122, ODQ and nicotinamide all (Table 6.2) indicate that protein kinases, PLC, guanyl cyclase and cADPR are important during NO-production and subsequent stomatal closure. Further experiments are necessary to ascertain and identify the precise role of these different signaling components during NO-production in response to MJ or ABA.

6.4 Conclusions

1. MJ or ABA induced stomatal closure is associated with an increase in the levels of the NO in guard cells. Suppression of NO levels, by either scavenging in the presence of c-PTIO or inhibiting NOS (by L-NAME) reversed the stomatal closure by ABA or MJ.
2. The change in the NO-levels of guard cells and responses to NO-modulators was more pronounced in case of MJ than that of ABA.
3. Real time monitoring with the help of fluorescent dyes indicated that NO production in guard cell peaked at 18 min, compared to 30 min incase of ROS and 15 min in case of pH. Thus changes in NO or pH preceded ROS production.
4. Modulation of pH resulted in changes in NO production as well, indicating that changes in guard cell pH and NO production are either related or interact with each other.
5. Rise in internal Ca^{2+} appears to be a final event after NO-increase, as EGTA reversed stomatal closure, despite sustaining the increase in NO-production by MJ or ABA.
6. Experiments using pharmacological compounds: ML-7 (MLCK inhibitor), staurosporine (broad-spectrum of PK inhibitor), wortmannin/LY294002 (PI_3K/PI_4K inhibitor), W7 (CaM antagonist), U73122 (PLC inhibitor), ODQ (GC inhibitor), nicotinamide (cADPR inhibitor), which effect different protein kinases or calcium mobilizing components were inconclusive. All these compounds decreased NO-levels and reversed stomatal closure caused by MJ or ABA.

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Chapter 7

Reactive Oxygen Species and pH Changes are Common Signaling Elements During Abscisic Acid or Methyl Jasmonate Induced Shrinkage of Guard Cell and Mesophyll Protoplasts

Chapter 7

Reactive oxygen species and pH changes are common signaling elements during abscisic acid or methyl jasmonate induced shrinkage of guard cell and mesophyll protoplasts

7.1 Introduction

There is growing interest in physiological and molecular basis of the action of plant hormones, with considerable progress in our understanding of the effects of ABA (Finkelstein and Rock, 2002, Kuhn and Schroeder, 2003). Another interesting growth hormone, which has marked inhibitory effect on plant processes is MJ (Turner et al., 2002, Devoto and Turner, 2003). Some of the MJ responses are similar to that of ABA such as wounding, elicitation of phytoalexins, desiccation, abscission, stomatal closure (Sembdner and Parthier, 1993, Armengaud et al., 2004). In certain instances JA was found to be more efficient than ABA, for e.g. promotion of leaf senescence (Ueda et al., 1981), or stomatal closure (Raghavendra and Reddy, 1987). The vegetative storage proteins (Mason and Mullet, 1990) and lipoxygenase (LOX) gene in soybean are markedly induced after jasmonate treatment (Bell and Mullet, 1991).

Despite the extensive efforts, our knowledge of plant signal transduction is still incomplete. Several signaling components are identified during transduction of hormonal signals such as receptors, ion channels, G-proteins and other secondary messengers including protein kinases and protein phosphatases (Jones et al., 1998, Fairchild and Quail, 1998, Sanders et al.,

1999), but the exact sequence is still ambiguous. During ABA induced stomatal closure, cytosolic Ca^{2+} increases, cytosolic pH gets alkaline and reactive oxygen species (ROS) accumulates but the role of these signals during MJ-action is still unclear (MacRobbie, 1998, Assmann and Shimazaki, 1999). In the present work, the importance of pH shifts and ROS production was studied with respect to both ABA and MJ.

Protoplasts have been useful for diverse studies on physiology, biochemistry, molecular biology and biotechnology, including plant regeneration and genetic engineering (Maurel et al., 1995). The system of protoplasts is ideal for experiments to examine plant metabolism (e.g. photosynthesis, respiration) and responses to different stress conditions (e.g. chilling, supra-optimal light, drought) (Saradadevi and Raghavendra, 1992, 1994, Raghavendra et al., 1994).

Protoplasts could be a simple system to study plant cell responses to osmotic stress, as their volume changes can be monitored by using either their diameter or their absorbance at 440 nm, corrected for turbidity at 750 nm (Willmer et al., 1999). The decrease in protoplast volume or contraction was strongly correlated with the extent of inhibition of photosynthesis (Saradadevi et al., 1995) and stomatal closure (Willmer and Fricker, 1996). Isolated protoplasts provide also an opportunity to study the physiological and molecular properties, such as membrane fluidity and gene expression (Sheen, 2001).

The swelling and shrinkage of the GCP of *C. communis, A. cepa, V. faba* have been considered to be equivalent to the opening and closure of

stomata, as the stomata open when the guard cells are turgid (and swell) and stomata close when the guard cells are flaccid (and shrink) (Willmer and Fricker, 1996). Such swelling or shrinkage is due to the modulation of turgor in guard cells (Assmann, 1993). The changes in the turgor of guard cells are due to marked modulation of both inward and outward K^+ channels (MacRobbie, 1998, 2000). Typical factors or signals, which trigger K^+ influx or Ca^{2+} influx, lead to shrinkage of guard cells.

In this report, we demonstrate that guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) of *Arabidopsis* and *Pisum sativum* swell or shrink in response to MJ or ABA signaling. The role of pH and ROS production were also examined, using volume changes in GCP or MCP as a tool to study the responses to MJ or ABA.

7.2 Results

7.2.1 Volume changes of MCP or GCP on exposure to MJ or ABA

When GCP or MCP were exposed to MJ or ABA, there was a marked decrease in their volume. The decrease in volume of GCP or MCP was dependent on the concentration of hormone. The volume of GCP decreased by >60% at 20 μM MJ or ABA (Fig. 7.1A). The mesophyll protoplasts also decreased as the concentration of MJ or ABA increased. However, the decrease in volume of MCP was only about 20% at 20 μM MJ or ABA (Fig. 7.1B). Thus, the decrease in volume of MCP by MJ or ABA was much less than that of GCP.

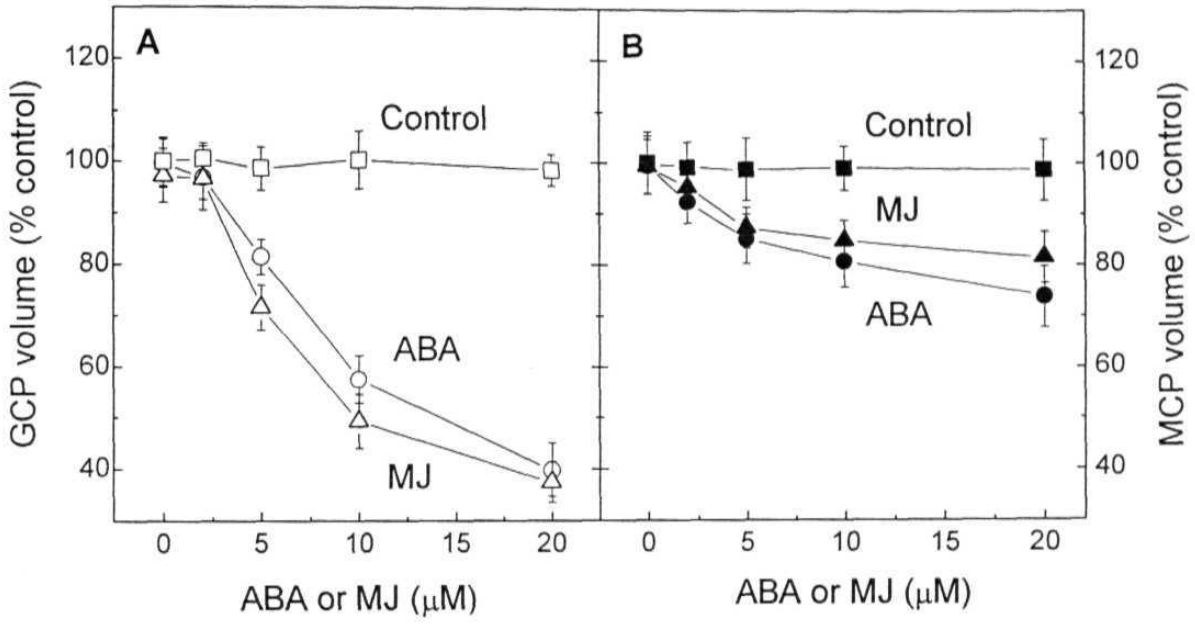

Figure 7.1: Shrinkage of protoplasts from *Arabidopsis* in response to increasing concentration of MJ or ABA. (A) Guard cell protoplasts (GCP); (B) Mesophyll cell protoplasts (MCP). The protoplasts were incubated at room temperature of 22 ± 2 ^{0}C for 30 minutes in the absence or presence of MJ or ABA. The volume changes are expressed in relation to the volume at the beginning of the experiment (taken as 100%). The average volumes of GCP were 3.3 ± 0.1 fl and MCP were 56.7 ± 3.3 pl. The data are the averages ± SE of three independent experiments. Further details are described in Materials and Methods.

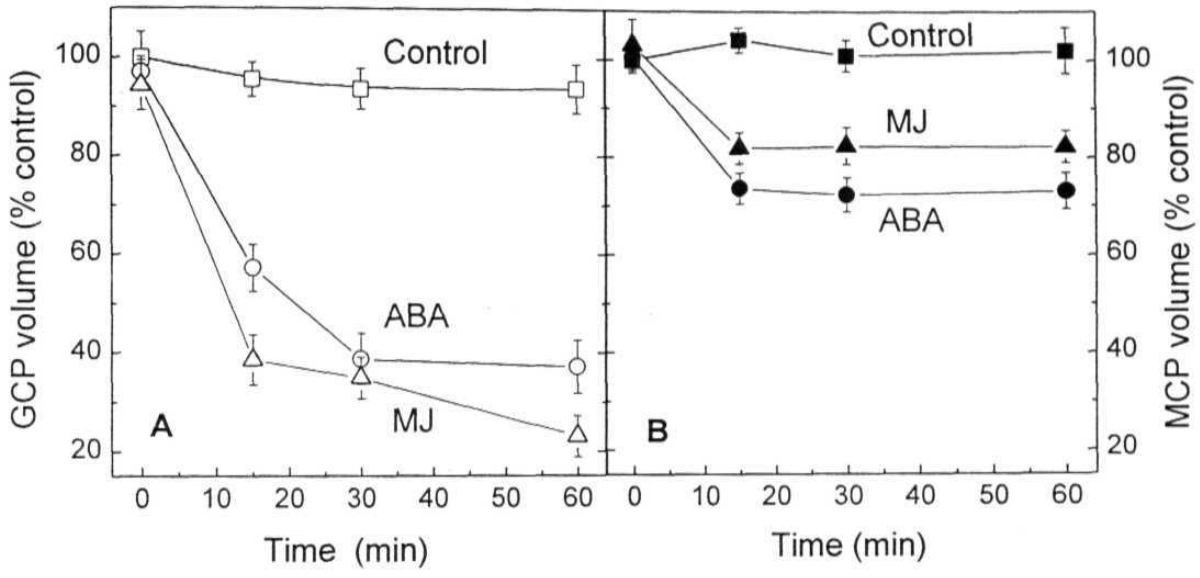

Figure 7.2: The time course of changes in volume of *Arabidopsis* protoplasts in response to 20 μM ABA or MJ. (A) GCP; (B) MCP. The average volumes of GCP were 3.4 ± 0.1 fl and MCP were 55.4 ± 2.7 pl. Further details are as in Fig. 7.1.

The volume changes in GCP were not spontaneous but were gradual and required a few minutes. Maximum shrinkage occurred by 30 min, in case of GCP (Fig. 7.2A) but was accomplished by 15 min in case of MCP (Fig. 7.2B). Again the extent of shrinkage due to MJ or ABA was more pronounced in GCP that that of MCP. Further, the GCP was more sensitive to MJ than that to ABA, in relation to shrinkage. Similarly, the MCP was more responsive to ABA than to MJ.

7.2.2 Changes in pH of MCP or GCP on exposure to MJ or ABA

The pH of protoplasts can be determined by the null point method, developed to measure pH in aluerone protoplasts (van der Veen et al., 1992). Using this null point method, the pH of GCP and MCP were monitored with respect to MJ and ABA treatment. Treatment of GCP and MCP with 20 μM MJ or ABA resulted in an increase in pH. The increase in pH induced by ABA was more in MCP than that of in case of GCP. In contrast, the rise in pH in response to MJ was much more pronounced in case of GCP that that with MCP. The increase in pH in case of both GCP and MCP peaked by 15 min and pH decreased thereafter (Fig. 7.3).

The alkalization of cells or protoplasts can be restricted by adding externally weak acids such as butyrate or propionate or acetate. Therefore, the effect of butyrate was examined on pH and volume changes of GCP and MCP after exposure to ABA or MJ. Butyrate at 0.5 mM reversed the MJ/ABA induced increase in pH of both MCP and GCP (Fig. 7.4). These observations are summarized in Table 7.1.

7.2.3 Changes in ROS levels of MCP or GCP

Dihydrochlorofluorescein diacetate (H_2DCF-DA), a fluorescent dye was used to measure the changes in ROS levels. As indicated by the fluorescence of H_2DCF-DA, with ABA or MJ resulted in a marked increase in ROS levels (Fig. 7.5). The ROS production peaked by 30 min in both GCP and MCP. As in the case of pH, ABA induced ROS production was more than that of MJ in MCP, while MJ induced ROS induction was greater than that by ABA in GCP.

The enzyme NAD(P)H oxidase, which mediates ROS production can be inhibited by DPI. The effect of DPI [NAD(P)H oxidase inhibitor] was therefore examined on volume changes of MCP and GCP after exposure to ABA or MJ. DPI at 12.5 μM reversed the MJ/ABA induced shrinkage of protoplasts the effect was marginally low in comparison to the effect of pH modulator butyrate (Fig. 7.6). The volume changes had shown variation in GCP and MCP in case of mutant the shrinkage of protoplasts was similar to the wild type MCP, but the GCP were insensitive to the hormones (Fig. 7.7).

Table 7.1: Absolute measurements of pH in protoplasts of *Arabidopsis* wild type (Columbia) in response to 20 μM ABA or 20 μM MJ. The pH was determined by "null-point" method, using digitonin for permeation of GCP or MCP. Further details are described in Materials and Methods.

Hormone	MCP	GCP
None	7.18 ± 0.03	7.33 ± 0.02
ABA	7.58 ± 0.03	7.51 ± 0.04
MJ	7.42 ± 0.02	7.74 ± 0.03

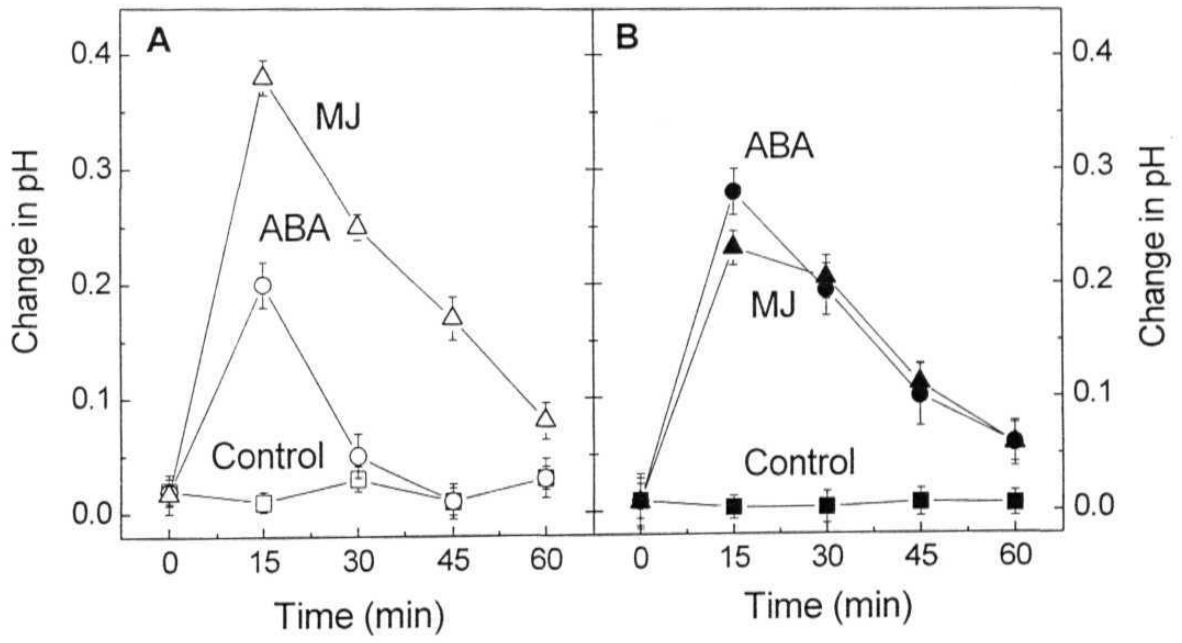

Figure 7.3: Kinetics of pH changes in the suspension of protoplasts from *Arabidopsis*, in response to 20 μM ABA or MJ (A) GCP; (B) MCP. The initial pH of GCP without hormone was 7.3 ± 0.02 and of MCP 7.2 ± 0.03. The pH was determined using "null point" method, as described in Materials and Methods.

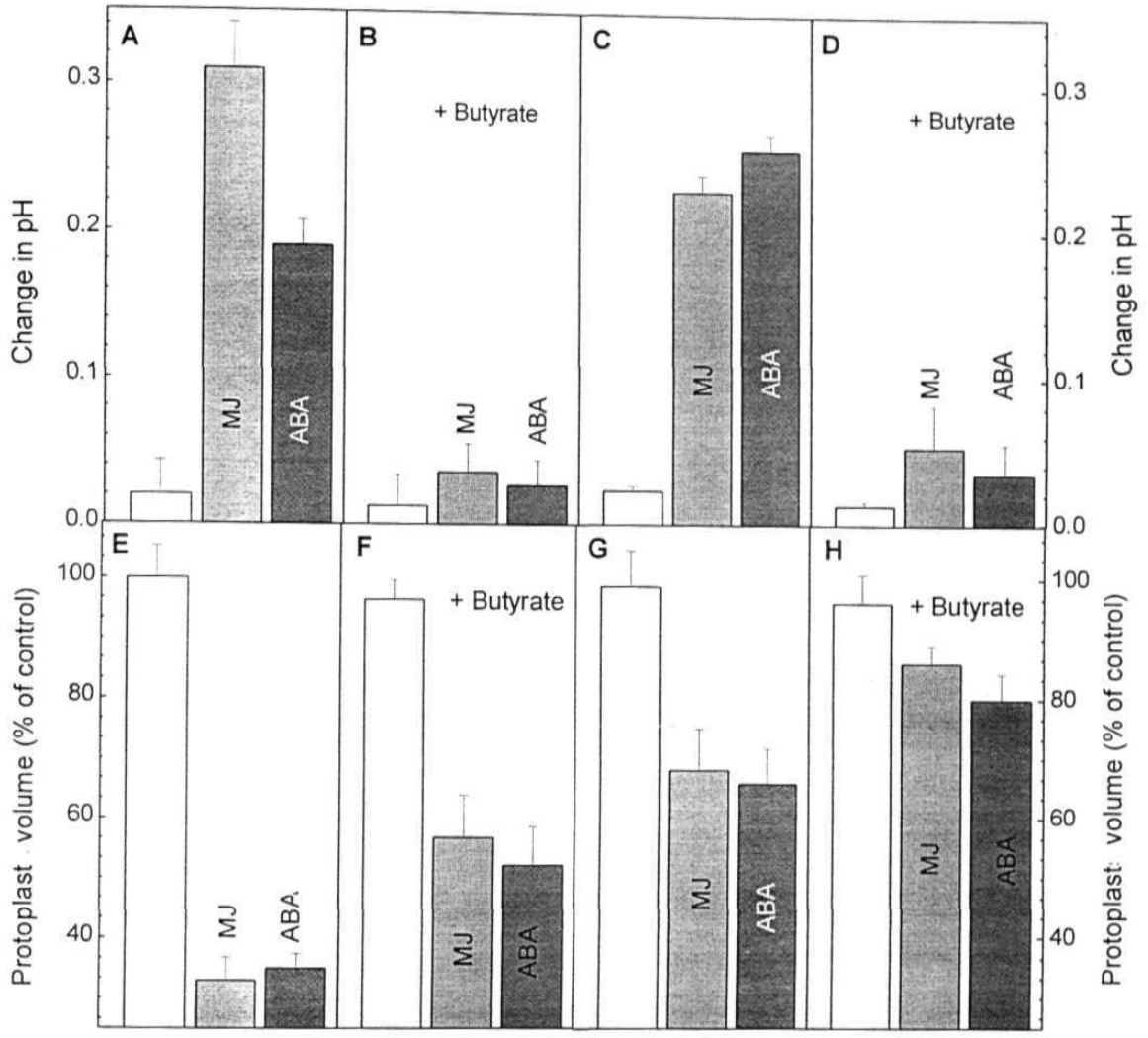

Figure 7.4: Change in pH of protoplast suspension of *Arabidopsis* after permeation with digitonin and volume changes in relation to the values at the beginning of the experiment (taken as 100%), in the absence or presence of 20 μM MJ or ABA. A, B, E and F: GCP and C, D, G and H: MCP. The change in pH due to MJ or ABA was examined in the absence (A, C, E, G) or presence of 0.5 mM butyrate (B, D, F, H). The initial pH of GCP was 7.3 ± 0.04 and MCP 7.2 ± 0.03. The initial volumes of GCP were 3.1 ± 0.2 fl and of MCP were 54.7 pl. Further details are described in Materials and Methods.

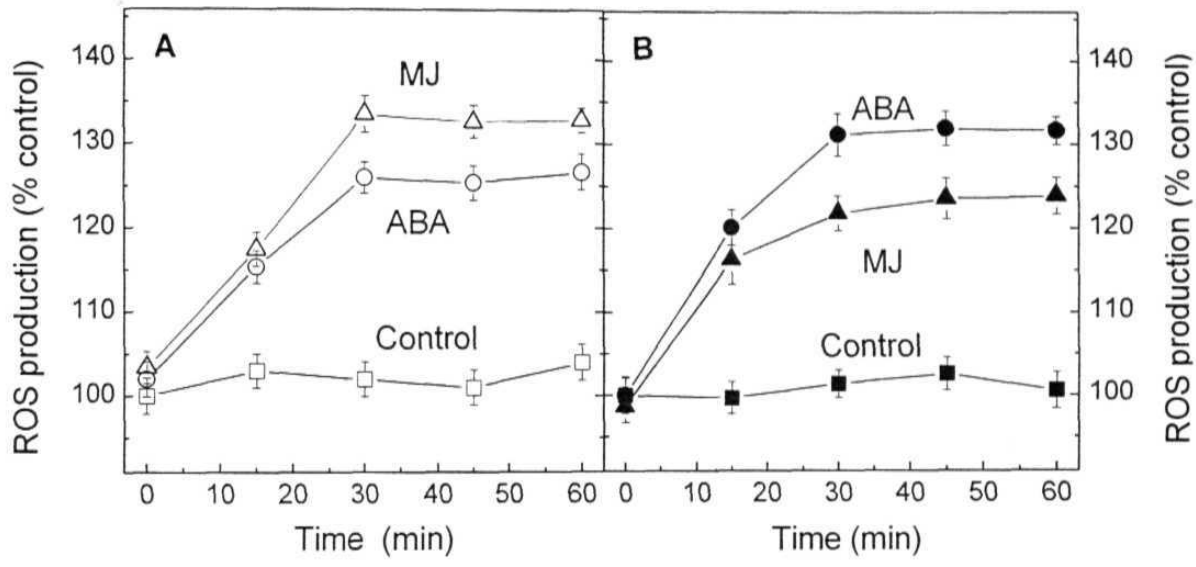

Figure 7.5: The time course of ROS production in protoplasts of *Arabidopsis* in response to 20 μM ABA or 20 μM MJ (A) GCP and (B) MCP. The ROS production was monitored using the fluorescent dye, H_2DFCDA, as described in Materials and Methods. The data are all the means ± SE from at least three independent experiments.

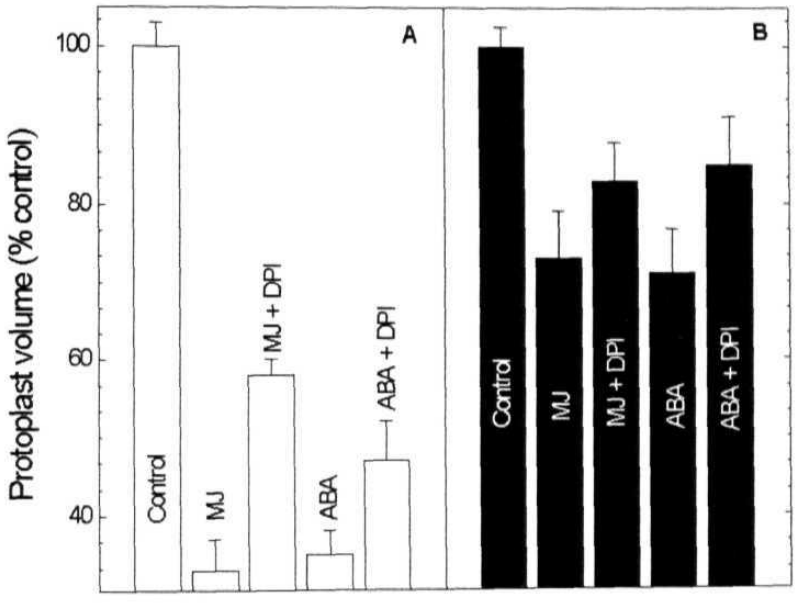

Figure 7.6: Volume changes in (A) GCP and (B) MCP of pea induced by 20 μM ABA or MJ in the absence or presence of 12.5 μM DPI (inhibitor of ROS). The volumes were calculated as % of control i.e., without hormone. Average volumes of GCP were 1.7 ± 0.05 fl and of MCP were 49.4 ± 2.7 pl and other details are as in Fig. 7.1.

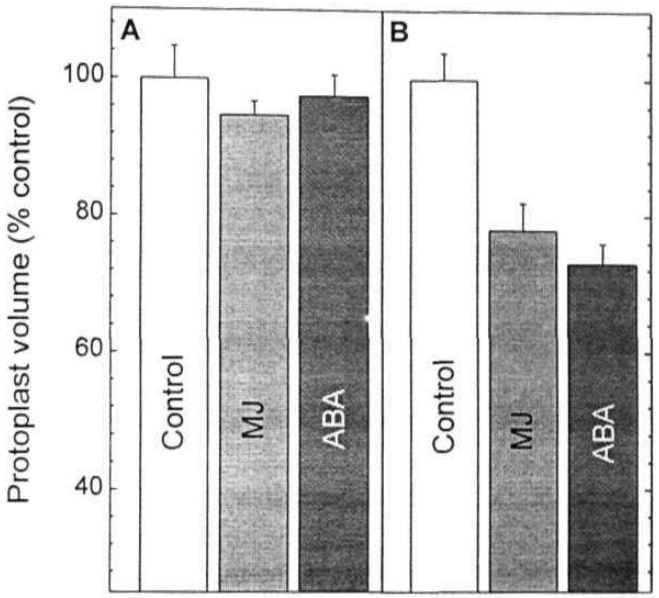

Figure 7.7: MJ or ABA (both at 20 μM) induced volume changes in *Arabidopsis* mutant (*atrbohD/F*) protoplasts (A) GCP; (B) MCP. The average volumes of GCP were 3.2 ± 0.4 fl and of MCP were 56.2 ± 3.8 pl. Further details are as in Fig. 7.1.

7.3 Discussion

Our results establish that the protoplasts can be a simple and sensitive system to study plant cell responses to hormones. The shrinkage or swelling of protoplasts on exposure to changes in osmotic strength of the suspension medium are known (Saradadevi et al., 1995, Willmer et al., 1999). A decrease in volume of mesophyll protoplasts by MJ or ABA has earlier been briefly reported (Kolla et al., 2004). The present study points out that GCP are not only sensitive to MJ or ABA, but also respond more vigorously to these hormones than the MCP (Fig. 7. 1 and 7.2).

The marked responses of both GCP and MCP to not only ABA but also MJ, in terms of volume changes, pH changes or ROS production are not surprising. The levels of ABA as well as jasmonic acid increase significantly on the exposure of plant cell to drought or water deficit (Creelman and Mullet, 1997). Similarly MJ has been reported to modulate growth and development responses, stomatal opening, desiccation and responses to water/osmotic stress as is the case of ABA (Raghavendra and Reddy, 1987, Sembdner and Parthier, 1993, Turner et al., 2002).

The participation of multiple secondary messengers in plant tissues during the transduction of hormonal signals is expected (Blatt and Armstrong, 1993, Cheong et al., 2002). In the present work, attempt is made to examine the role of pH during the effects of ABA and MJ on GCP and MCP. The changes in pH were determined and these observations were complemented by experiments to modulate pH by butyrate, a weak acid. Butyric acid is frequently used in plant studies to evoke an acidification

of cytoplasm (Guern et al., 1991). The marked alkalization of GCP and MCP on exposure to MJ or ABA (Fig. 7.3) and the reversal of the ABA or MJ effects by butyrate confirm that the cytosolic alkalinization is an important event during the ABA or MJ induced shrinkage of protoplasts. Cytosolic alkalinization of plant cells on treatment with ABA has been widely demonstrated (Blatt, 2000a, Knight and Knight, 2001). The reports on MJ induced cytosolic alkalinization is however are very few (Gehring et al., 1997, Suhita et al., 2004).

Increase in ROS levels were recorded in plant cells on exposure to either ABA or MJ (O'Donnell et al., 1993, Lee et al., 1999). Such increase in ROS may lead to mobilization of internal calcium and changes in gene expression. Very similar to other plant cells, marked increase in ROS occurred in both GCP and MCP on exposure to MJ or ABA (Fig. 7.5). The presence of DPI, an inhibitor of NAD(P)H oxidase, restricted the ROS production and prevented the shrinkage of protoplasts (Fig. 7.6). These observations suggest that the rise in ROS occurs during protoplast shrinkage (Fig. 7.5 and 7.6), as in the case of stomatal closure (Pei et al., 2000, Murata et al., 2001), leaf elongation (Rodriguez et al., 2004), and responses to biotic/abiotic stress (e.g. chilling, heat and pathogens) (Prasad et al., 1994, Alvarez et al., 1998).

The study on the double mutant of *Arabidopsis* (*AtrbohD/F*) deficient in both isoforms of plasma membrane NAD(P)H oxidase impaired of ABA induced stomatal closure, ABA promotion of ROS production (Kwak et al., 2003). These genes are highly expressed has been shown limited specifically

to guard cells. Due to expression of one NAD(P)H oxidase isoform in mesophyll cell there could be retardation of change in volume of MCP similar to the wild type response (Fig. 7.7).

The data on temporal patterns of protoplasts shrinkage (Fig. 7.1and 7.2), pH changes (Fig. 7.3) and ROS production (Fig. 7.5) reveals two important points. First point is that the increase in pH is transient and precedes the decrease in protoplast volume. The second point is that ROS production occurs later and continues to reach a plateau. The cytosolic alkalinization as an early event during stomatal responses to MJ or ABA is now well documented (Blatt and Grabov, 1997, Suhita et al., 2004). The later start of ROS production during MJ or ABA action is reported recently (Suhita et al., 2004). Further experiments are necessary to understand the interaction of cytosolic alkalinization and ROS production. It is possible that the increase in cytosolic pH triggers ROS production but this point requires validation.

The present results highlight the usefulness of protoplasts, to study the responses of plant cells to hormonal signals such as MJ or ABA and also indicate that ROS and pH changes are common elements during MJ or ABA signaling even in different cell types. There was an important difference between GCP and MCP in their responses to MJ and ABA. The increase in pH or ROS and decrease in volume all were much more prominent with MJ, than that with ABA (Fig. 7.1, 7.3 and 7.5). Such high sensitivity of guard cells to MJ has not been reported earlier. Further experiments are needed to identify the factors, which contribute to the high sensitivity of guard cells to MJ.

7.4 Conclusion

1. MJ and ABA caused shrinkage of protoplasts and the extent of such shrinkage, was much more dramatic in case of GCP than that of MCP.
2. Exposure to ABA or MJ caused marked alkalization and the production of ROS in both GCP and MCP.
3. Presence of butyrate, a weak acid, restricted the pH changes, as well as shrinkage in GCP or MCP induced by MJ or ABA. DPI, inhibitor of NAD(P)H oxidase, decreased the ROS production and prevented partially the shrinkage of both MCP and GCP.
4. These results demonstrate that the alkalization and ROS production are among the components of common signaling pathways in the responses to ABA or MJ of both GCP and MCP.
5. There is a difference in the relative sensitivity of GCP or MCP to MJ or ABA. The extent of decrease in protoplasts volume, pH changes and ROS production in presence of MJ was more in GCP than those in MCP. In contrast, MCP responded more to ABA than that to MJ.

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Chapter 8

General Discussion

Chapter 8
General Discussion

The stomatal guard cells respond quickly and efficiently to several internal and external signals. The guard cells have therefore become a model system to study transduction of plant hormones. Among the growth hormones abscisic acid (ABA) and methyl jasmonate (MJ) suppress stomatal opening, while cytokinins and auxins promote stomatal opening (Mansfield et al., 1990, Raghavendra and Reddy, 1987). The mechanism of ABA effects on guard cells has been studied extensively (Leung and Giraudat, 1998, Schroeder et al., 2001a, Desikan et al., 2004, Wang et al., 2004). The modulation of cytosolic free Ca^{2+} or release from internal stores such as vacuole or endoplasmic reticulum into the cytosol of guard cells seems to be a major factor during ABA-induced stomatal closure. Calcium then can act on guard cells in several ways: inhibition of inward K^+ channels, stimulation of Cl^- efflux leading to depolarization of the plasma membrane, activation of outward K^+ channels and interaction with calmodulin (CaM) (Schroeder et al., 2001a).

Compared to the extensive literature available on the effects of ABA on guard cells, studies on MJ-induced stomatal closure are quite limited. Jasmonates, play an important role in metabolic, developmental and defensive processes in plants (Wasternack and Hause, 2002, Farmer et al., 2003, Thaler et al., 2004). MJ is reported to participate in processes, such as promotion/inhibition of growth, wounding in response to pathogens, promotion of senescence, tuberization and bulb formation

(Sembdner and Parthier, 1993, Creelman and Mullet, 1997, Devoto and Turner, 2003, Ryu et al., 2004).

Several responses of plants to MJ are similar to that of ABA, and both these hormones form a part of signal transduction chain between stress signals and stress responses (Wiedhase et al., 1987, Creelman and Mullet, 1997, Casaretto et al., 2004). JA-dependent wound signaling in tobacco appears to involve kinase-dependent JA accumulation, the effects of which are transduced by Ca^{2+} (Kenton, 1999). The induction levels of NtCDPK1 mRNA, DAHPS mRNA's and ATPase activity of Ca^{2+}-ATPase went up after treatment with MJ (Starling et al., 1994, Suzuki et al., 1995, Yoon et al., 1999). Most of the research, on the effects of ABA and MJ on plant tissues was found on regulation of gene expression. Nevertheless there have been detailed studies on nitric oxide (NO) and reactive oxygen species (ROS) and their involvement in signal transduction. It is likely that NO does not act alone, but interacts with other signaling molecules such as hydrogen peroxide (H_2O_2) to effect stomatal closure (Neill et al., 2003).

It is now clear that NO and ROS function as signaling molecules during stomatal closure (Desikan et al., 2004). Sodium nitroprusside (SNP), a NO donor, promotes stomatal closure in presence of ABA, while stomatal closure is prevented by cPTIO (2-phenyl-4, 4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide) a specific NO scavenger. Recent work has demonstrated that NO burst after wound responses or after treatment with JA in *Arabidopsis thaliana* (Huang et al., 2004). Further NO and ROS are

essential intermediates in ABA induced stomatal closure in *Pisum sativum* and *Vicia faba* (Zhang et al., 2001, Neill et al., 2002, Garcia-Mata and Lamattina, 2002). However, despite these emerging new roles for NO it's biosynthetic route has not yet been resolved. Similarly the role of NO during stomatal closure by MJ is not examined.

The present work examined the mechanisms involved in the suppression of stomatal opening by MJ or ABA. The importance of intracellular Ca^{2+} in guard cells during stomatal closure by ABA, CO_2 or H_2O_2 has been demonstrated by several workers (McAinsh et al., 1997, Webb and Hetherington, 1997, Assmann, 1999, Blatt, 2000). Our observations on promotion of stomatal closure by external Ca^{2+} (Fig. 4.1) and reversal by EGTA (Fig. 4.2) imply that the intracellular Ca^{2+} in guard cells may increase on exposure to MJ. These results are in agreement with previous studies on strong modulation by Ca^{2+} response to MJ in case of plant pathogen interaction and leaf senescence (Hung and Kao, 1998, Leon et al., 1998, Kenton et al., 1999, Leon et al., 2001).

However, there were some differences in the signaling components mediating stomatal closure by MJ or ABA. These appear to be due to differences in Ca^{2+} mobilization through Ca^{2+} channels or other processes by ABA or MJ. Several secondary messengers are known to be involved while responding to ABA e.g. phosphoinositides, G-proteins, PLC, Ca^{2+} channels, protein kinases including PKC or myosin light chain kinases (Hetherington, 2001, Fedoroff, 2002, Wang et al., 2004). The presence of PMA (stimulator of PKC) or U73122 or 1-butanol (inhibitors of PLC/PLD)

reversed the ABA-induced stomatal closure, but not that of MJ (Figs. 4.8, 4.9 and 4.10) indicating that the effect of ABA involved the modulation of PLC/PLD and PKC. These results are consistent with the earlier observations of the effect of inhibitors of PLC/PLD or protein kinases in reversal of ABA induced stomatal closure (Jacob et al., 1999, Schroeder et al., 2001).

In contrast with the response to ABA, the stomatal closure caused by MJ does not seem to depend much on PLC or PKC. The action of MJ appears to be strongly dependent on the Ca^{2+} channels as indicated by the complete reversal of MJ effect by three Ca^{2+} channel blockers: lanthanum, ruthenium red or verapamil (Figs. 4.3, 4.4 and 4.5). The ABA induced stomatal closure may involve both Ca^{2+} dependent and independent signal transduction pathways (Allan et al., 1994, Leung and Giraudat, 1998). Our studies confirm that Ca^{2+} plays a vital role to mediate the effect of both ABA/MJ during stomatal closure (Fig. 4.11). The Ca^{2+} dependence of MJ action appears to be greater than that in case of ABA, as indicated by the pronounced effect of EGTA in reversing the closure caused by MJ (Fig 4.2).

The use of *Arabidopsis* has been instrumental in the understanding of stomatal function. Using a novel approach of infrared thermography, several mutants of *Arabidopsis*, with altered stomatal function, were isolated. Studies on the mutants revealed the importance of different signaling components in stomatal movements, for e.g. ABI1 & ABI2 (protein phosphatases type 2C), OST1 (ABA activated proteinkinase), *atrbohD/F*

(plasmamembrane NAD(P)H oxidase) (Kwak et al., 2003), *GORK* (outward K^+-channel) (Mustilli et al., 2002, Schroeder, 2003, Hosy et al., 2003).

The pattern and sequence of signaling events involved in ABA or MJ-induced stomatal closure were examined in *Arabidopsis* wild type and ABA or MJ insensitive mutants *ost1*-2 and *jar1*-1, respectively (Fig. 5.1). The stomatal response to MJ was more sensitive to Ca^{2+}-calmodulin (CaM) protein kinase inhibitors (e.g. W7) than that of ABA (Fig. 5.2). These inhibitors were able to reverse the response to MJ, while the response to ABA was only partially affected. These findings suggest that at least one protein kinase with a Ca^{2+}-CaM like regulatory domain plays an essential role in MJ response, while such activity appears to participate to a limited extent in the ABA cascade. In contrast, a broad range inhibitor of protein kinase (K252A) was able to suppress both responses suggesting that Ca^{2+}-dependent and Ca^{2+}-independent protein kinases are involved parallely during the ABA or MJ signaling.

Stomatal closure by MJ or ABA was associated with alkalinization of the guard cell cytoplasm and elevation of ROS production. Interestingly, K252A was able to suppress also pH changes and ROS production induced by MJ or ABA (Table 5.1), suggesting that a protein phosphorylation event is essential and located upstream of pH change responses. An analysis of the kinetics of pH changes and ROS production revealed that alkalization preceded ROS production during the stomatal closure caused by both ABA and MJ (Fig. 5.6). Alkalization of the guard cells occurred in the presence of MJ and stomatal closure by

MJ was suppressed in presence of the weak acid butyrate (Fig 5.7 and Table 5.3). Thus, our results confirm the earlier observations on alkalinization of the guard cell cytoplasm observations by Gehring et al. (1997).

A partial scheme of events, which occur in guard cells after exposure to MJ or ABA, can be constructed based on our results (Fig 5.9). The limited responses of *jar1*-1 and *ost1*-2 allow placing JAR1 and OST1 upstream of cytoplasmic alkalization in the MJ and ABA-signaling pathways, respectively. The sequence of events during signal transduction appears broadly similar for MJ and ABA. At least one protein phosphorylation event is necessary for the cytoplasmic alkalization, which leads to ROS production by the NAD(P)H oxidase. In turn, ROS would activate Ca^{2+} channels and elevate cytoplasmic Ca^{2+}.

Among the last steps in the cascade leading to stomatal closure is the activation of the outward K^+ rectifier from guard cells (Armstrong et al., 1995), allowing potassium efflux and loss of turgor. These outward K^+-channels are down regulated by H_2O_2 (Köhler et al., 2003), and are up regulated by a rise in cytoplasmic pH (Miedema and Assmann, 1996). Furthermore, the insensitivity of the *gork1* mutant to MJ demonstrates that GORK is an essential component in the MJ signaling cascade, leading to stomatal closure (Fig. 5.8).

It is well established that ROS and NO are essential signaling components in mediating ABA induced stomatal closure (Neill et al., 2002). The presence of SNP (a NO donor) promoted stomatal closure while c-PTIO and L-NAME, which are expected to decrease NO, reversed

stomatal closure by ABA or MJ (Fig. 6.2 and 6.3). Thus, the present study demonstrates, for the first time, the importance of NO mediated signaling in also MJ dependent stomatal closure. At least three enzymes could mediate NO production in plants: nitrate reductase (NR) (Desikan et al., 2002, Kaiser et al., 2002), inducible NOS (iNOS) (Chandok et al., 2003) and constitutive NOS that are activated by pathogen-derived elicitors (Fossiner et al., 2000). Only a partial reversal by L-NAME of MJ or ABA induced stomatal closure suggests that NOS is important but it is possibly not the sole source of NO during MJ or ABA effects on guard cells.

Kinetic studies using DAF-2DA revealed that ABA or MJ induced increase in NO reached maximum by 20 minutes (Fig. 6.7). This is similar to the time observed for the rise in NO, along with accumulation of large amounts of H_2O_2 in soybean suspension cells (Delledonne et al., 2001). Compared to the increase in NO levels, the ROS accumulation in guard cells continued until 30 min (Fig. 6.9). The time of 30 min for ROS accumulation is quite similar to the pattern observed in *Arabidopsis thaliana* and *Pisum sativum* (Suhita et al., 2004).

Changes in cytoplasmic pH are involved in stomatal regulation. Substances that raise cytoplasmic pH (ABA, MJ) result in stomatal closure (Blatt and Armstrong, 1993, Suhita et al., 2004) and substances lowering cytoplasmic pH (auxin, fusicoccin) open stomata (Irving et al., 1992). Butyrate can decrease cytoplasmic alkalinization and modulate related events, e.g. K^+ channels (Blatt and Theil, 1994). The presence of butyrate not only decreased the extent of stomatal closure, but also decreased NO

production caused by ABA or MJ (Table 6.1). We therefore suggest that cytoplasmic pH is an important feature in promoting or maintaining NO action/synthesis. There are reports that the NO-molecule could be very active at alkaline pH (Beckman et al., 1990) while at neutral pH being unreactive (Reiter et al., 2000). Further experiments are needed to study in detail the interrelationship of cytosolic pH changes and NO production in guard cells.

Signals such as ABA or high CO_2 cause stomatal closure, by elevating cytosolic free Ca^{2+} (Webb et al., 1996, Allen et al., 1999). Our results emphasize that the effects of ABA or MJ converge at the level of cytosolic Ca^{2+}. The marked reversal of stomatal closure by EGTA without affecting the NO-levels (Table 6.3) indicates that the rise in cytosolic Ca^{2+} possibly occurs after the changes in NO-levels, due to either ABA or MJ. A similar situation on the role of Ca^{2+} was observed during cytosolic alkalinization and ROS-production by MJ or ABA (Suhita et al., 2004).

Another interesting and useful observation from the present study is that the protoplasts can be a simple and sensitive system to study plant cell responses to hormones. The shrinkage or swelling of protoplasts on exposure to changes in osmotic strength of the suspension medium are known (Saradadevi et al., 1996, Willmer et al., 1999). A decrease in volume of mesophyll protoplasts by MJ or ABA has been briefly reported (Kolla et al., 2004). Our study points out that GCP are not only sensitive to MJ

or ABA, but also respond vigorously to these hormones as is the case of MCP (Fig. 7. 1 and 7.2).

The marked responses of both GCP and MCP to not only ABA but also MJ, in terms of volume changes, pH changes or ROS production are not surprising. The levels of ABA as well as JA increase significantly on the exposure of plant cells to drought or water deficit (Creelman and Mullet,, 1997). Similarly MJ has been reported to modulate growth and development responses, stomatal opening, desiccation and responses to water/osmotic stress as in the case of ABA (Raghavendra and Reddy, 1987, Sembdner and Parthier, 1993, Turner et al., 2002).

The present work demonstrated that the system of GCP or MCP can be used to study the role of pH or ROS during the effects of ABA and MJ. The marked alkalization of GCP and MCP on exposure to MJ or ABA (Fig. 7.3) and the reversal of the ABA or MJ effects by butyrate confirm that the cytosolic alkalinization is an important event during the ABA or MJ induced shrinkage of protoplasts. Cytosolic alkalinization occurs in plant cells on treatment with either ABA (Blatt, 2000, Knight and Knight, 2001), or MJ (Gehring et al., 1997, Suhita et al., 2004). Very similar to other plant cells, marked increase in ROS occurred in both GCP and MCP on exposure to MJ or ABA (Fig. 7.5). The presence of DPI, an inhibitor of NAD(P)H oxidase, restricted the ROS production and prevented the shrinkage of protoplasts (Fig. 7.6). These observations suggest that the rise in ROS occurs during protoplast shrinkage as in the case of stomatal closure (Pei et al., 2000, Murata et al., 2001).

The data on temporal patterns of protoplasts shrinkage (Fig. 7.1, 7.2), pH changes (Fig. 7.3) and ROS production (Fig. 7.5) offers two important points. First, the increase in pH is transient and precedes the decrease in protoplast volume. Secondly, ROS production occurs later and continues to reach a plateau. The cytosolic alkalinization as an early event during stomatal responses to MJ or ABA is now well documented (Blatt and Grabov, 1997, Suhita et al., 2004). The later start of ROS production during MJ or ABA action has been reported recently (Suhita et al., 2004). Further experiments are necessary to understand the interactions of cytosolic alkalinization and ROS production.

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Chapter 9

Summary and Conclusions

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Summary and Conclusions

Stomata are important organs regulating the gas exchange of leaves. Due to the extreme sensitivity of guard cells to several stimuli, stomatal movement helps in regulating transpiration and photosynthesis. Stomatal guard cells have therefore become a model system to study the mechanism of signal transduction. Among the plant hormones, ABA and MJ, were used extensively to study the signal transduction cascade, during the modulation of stomatal movements. Our observations of promotion by external Ca^{2+} and reversal by EGTA of stomatal closure imply that the intracellular Ca^{2+} in guard cells may increase on exposure to MJ or ABA. Thus, Ca^{2+} was an important messenger during the stomatal closure induced by not only ABA but also MJ. Strong modulation by Ca^{2+} of response to MJ has been observed in case of plant pathogen interaction and leaf senescence (Pieterse and van Loon, 1999). However, there were differences in the mode of action by ABA or MJ. The closure induced by ABA was dependent on the functioning of PLC/PLD and PKC. In contrast, the action by MJ was strongly dependent on Ca^{2+} channels and CaM.

The varied responses to Ca^{2+} in relation to stomatal closure by MJ or ABA could be due to differences in Ca^{2+} mobilization through Ca^{2+} channels or other processes. For example, several secondary messengers are known to be involved while responding to ABA, e.g. phosphoinositides, G-proteins, PLC, Ca^{2+} channels, protein kinases including PKC or MLCK (Schroeder et al., 2001a,b). The action of MJ appeared to be strongly dependent on the Ca^{2+}

channels as indicated by the complete reversal of MJ effect by three Ca^{2+} channel blockers: lanthanum, verapamil and ruthenium red. Some of these are published (Suhita et al., 2003).

The effects of Ca^{2+} are often mediated through CaM and can be assessed by using CaM antagonists, such as TFP or W7 (Shimazaki et al., 1992). The reversal of stomatal closure by TFP or W7 suggests that the effects of MJ are mediated by calmodulin, while such dependence on CaM appears to be low in case of ABA. Further, the dependence on Ca^{2+} appears to be much stronger in case of MJ than that of ABA as the presence of EGTA reversed completely the stomatal closure caused by MJ while being only partial in case of ABA. Thus, the involvement of Ca^{2+} and CaM was more pronounced during stomatal closure induced by MJ but not by ABA. All together, these observations suggest the existence of a cross talk between MJ and ABA signaling cascades.

To analyse further the role of other signaling components playing significant role in MJ and ABA pathways, mutants of *Arabidopsis* have been used. These were mutants affected in ABA signaling (*ost1*-2), or MJ signaling (*jar1*-1), or the plasma membrane NAD(P)H oxidases (*AtrbohD* x *AtrbohF)* or guard cell outward K^+-channel (*gork1*). The mutants responded differently when examined for MJ or ABA induced stomatal closure.

The stomatal response to MJ was more pronounced to Ca^{2+}-CaM protein kinase inhibitors than the stomatal response to ABA. This suggested that at least one protein kinase with a Ca^{2+}-CaM like regulatory domain played an essential role in MJ response. Interestingly, K252A (protein kinase inhibitor)

suppressed MJ- or ABA-induced pH changes and ROS production, suggesting that a protein phosphorylation event was essential and located upstream of these responses.

ABA is known to cause cytoplasmic alkalization in plant tissues (Irving et al., 1992, Gehring et al., 1997). There were marked changes also in the pH of the cytosol of guard cells, on exposure to MJ or ABA, within a span of 15 minutes. The patterns of pH changes were similar during measurements of pH by the null-point method or by using fluorescence probe. The ROS production peaked at 30 min in presence of MJ or ABA. The amplitude of responses of ROS production and pH changes were higher in response to MJ than to ABA. Additionally, the limited responses of *jar1*-1 and *ost1*-2 allowed a placing of JAR1 and OST1 upstream of cytoplasmic alkalinization in the MJ and ABA-signaling pathways, respectively.

ROS production would activate hyperpolarization-activated Ca^{2+}-channels (Murata et al., 2001) and also inhibit inward-rectifying K^+-channels (Köhler et al., 2003). Among the last steps in the cascade leading to stomatal closure is the activation of the outward K^+ rectifier from guard cells allowing K^+ efflux. These channels are down regulated by H_2O_2 and up regulated by a rise in cytoplasmic pH that occurs after application of MJ or ABA. H_2O_2 production in *gork1* in response to MJ or ABA was in the range of levels observed in the wild type, confirming that GORK is involved in downstream of H_2O_2 production during the signaling cascade.

Our results suggested that the guard cell signaling was mediated by numerous factors including Ca^{2+}, pH, ROS, protein kinases and ion channels.

The next focus was set on the role of NO, another interesting signaling molecule. Previous studies have indicated that NO, similar to ABA, regulates stomatal movement in response to ABA via nitric oxide synthase (NOS) like activity (Neill et al., 2002). We now provide strong evidence of NO mediated action during MJ dependent stomatal closure. The presence of SNP (a generator of NO) in incubation medium enhanced stomatal closure in presence of MJ or ABA. Inhibition of these effects by incubation with cPTIO (2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl3-oxide, a NO scavenger), demonstrated that the effect of the MJ or ABA induced stomatal closure was associated with NO release within guard cells. The effect of NOS inhibitor on stomatal response to MJ treatments was much more pronounced than that with ABA. Our studies suggest that NOS could participate in the MJ signaling cascade in tobacco and pea guard cells.

The role of NO during MJ or ABA induced stomatal closure was further checked by modulating NO levels in guard cells. Suppression of NO levels, by either scavenging in the presence of c-PTIO or inhibiting NOS (by L-NAME) reversed the stomatal closure by ABA or MJ. The changes in the NO-levels of guard cells and responses to NO-modulators were more pronounced in case of MJ than that of ABA. Real time monitoring with the help of fluorescent dyes indicated that NO production in guard cell peaked at 18 min, compared to 30 min incase of ROS and 15 min in case of pH. Thus, changes in NO or pH preceded ROS production. Modulation of pH resulted in changes in NO production as well, indicating that changes in guard cell pH and NO production are either related or interact with each other.

Rise in internal Ca^{2+} appears to be a final event after NO-increase, as EGTA reversed stomatal closure, despite sustaining the increase in NO- production by MJ or ABA. Experiments using pharmacological compounds: ML-7 (MLCK inhibitor), staurosporine (broad-spectrum of PK inhibitor), wortmannin/LY294002 (PI_3K/PI_4K inhibitor), W7 (CaM antagonist), U73122 (PLC inhibitor), ODQ (GC inhibitor), nicotinamide (cADPR inhibitor), which effect different protein kinases or calcium mobilizing components were inconclusive. All these compounds decreased NO-levels and reversed stomatal closure caused by either MJ or ABA.

The presence of butyrate, a weak acidifying agent, prevented stomatal closure induced by MJ or ABA. Similarly, butyrate decreased also the levels of NO in guard cells as indicated by DAF-2DA fluorescence. Fusicoccin did not alter much the levels of NO or ROS but caused a change in pH. We, therefore conclude that NO influenced the signal pathway down stream of MJ synthesis and upstream of H_2O_2 synthesis (Orozco-Cardenas and Ryan, 2002).

Protoplasts can be a useful model system for studying signal transduction and transient gene expression. Simple and efficient techniques are available for isolating MCP and GCP. There was a brief report on the signaling components involved during MJ or ABA induced shrinking of MCP of *Pisum sativum* (Kolla et al., 2004). In the present work, shrinkage of protoplasts on treatment with MJ or ABA was found to be more dramatic in case of GCP than that of MCP. During the course of shrinkage, MJ or ABA caused marked alkalization and elevated the production of reactive oxygen species (ROS). Interestingly, the GCP and MCP exhibited a difference in their

relative sensitivity to MJ or ABA. The extents of decrease in protoplast volume, pH change and the ROS production in GCP were all more in response to MJ than to ABA. In contrast, the MCP responded more to ABA than to MJ. DPI, an inhibitor of NADH oxidase, decreased ROS production and suppressed shrinkage of both MCP and GCP by MJ or ABA.

Butyrate decreased the MJ or ABA-induced shrinking of both MCP and GCP. The responses of GCP to MJ or ABA were suppressed in the NADPH oxidase double mutant (*atrbohD/F*) of *Arabidopsis*, while the responses stayed unaffected in their MCP. These results demonstrate that the alkalization and ROS production are among the components of common signaling pathways in the responses to MJ or ABA of both GCP and MCP. We suggest that the decrease in cell turgor induced by MJ or ABA in mesophyll and guard cells may be a significant phenomenon during the growth reduction and adaptation to adverse conditions such as water stress or pathogen attack.

The following are the major conclusions, in relation to the objectives set at the beginning of the present study.

1. Ca^{2+} played a vital role in MJ and ABA signaling cascade of both ABA and MJ. The action of ABA showed dependency on phospholipases, while the action of MJ was dependent on calcium dependent calmodulin kinases. The dependence on Ca^{2+} appeared to be more in MJ-signaling, while ABA responses seem to operate in both Ca^{2+} dependent and independent manner.
2. The experiments on various mutants were useful to evolve a scheme of signaling events during stomatal response to MJ or ABA. In response to MJ

or ABA, the sequence of events appears to be the alkalinization, H_2O_2 production, activation of Ca^{2+} channels and elevation of cytosolic Ca^{2+} occurs down stream of phosphorylation events. Our results also point out some specificity in the ABA cascade, which appears to involve in parallel with Ca^{2+}-dependent and Ca^{2+}-independent pathways. Ca^{2+}-CaM protein kinase seems to be involved in a very early MJ signaling.

3. Pharmacological study demonstrate that MJ or ABA mediated NO production is due to increase in pH as well as involvement of guanyl cyclase, phosphoinositide kinases, PLC and cADPR. It is shown, that NOS seems to be the potential source for NO production in MJ induced signaling cascade. In contrasts, ABA perception showed marginal involvement of NOS in releasing NO.
4. Alkalization of protoplasts and ROS production are among the components of common signaling pathways in response to MJ or ABA of both GCP and MCP. MCP was more sensitive to ABA than MJ, where as the GCP showed more sensitivity with MJ than ABA.

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Chapter 10

Literature Cited

Chapter 10

Literature Cited

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Appendix: Research Papers Published

(First page of the article is attached)

List of Publications/Under Preparation in Referred Science Journals

1. Kolla VA, **Suhita D**, Vavasseur A and Raghavendra AS (2004) Reevaluation of stomatal responses to bicarbonate in abaxial epidermis of *Commelina benghalensis* in comparison to the effects of abscisic acid. ***Journal of Plant Biology*** **31:** (In Press)

2. Kolla VA, **Suhita D**, Raghavendra AS (2004) Marked changes in volume of mesophyll protoplasts of pea *(Pisum sativum)* on exposure to growth hormones ***Journal of Plant Physiology*** **161:** 557-562.

3. **Suhita D**, Raghavendra AS, Kwak JM and Vavasseur A (2004) Cytoplasmic alkalization precedes ROS production during methyl jasmonate- and abscisic acid-induced stomatal closure. ***Plant Physiology*** **134:** 1536-1545.

4. **Suhita D**, Kolla VA, Vavasseur A and Raghavendra AS (2003). Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid. ***Plant Science*** **164:** 481-488.

Articles under preparation

5. **Suhita D**, Vavasseur A and Raghavendra AS. The reactive oxygen species and pH changes are common signaling elements during ABA or MJ-induced shrinking of guard cell and mesophyll protoplasts of *Arabidopsis thaliana* and *Pisum sativum*.

6. **Suhita D**, Kolla VA and Raghavendra AS. NO production is a common event during the transduction by guard cells of both MJ and ABA.

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Reevaluation of Stomatal Responses to Bicarbonate in Abaxial Epidermis of *Commelina benghalensis* in Comparison to the Effects of Abscisic Acid

Venkat Apparao Kolla[1], Dontamala Suhita[1], Alain Vavasseur[2] and Agepati S. Raghavendra[1,*]

[1]Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India
[2]CEA/Cadarache-DSV-DEVM, Laboratoire des Echanges Membranaires et Signalisation, UMR 163 CNRS-CEA, 13108 St. Paul lez Durance Cedex, France

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Stomatal opening in abaxial epidermis of *Commelina benghalensis* L. was monitored at varied concentrations of bicarbonate (10 μM to 5 mM) and ABA (1 μM to 1 mM). Stomatal opening was progressively suppressed by increasing concentrations of ABA. In contrast, bicarbonate stimulated stomatal opening at low concentrations (< 0.5 mM) and caused stomatal closure at concentrations above 2 mM. At low levels of external calcium (< up to 10 μM), the stimulation by bicarbonate was evident, whereas the suppression of stomatal opening was pronounced at higher calcium (above 50 μM). Thus, stimulation by bicarbonate required low calcium and suppression by bicarbonate preferred high calcium. The effect of bicarbonate was reduced in the presence of ABA. Experiments using pharmacological compounds revealed that the Ca^{2+} channels mediate bicarbonate-induced stomatal closure, whereas calmodulin or phospholipases appears to be important during ABA-induced stomatal closure. These observations suggest that the inhibition of stomatal opening by ABA was more pronounced than that by HCO_3 and that the signalling components during bicarbonate-induced stomatal closure differ partly from that of ABA.

Keywords: Stomatal response, bicarbonate, abscisic acid, calcium, signal transduction, secondary messenger, pharmacological compounds.

Introduction

The stomata offer an attractive model system for investigating the mechanism of responses to different environment signals. The stomatal guard cells respond to an array of stimuli, such as CO_2, or light or hormones, by an alteration in their cell turgor, which in turn results in change in stomatal aperture (Willmer and Fricker, 1996; Assmann, 1999; Cousson, 2000; Outlaw, 2003).

Stomatal movements are regulated by internal and external factors. The most important external/environment factors are light, CO_2 and temperature (Willmer and Fricker, 1996). The stomatal opening is stimulated by light while stomata close in response to hormone such as ABA or atmospheric levels or higher CO_2. Depending on the ambient concentration, CO_2 can either stimulate opening or cause a closure of stomata (Assmann, 1999). Such contrasting effects of CO_2 are highly intriguing and interesting, but are not examined in detail. Abscisic acid (ABA), a hormone and signal of water stress in plants, also causes marked stomatal closure. During the past two decades extensive work has been done to study various aspects of stomatal closure induced by ABA (Assmann, 1993; Giraudat, 1995; Schroeder *et al.*, 2001). In contrast to studies with ABA, only limited information is available on the mechanism of reception and participation of various signalling components during stomatal closure induced by CO_2.

There has been a debate on the interaction between ABA and CO_2 in the regulation of stomatal movements. An interdependence of CO_2 and ABA was reported during stomatal closure in *Xanthium stromarium* (Raschke, 1975). However, no significant interaction between CO_2 and ABA was found in stomatal responses of *X. stromarium* (Mansfield, 1976) and in *Commelina* (Wilson, 1981). Experiments with *Arabidopsis thaliana* and ABA-insensitive mutants have also been ambiguous. Leymarie *et al.* (1998) reported lack of complete convergence of the effects of ABA and CO_2, while Webb and Hetherington (1997) suggested a convergence of signalling components during the stomatal responses to CO_2, ABA and external calcium.

*For correspondence. (e-mail: asrsl@uohyd.ernet.in)

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Marked changes in volume of mesophyll protoplasts of pea (*Pisum sativum*) on exposure to growth hormones

Venkat Apparao Kolla, Dontamala Suhita, Agepati S. Raghavendra*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

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Summary

The present study reports quick and significant changes induced by plant hormones in the volume of mesophyll protoplasts of pea (*Pisum sativum*). Four plant hormones: gibberellic acid (GA_3), indole 3-acetic acid (IAA), abscisic acid (ABA)(±) and methyl jasmonate (MJ), caused marked changes in the volume of mesophyll protoplasts. GA_3 and IAA increased the volume of the protoplasts (up to 90 %) whereas the ABA and MJ decreased (by about 40 %) the volume. Aquaporins or water channels appear to play an important role in swelling/shrinkage of the protoplasts as indicated by the suppression of volume changes by $HgCl_2$ and reversal by mercaptoethanol. The possible role of secondary messengers in volume changes induced by GA_3 was investigated by using selected pharmacological reagents. The GA_3 induced swelling was restricted by GDP-β-S (G-protein antagonist), U73122 (phospholipase C inhibitor), and TFP (calmodulin antagonist), but was not affected by 1-butanol (phospholipase D inhibitor), GTP-γ-S (G-protein agonist), or verapamil (calcium channel blocker). The results suggest that the mesophyll protoplasts can be a simple and useful system for further studies on volume changes in plant tissues.

Key words: abscisic acid – gibberellic acid – indole 3-acetic acid – methyl jasmonate – plant hormones – shrinkage – swelling

Abbreviations: GDP-β-S = Guanosine 5′-O-(2-thiodiphosphate). – GTP-γ-S = Guanosine 5′-O-(3-thiotriphosphate). – PLC = phospholipase C. – PLD = phospholipase D. – TFP = Trifluoperazine. – U73122 = 1-(6-[([17β]-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione

Introduction

Plant protoplasts can be prepared from a wide range of tissues (e.g., cells, suspension cultures, epidermis, coleoptiles, or root tips) by simple enzymatic methods involving the combined use of cellulase and macerozyme (Evans 1976, Schlangstedt et al. 1992, Devi et al. 1992). Protoplasts have been extensively used for plant regeneration and genetic engineering, e.g. including tissue culture (Takeuchi et al. 1998, Maurel et al. 1995) and generation of somatic hybrids (Cai 1988). Besides their use in plant biotechnology, protoplasts have been model systems to study various aspects of plant metabolism, such as photosynthesis, respiration and

* E-mail corresponding author: asrsl@uohyd.ernet.in, as_raghavendra@yahoo.com

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Cytoplasmic Alkalization Precedes Reactive Oxygen Species Production during Methyl Jasmonate- and Abscisic Acid-Induced Stomatal Closure[1]

Dontamala Suhita, Agepati S. Raghavendra, June M. Kwak, and Alain Vavasseur*

CEA/Cadarache-DSV-DEVM, Laboratoire des Echanges Membranaires et Signalisation, UMR 163 CNRS-CEA, Université de la Méditerranée, 13108 St Paul lez Durance cedex, France (D.S., A.V.); Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India (D.S., A.S.R.); and Cell and Developmental Biology Section, Division of Biological Sciences, University of California San Diego, La Jolla, California 92093–0116 (J.M.K.)

Signaling events during abscisic acid (ABA) or methyl jasmonate (MJ)-induced stomatal closure were examined in Arabidopsis wild type, ABA-insensitive (*ost1*-2), and MJ-insensitive mutants (*jar1*-1) in order to examine a crosstalk between ABA and MJ signal transduction. Some of the experiments were performed on epidermal strips of *Pisum sativum*. Stomata of *jar1*-1 mutant plants are insensitive to MJ but are able to close in response to ABA. However, their sensitivity to ABA is less than that of wild-type plants. Reciprocally, the stomata of *ost1*-2 are insensitive to ABA but are able to close in response to MJ to a lesser extent compared to wild-type plants. Both MJ and ABA promote H_2O_2 production in wild-type guard cells, while exogenous application of diphenylene iodonium (DPI) chloride, an inhibitor of NAD(P)H oxidases, results in the suppression of ABA- and MJ-induced stomatal closure. ABA elevates H_2O_2 production in wild-type and *jar1*-1 guard cells but not in *ost1*-2, whereas MJ induces H_2O_2 production in both wild-type and *ost1*-2 guard cells, but not in *jar1*-1. MJ-induced stomatal closing is suppressed in the NAD(P)H oxidase double mutant *atrbohD/F* and in the outward potassium channel mutant *gork1*. Furthermore, MJ induces alkalization in guard cell cytosol, and MJ-induced stomatal closing is inhibited by butyrate. Analyses of the kinetics of cytosolic pH changes and reactive oxygen species (ROS) production show that the alkalization of cytoplasm precedes ROS production during the stomatal response to both ABA and MJ. Our results further indicate that JAR1, as OST1, functions upstream of ROS produced by NAD(P)H oxidases and that the cytoplasmic alkalization precedes ROS production during MJ or ABA signal transduction in guard cells.

Methyl jasmonate (MJ), a linolenic acid derivative, is involved in plant development and defense and is overproduced during wounding, fruit ripening, and drought stress (Creelman and Mullet, 1997). MJ affects plant transpiration (Lee et al., 1996; Wang, 1999) by promoting stomatal closure (Raghavendra and Reddy, 1987; Gehring et al., 1997; Suhita et al., 2003). MJ-induced stomatal closure is accompanied by an alkalization of the guard cell cytoplasm in *Paphiopedilum* spp. (Gehring et al., 1997). A recent study has shown that this response to MJ requires external calcium and involves a calmodulin-like domain, obviously of a protein kinase (Suhita et al., 2003). Interestingly, Evans (2003) demonstrated that MJ activates the outward potassium channel from guard cell protoplast of *Vicia faba*, the main conductance allowing K^+ efflux and loss of turgor. These steps of cytoplasmic pH modification (Irving et al., 1992) and modulation of potassium channels at the guard cell plasma membrane (Armstrong et al., 1995) are also involved in abscisic acid (ABA)-induced stomatal closure.

In addition, cytoplasmic calcium waves (Allen et al., 2000), protein (de)phosphorylation (Leung et al., 1994, 1997; Meyer et al., 1994; Li et al., 2000; Merlot et al., 2001; Kwak et al., 2002; Mustilli et al., 2002) and reactive oxygen species (ROS) have all been identified to participate in ABA signaling (Gomez-Cadenas et al., 1999; Guan et al., 2000; Pei et al., 2000; Zhang et al., 2001a, 2001b; Klüsener et al., 2002). In guard cells, ABA induces ROS production, which in turn activates Ca^{2+} channels at the plasma membrane (Pei et al., 2000; Murata et al., 2001). Further, ABA-induced elevation in cytosolic Ca^{2+} leads to activation of slow anion channels and inactivation of inward rectifying K^+ channels. The consequences are K^+ efflux, guard cell turgor reduction, and stomatal closure. Interestingly, MJ together with various elicitors also induces an accumulation of H_2O_2 in leaves (Orozco-Cardenas and Ryan, 1999). Thus, it is likely that ABA and MJ transduction pathways leading to stomatal closure involve overlapping signaling elements. Such interaction has already been suggested by Herde et al. (1997) who

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* Corresponding author; email avavasseur@cea.fr; fax 33–4–42252364.

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Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid

Dontamala Suhita [a], Venkat Apparao Kolla [a], Alain Vavasseur [b], Agepati S. Raghavendra [a,*]

[a] *Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India*
[b] *CEA/Cadarache-DSV-DEVM, Laboratoire des Echanges Membranaires et Signalisation, UMR 163 CNRS-CEA, 13108 St. Paul lez Durance Cedex, France*

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Abstract

The stomatal opening in abaxial epidermal strips of *Nicotiana glauca* is suppressed by the presence of abscisic acid (ABA) or methyl jasmonate (MJ). The role of calcium and related secondary messengers/signaling compounds during the restriction of stomatal opening by ABA or MJ was assessed. External calcium promoted, while EGTA prevented the process of stomatal closure, in case of both ABA and MJ. However, the effect of EGTA was more pronounced in the case of MJ than that of ABA. These observations established that the intracellular calcium is an important factor during stomatal closure induced not only by ABA but also by MJ. Lanthanum, verapamil, ruthenium red (Ca^{2+} channel blockers), trifluoperazine and W7 (calmodulin (CaM) antagonists) prevented MJ-induced stomatal closure but had only a partial effect on ABA-mediated stomatal closure. In contrast, U73122, 1-butanol (phospholipase C/D inhibitors) and phorbol myristate acetate (PMA, protein kinase C activator) reversed the stomatal closure caused by ABA but not that by MJ. Thus, the suppression of stomatal opening by ABA appears to be more dependent on phospholipases and protein kinase C than that by MJ. Similarly, the action of MJ on stomata seems to be crucially dependent on CaM and calcium channels of the guard cells. We suggest that MJ could be a useful tool, besides ABA, to analyze the sequence and pattern of signal transduction in stomatal guard cells.

Keywords: Abscisic acid; Calcium channel; Calmodulin; Guard cell; Methyl jasmonate; Phospholipase C/D

1. Introduction

Stomatal movement is regulated markedly by plant hormones. Abscisic acid (ABA) and methyl jasmonate (MJ) suppress stomatal opening, while cytokinins and IAA promote stomatal opening [1–4]. The mechanism of ABA effects on guard cells has been studied extensively [5,6]. The modulation of cytosolic free calcium (Ca^{2+}) in guard cells seems to be a major factor during ABA-induced restriction of stomatal opening [5–7]. ABA induces an increase in cytosolic free calcium of guard cell possibly by the influx as well as the release from internal stores such as vacuole or endoplasmic reticulum [8–10].

The involvement of Ca^{2+} during signal transduction pathway in guard cell responses has been of great interest. Elevated levels of extracellular Ca^{2+} lead to stomatal closure even in the presence of high K^+ or low CO_2 [11–14]. Similarly, stomatal closure in response to darkness, or elevated CO_2 or ABA is prevented by the presence of EGTA, a Ca^{2+}-chelator [11–13]. Ca^{2+} may act on guard cells in several ways: inhibition of inward K^+ channels, stimulation of Cl^- efflux leading to depolarization of plasma membrane, and interaction with calmodulin (CaM) [6,14,15].

Abbreviations: ABA, abscisic acid; CaM, calmodulin; DAG, diacylglycerol; $InsP_3$, inositol-1,4,5-triphosphate; MJ, methyl jasmonate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol myristate acetate; TFP, trifluoperazine; U73122, 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl]-1H-pyrrole-2,5-dione; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

* Corresponding author. Fax: +91-40-23010-145/120.
E-mail address: asrsl@uohyd.ernet.in (A.S. Raghavendra).

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