Signalling components and their integration during stomatal closure by abscisic acid in *Pisum sativum* and *Arabidopsis thaliana*

DOCTOR OF PHILOSOPHY

By

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Thesis submitted to the University of Hyderabad for the degree of
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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Signalling components and their integration during stomatal closure by abscisic acid in *Pisum sativum* and *Arabidopsis thaliana*"** 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.

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CERTIFICATE

This is to certify that **Mr. Vijaya Kumar Gonugunta** has carried out the research work embodied in the present thesis entitled **"Signalling components and their integration during stomatal closure by abscisic acid in *Pisum sativum* and *Arabidopsis thaliana*"** for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

Prof. A.S. Raghavendra	**Head**	**Dean**
Supervisor	**Department of Plant Sciences**	**School of Life Sciences**

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Vijaya Kumar Gonugunta

List of abbreviations used

ABA	=	abscisic acid
BAPTA	=	1, 2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid
BAPTA-AM	=	1, 2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid acetoxymethyl ester
BCECF-AM	=	3'-O-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester
cADPR	=	cyclic adenosine di phosphate ribose
CaM	=	calmodulin
CDZ	=	calmidazolium chloride
cPTIO	=	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF-2DA	=	4, 5-diaminofluorescein diacetate
H_2DCFDA	=	dichlorofluorescein diacetate
IAA	=	indole acetic acid
L-NAME	=	N^G-nitro-L-Arg-methyl ester
LY294002	=	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MCP	=	mesophyll cell protoplast
MJ	=	methyl jasmonate
NOS	=	nitric oxide synthase
NR	=	nitrate reductase
PI3K	=	phosphatidylinositol 3 kinase
PI3P	=	phosphatidylinositol 3-Phosphate
PLC	=	phospholipase C
PLD	=	phospholipase D
PMSF	=	phenyl methyl sulfonyl fluoride
PP2C	=	type 2 protein phosphatase
ROS	=	reactive oxygen species
W-7	=	N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide

hydrochloride

WM = Wortmannin

All the remaining abbreviations are all standard ones, and as per Plant Physiology issue, 2009, Instructions for contributors, website:http://www.aspb.org

Dedicated to my best friend

Dinakar

Chapter 1

Introduction and review of literature

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Introduction and review of literature

Stomata, the tiny pores, located on the epidermis of leaves, represent the common gateways for entry and exit of water vapor, carbon dioxide as well as oxygen (Willmer and Fricker, 1996). When stomata are open, CO_2 diffuse through the stomata and can enter into the leaves, for photosynthetic carbon fixation. At the same time transpirational water escape out. During unfavorable conditions, stomata close to minimize water loss and avoid wilting of the plant. Besides the involvement in gas exchange, stomata are entry points for also plant pathogens. Effecting stomatal closure can therefore limit the penetration of pathogens, thereby conferring resistance to plants (Melotto et al., 2008). Thus regulation of stomata is a very important phenomenon, to optimize plant growth and development.

Each stoma is constituted by two modified epidermal cells called guard cells, whole turgor determines the stomatal aperture. An interesting feature of guard cells, is that despite being connected to neighboring cells via their dorsal walls, do not possess functional plasmodesmata, at maturity (Willmer and Fricker, 1996; Kolla and Raghavendra, 2006). These cells sense and integrate environmental signals to modulate stomatal aperture. Guard cells have become popular model systems to study the signalling mechanisms and secondary messengers in plants (Fan et al., 2004; Israelsson et al., 2006).

In view of the versatility and dynamic responses to abiotic and biotic signals, the stomatal function and guard cell features have been studied extensively. Several reviews on different aspects of stomatal function in relation to signal transduction in guard cells have appeared within the last five years (Fan et al., 2004; Hetherington and Brownlee, 2004; Yang et al., 2004; Buckley, 2005; Pei and Kuchitsu, 2005; Paoletti and Grulke, 2005; Roelfsema and Hedrich, 2005;

Vavasseur and Raghavendra, 2005; Christmann et al., 2006; Israelsson et al., 2006; Pandey et al., 2007; Shimazaki et al., 2007; Neill et al., 2008; Wang and Song, 2008; Acharya and Assmann, 2009; Lawson, 2009; Sirichadra et al., 2009).

Stomatal Movement: Basics and modulating factors

The stomatal movement is facilitated by a combination of mechanistical components and water status of guard cells. Stomata open when the guard cells are turgid and close when the guard cells become flaccid. Such increase in stomatal guard cells is driven by the accumulation of K^+ salts, particularly chloride/malate and sugars (Outlaw, 2003; Vavasseur and Raghavendra, 2005). The resulting increase in osmotic components and marked decrease in water potential drives water into guard cells, making them turgid. As a result, guard cells increase their volume. The thick inner walls and the asymmetric positioning of micro fibrils within the cells make the guard cells pull towards periphery leading to the expansion of stomatal pore. Stomatal closure is the result of flaccidity of guard cells, caused by the release/efflux of osmotic components.

Several technical advantages make stomatal guard cells among the best model systems for signal transduction studies. For example, stomata can be separated from other tissues/cells by peeling the epidermis or by blending whole leaves (MacRobbie, 1981; Kruse et al., 1989). The response of stomata to various stimuli can be simulated under *in-situ* or *in-vitro* conditions, because guard cells are highly differentiated, functionally limited, and physically isolated (due to the lack of plasmodesmatal connections) and yet functional (Willmer and Fricker, 1996). The separation of stomatal guard cells from their surrounding cells allows the application of cell biological and electrophysiological imaging. In fact, several early ABA-signaling events in guard cells were discovered using isolated guard cells and their protoplasts. Furthermore, the epidermis being a single, fairly

transparent layer, simple microscopic measurements of stomatal apertures as well as guard cells provide reliable assays of the biological functioning of guard cells.

Stomatal movements are influenced directly or indirectly by a wide range of environmental variables, which include both abiotic and biotic factors. Examples of biotic factors are light, CO_2, water status, temperature. The biotic factors are illustrated by the internal hormonal status and the challenges imposed by plant pathogens or insects. Further, circadian rhythms and other factors, e.g. nutrient availability, can also influence guard cell responses and guard cells. Frequently, some or several of these signals are integrated to produce the net stomatal response.

Environmental/External factors

Light

In response to light stomata of the most plants open, and close in response to dark. Stomata in CAM plants are an exception to this role, as they can open in dark to varying extent, according to the degree of CAM. Stomata respond to two separate wavelengths in the visible spectrum, one that shows an action spectrum with a peak near 450 nm, and one that shows an action spectrum that coincides with the absorption spectrum of chlorophyll. These have been called the 'blue light' and 'red light' responses, respectively (Shimazaki et al., 2007). The blue light response saturates at low light fluxes and is usually studied by adding low fluxes of blue light in the presence of a high-intensity red light to say, blue light 2-20 fold more efficient than the red light in most species. Thus, although blue light is more efficient per photon than red light in opening stomata, most of the stomatal response to white light is caused by the red light effect (Willmer and Fricker, 1996; Shimazaki et al., 2007).

Blue light acts as a signal and red light both as a signal and an energy source. Blue light activates the plasma membrane H^+-ATPase (Kinoshita and Shimazaki, 1999), hyperpolarizing the membrane potential with simultaneous apoplast acidification, and drives K^+ uptake through voltage-gated K^+ channels. Red light drives photosynthesis in mesophyll and guard cell chloroplasts and decreases the intercellular CO_2 concentration. Red-light-induced stomatal opening may result from a combination of guard cell response to the reduction in intercellular CO_2 and a direct response of the guard cell chloroplasts to red light (Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005).

The accumulation of positively charged K^+ ions in guard cells must be compensated by anions, mainly in the form of malate (Willmer and Fricker, 1996). Malate forms in response to weak blue light under a red-light background, and the formation does not occur without red light. Guard cell chloroplasts are responsible for malate formation (Sharkey and Ogawa, 1987), and the chloroplasts also act as a reservoir for starch and catabolize it as a precursor of malate (Willmer and Fricker, 1996; Vavasseur and Raghavendra, 2005). By afternoon, sugars also accumulate in guard cells as osmotic and maintain stomatal opening (Talbott and Zeiger, 1998).

In Arabidopsis, *PHOT1* and *PHOT2* (phototropins), which exhibit serine/threonine kinase activity are demonstrated to be key photoreceptors for promoting stomatal opening, by activating the ATPase and acts redundantly. These phototropins undergo auto phosphorylation under blue light irradiation, leading to phosphorylation of ATPase and enhanced interaction with 14-3-3 protein(s) during stomatal opening (Shimazaki et al., 2007).

Another photoreceptor which could mediate the stomatal responses to blue light is zeaxanthin, as inhibition of zeaxanthin formation suppressed blue-light-stimulated stomatal opening (Zeiger et al., 2002), the zeaxanthin-less mutant of Arabidopsis, *npq1*, failed to respond to blue light (Eckert and Kaldenhoff, 2000).

Further confirmation is needed because in *npq1* mutant stomatal aperture responses could not be reproduced in leaves, epidermis, or guard cell protoplasts (Shimazaki et al., 2007).

Carbon dioxide

Guard cells can perceive and respond to the CO_2 concentration either at the external surface of the plasma membrane or within the guard cells. Stomata in the leaf surface open at low CO_2 concentrations (below 100 ppm) and close at CO_2 of above 100 ppm (Assmann, 1999). Aquaporins could mediate the transport of CO_2 into guard cells (Uehlein et al., 2003). Studies in our laboratory, have established that stomatal responses to CO_2 can be studied by adding HCO_3 to the incubation medium (Kolla et al., 2007).

Despite several studies, the CO_2-sensing mechanism of guard cell is not completely understood. Some of the events that occur in stomatal guard cells on exposure to CO_2 are membrane depolarization, deactivation of plasma membrane ATPase, activation of the anion channel (Brearly et al., 1997; Hedrich et al., 2001; Roelfsema et al., 2002), and increase in cytosolic Ca^{2+} concentrations (Webb and Hertherington, 1997). High CO_2 levels caused an increase in the bulk-leaf apoplastic malate concentration, which can affect anion-channels. Therefore, it was proposed that guard-cell anion channels were CO_2 sensors (Hedrich et al., 2001). This appears to be an attractive hypothesis, in view of the drought-induced increase in apoplastic malate-concentration in leaves (Patonnier et al., 1999).

Humidity and water status

Stomata appear to respond to perturbations of many aspects of the soil–plant–atmosphere hydraulic continuum, but there is little agreement regarding the mechanism by which stomata sense such perturbations. The epidermis has a high

hydraulic conductance and could act as a major route for water movement. Stomatal responses to humidity can be rapid and can occur, even when the bulk leaf water potential does not alter, suggesting that the turgor relations of the epidermal cells can be independent of the mesophyll tissue (Edwards and Meidner, 1978; Franks, 2003). Under water stress, guard cells display a short-term response based on osmoregulation and a long-term response involving major modification of metabolism, possibly mediated by guard cell gene regulation (Vavasseur and Raghavendra, 2005). Mott and Parkhurst (1991) concluded that stomatal responses to humidity simply involve decreased water availability leading to decreased guard cell turgor. It is expected that the guard cells can sense humidity, but such humidity responses remain mysterious (Assmann, 1993).

Temperature

As the leaf temperature is raised the metabolic activity within the guard cells as well as the leaf will increase. The effect of the increased metabolic activity within guard cells tend to stimulate opening (Shope et al., 2009). There are also indirect effects of temperature on stomatal behavior. For e.g. rise in temperature can affect carbon assimilation and in turn the intercellular CO_2 concentration, leading to changes in stomatal aperture. If the respiration and photorespiration outpace photosynthesis as the temperature increases, CO_2 levels will increase within the leaf, bringing out stomatal closure. In addition, an increase in leaf temperature will result in an increase of water vapor pressure gradient between the leaf and the surrounding air, which may ultimately cause stomatal closure through both direct and indirect effects (Shope et al., 2008).

Hormonal regulation

Stomatal function is also regulated differently and to a varying extent by phytohormones such as auxins, cytokinins, ethylene, brassinosteroids, jasmonates,

and salicylic acid besides ABA. The effects of other hormones, namely gibberellines ethylene and brassinosteroids are not clearly established. The stomatal closure by ABA is well established and stimulation by cytokinins or auxins seen mostly on stomata of certain plants and in adaxial epidermis. It is possible that these hormones interact during stomatal regulation. For e.g. a balance between cytokinins and ABA in the plant may cause the final response (Acharya and Assmann, 2009).

Auxin activates the plasma membrane H^+-ATPase which promotes stomatal opening (Coenen et al., 2002), as the proton extrusion via the H^+-ATPase leads to hyperpolarization of the membrane, which in turn facilitates K^+-uptake. Auxin at low concentrations, could promote the activity of the inward K^+ channels, while at higher concentrations activating the outward K^+ channels (Blatt and Thiel, 1994). The intriguing aspect is that the stimulation by auxin is more prominent on stomata of adaxial (upper) epidermis than that of abaxial (lower) epidermis. Antagonistic stomatal regulation has been observed between ABA and auxin. Auxin restricted stomatal closure caused by ABA in epidermal peels of *Commelina communis* and Arabidopsis (Tanaka et al., 2006).

Increased cytokinin concentration in the xylem sap promoted stomatal opening and decreased sensitivity to ABA (Wilkinson and Davies, 2002). Transgenic tobacco, overexpressing trans-zeatin o-glucosyltransferase, had increased levels of cytokinins, and showed delayed stomatal closure in response to water deficit (Havlova et al., 2008). Cytokinins could induce stomatal opening particularly in the grasses, or monocots (Jewer and Incoll, 1980) and could also reverse/relieve ABA induced stomatal closure mainly in monocot species (Stoll et al., 2000; Tanaka et al., 2006). However, there were reports that cytokinins inhibited stomatal opening, Commelina and Anthephora, at high concentrations (Blackman and Davies, 1983). Further experiments are needed to ascertain the stomatal responses to cytokinins.

Exogenous application of GA, had only a partial or no effect on stomatal apertures in Arabidopsis (Tanaka et al., 2006). The leaves of GA-deficient tomato plants did not show significant difference in transpiration in comparison to that of control leaves (Cramer et al., 1995), suggesting that GAs might not be a direct player during water stress. At best, in one report, GA application led to transient stomatal opening in *Vicia faba* and *Fritilaria imperialis* (Goring et al., 1990).

The reported effects of ethylene on stomata are either ambiguous or intriguing. Ethylene has been linked to promotion of both stomatal closure (Jackson, 2002; Dat et al., 2004) and stomatal opening (Merritt et al., 2001). Exogenous application of ethylene as ethephon or ACC, promote stomatal closure in Arabidopsis leaves (Desikan et al., 2006). Ethylene-induced stomatal closure was inhibited by 1-methylcyclopropane (1-MCP), a competitive inhibitor of the ethylene receptor. Such ethylene induced stomatal closure could not be seen in *etr1* (ethylene receptor mutant) as well as in *ein2-1* (ethylene signaling mutant) (Desikan et al., 2006). Ethylene also could relieve/reverse stomatal closure by ABA in isolated epidermal peels of Arabidopsis (Tanaka et al., 2005, 2006). ABA induction of stomatal closure was suppressed in plants of the ethylene-overproducing mutant, *eto1-1* (Tanaka et al., 2005).

Pretreatment with homobrassinolide delayed stomatal closure in response to water stress in Jackpine seedlings (Rajasekaran and Blake, 1999). In contrast, brassinolide (BL), promoted stomatal closure and inhibited stomatal opening in epidermal peels of *Vicia faba*. In guard cell protoplasts of *Vicia faba*, BL inhibited the inwardly rectifying K^+ channels, imolicating a suppression of K^+ uptake, and perhaps stomatal opening. The BR-deficient mutant of Arabidopsis, *sax1*, showed an enhanced stomatal closure in response to ABA (Ephritikhine et al., 1999).

Like ABA, methyl jasmonate causes stomatal closure (Suhita et al., 2003, 2004; Munemasa et al., 2007). JA could be an important player for stomatal closure during drought stress as MJ accumulated during drought (Creelman and

Mullet, 1997). The stomatal closure by MeJA is a matter of debate, as MeJA was unable to cause stomatal closure in some cases. MeJA-mediated stomatal closure has been associated with cytoplasmic alkalinization in guard cells, production of ROS via AtrbohD/F and NO, and activation of K^+ efflux channels (Evans, 2003) and slow anion channels (Gehring et al., 1997; Suhita et al., 2003, 2004; Munemasa et al., 2007). These effects are similar to those of ABA.

The possible overlap of signaling components for stomatal closure by ABA and MeJA is demonstrated by other reports of reduced sensitivity of in *ost1* for both MeJA and ABA, and reduced sensitivity in *jar1* (MeJA insensitive) to ABA (Suhita et al., 2004). However, *coi1* mutants do not show stomatal closure, ROS or NO production, or activation of slow anion channels or Ca^{2+} permeable channels in response to MeJA, but do so in response to ABA, suggests that COI1 is required for MeJA signaling but not ABA signaling in guard cells (Munemasa et al., 2007). Stomatal closure is not observed in response to either MeJA or ABA in ABA-insensitive protein phosphatase 2C mutant (*abi2*), but production of ROS and NO in response to both MeJA and ABA are retained. These results suggested that COI1 could function upstream of ROS and NO in MeJA but not during ABA signaling, while ABI2 functions downstream of ROS and NO during the stomatal closure by both MeJA and ABA.

Among the phytohormones, ABA is the most important hormone in regulating stomatal function and is often regulated as the signal mediating even drought responses. In response to water deficiency, which limits plant growth and development, the concentration of ABA in the guard cell apoplast increased up to 30-fold (Zhang and Outlaw, 2001; Outlaw, 2003). The elevated ABA triggers a signaling cascade in guard cells, which results in stomatal closure, eventually decreasing transpirational water loss (Schroeder et al., 2001a, 2001b). The effect of certain environmental factors on stomatal behavior may be mediated by hormones. For example, water stress, salt stress and chilling of plants can result in

elevated ABA levels within leaves with subsequent stomatal closure. ABA potently inhibits stomatal opening and promotes stomatal closure. In view of the strong effects of ABA on stomatal movement, extensive work is done on the mechanisms of ABA action on stomata at various levels: plant, leaves, epidermis, guard cell protoplasts, and even invitro reconstitution systems at molecular level (Fujii et al., 2009)

Other components

Application of SA induces production of ROS (Dong et al., 2001; Mori et al., 2001) and leads to stomatal closure in *Vicia faba* and *Commelina communis* (Mori et al., 2001). SA-mediated production of ROS may lead to elevation of cytosolic Ca^{2+}, thereby promoting stomatal closure (Mori et al., 2001) and preventing the pathogen invasion via stomatal openings (Melotto et al., 2006). Stomatal closure in response to bacterial pathogens is compromised in transgenic NahG plants (deficient in SA) and in the SA biosynthetic mutant *eds16-2*, indicating that SA is required for stomatal defense (Melotto et al., 2008). A role for ABA in defense-evoked stomatal closure has also been confirmed: the ABA-insensitive *ost1* mutants do not show stomatal closure in response to flg22, a pathogen associated molecular pattern (PAMP) elicitor, and the ABA-deficient *aba-3* mutant does not show stomatal closure in response to the bacterial pathogen Pst DC3000 (Melotto et al., 2006). Chitosan and oligogalacturonic acid (OGA) also induced an increase in calcium and ROS production in guard cells of tomato, *P. sativum* (Lee et al., 1999; Klüsener et al., 2002; Srivastava et al., 2009)

Signal transduction/signalling components in guard cells in response to ABA

Signal transduction systems appear to be broadly similar in plant and animals, although minor variations do occur. Signalling intermediates in guard cells have been identified, using combined approaches of biophysical, biochemical,

molecular, and genetic methods. Activation of guard cell plasma membrane H^+-ATPase and hyper polarization of plasma membrane are among the initial events in stomatal opening, which culminate in the accumulation of required osmotic K^+, Cl^-, malate and sucrose with minimal levels of cytoplasmic free Ca^{2+} (Zhao et al., 2000). In contrast events triggering the polarization deactivate K^+ inward channels and activate K^+ outward channels, resulting in net K^+ efflux from guard cells. An underlying event during these processes is the elevation of cytosolic Ca^{2+}.

Any stimulus (external or internal) initiates the response in a guard cell, by binding to its receptor, causes a change in the modulation of downstream elements, which leads to the final response. These elements are termed as secondary messengers or signaling components. A consolidated action of these secondary messengers results in the final cellular response. The downstream signaling cascades leading to the stomatal closure involves several secondary messengers, including type 2C protein phosphatases (PP2C), G-proteins, protein kinases, SnRK2s, phospholipases, besides cytosolic pH, reactive oxygen species (ROS), calcium (Ca^{2+}) and nitric oxide (NO) (Bright et al., 2006; Zhang et al., 2007; Neill et al., 2008). However, the exact sequence of these components during ABA action on guard cells is not completely understood. The literature on secondary messengers and transduction of ABA signal in guard cells has been reviewed frequently (Fan et al., 2004; Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005; Christmann et al., 2006; Israelsson et al., 2006; Pandey et al., 2007; Neill et al., 2008; Acharya and Assmann, 2009; Lawson, 2009; Sirichadra et al., 2009; Wang and Song, 2008). A variety of compounds are known to act as secondary messengers/signaling components among them most important are described below.

ABA receptors

A multitude of cellular components that modulate ABA responses downstream of ABA sensing have been identified (Kwak et al., 2002; Himmelbach et al., 2003; Fan et al., 2004). Despite the intense efforts during the past few years, there has been a lot of confusion and uncertainty about the exact identity of ABA receptors (McCourt and Creelman, 2008; Wang and Zhang, 2008). A few proteins have been reported as ABA receptors, localized in different compartments of the cell. These are ABAR/CHLH (chloroplasts & nucleus), GCR2 (plasma membrane) and FCA (nucleus) besides GTG1/GTG2 (plasma membrane). However, several of these reports have all been questioned. The first convincing results for the ABA receptors came from the two independent groups identifying a class of proteins that link ABA binding with downstream ABA responses, which can be considered as a landmark discovery (Ma et al., 2009; Park et al., 2009).

Primarily, Ma et al. (2009) discovered and termed as regulatory components of ABA receptor (RCARs), that interacted with the type 2C protein phosphatases (PP2Cs, like ABI1 and ABI2), that are well established as negative regulators of ABA responses. Similarly, Park et al. (2009) used pyrabactin, a synthetic growth inhibitor, functions as a selective ABA agonist, which acts through a family of START proteins called PYRABACTIN RESISTANCE 1 (PYR1)/PYLs isolated by map-based cloning. PYR1 was shown to bind ABA, which in turn interacts and inhibits PP2Cs to shutdown the activity. RCARs/PYR1/PYLs belong to a 14-member subfamily of the Bet v1-like superfamily, structurally similar with class 10 pathogen-related in Arabidopsis.

K^+ Channels

Potassium channels in guard cells are of two major classes, inward K^+ channels or outward K^+ channels. The key feature of these two classes of K^+ channels is their sensitivity to membrane voltage. The inward K-channel is active only on hyper

polarization. At millimolar K^+-concentration outside the cell, it provides the main pathway for K^+-influx required to drive stomatal opening (Blatt and Armstrong, 1993; Schroeder et al., 1994). The second type of channel, the outward rectifier, is activated when the membrane is depolarized. This channel serves as pathway for K^+ efflux in the course of stomatal closure (Thiel et al., 1992; Blatt and Armstrong, 1993).

Membrane ATPase

ATPase located on the plasma membrane, is an important component that modulates the ion flux into guard cells. The importance of the ATPase was demonstrated by diminished stomatal function in plants in which the expression of the dominant guard cell H^+-ATPase was suppressed (Zhao et al., 2000). The guard cell plasma membrane ATPase hyperpolarizes the membrane by excreting H^+, producing three important effects on K^+ uptake. First, the driving force for passive permeation of K^+ through inward K^+ channels is increased. Second, the membrane potential becomes sufficiently negative to open voltage gated inward K^+ channel. Third, the increase in concentration of external H^+, which precedes stomatal opening, activates further the inward K^+ channel. This ATPase is a member of large gene family (Leigh and Sze, 2000) that exhibits guard cell-specific expression (Assmann, 1996). An interesting property of the guard cell plasma membrane ATPase is its interaction with and modulatory 14-3-3 proteins (Kinoshita and Shimazaki, 1999)

Protein kinases/protein phosphatases

Several involved growth and developmental processes, are regulated through phosphorylation and dephosphorylation. In guard cells, both K^+_{in} and K^+_{out} channels were sensitive to kinase antagonists during elicitor stimulation (Blatt et

al., 1999). Tonoplast ion channels also are targets for phosphorylation-mediated control (Allen and Sander, 1995; Pei et al., 1996), as is the plasma membrane H^+ ATPase (Kinoshita and Shimazaki, 1999). Li et al. (1998) have shown that the Arabidopsis *KAT1* K^+ channel can be phophorylated but by a Ca^{2+}-dependent protein kinase endogenous to *Vicia faba* guard cells. Phosphorylation of plasma membrane H^+-ATPases in *Vicia faba* guard cells depended on endogenous 14-3-3 protein and lead to activation of ATPase (Kinoshita and Shimazaki, 1999). AAPK and Open Stomata1 (*OST1*)/*SnRK2.6*-type protein kinase (SnRK2.6) of *Arabidopsis* are ABA-activated SnRKs and are probably orthologs (Mustilli et al., 2002; Yoshida et al., 2002). OST1/SnRK2.6 controls ABA-dependent stomatal closure and ABA-dependent inhibition of opening, whereas seed dormancy and the inhibition of growth by ABA are not affected in *OST1/SnRK2.6*-deficient mutants. The key components of ABA signaling, SnRK2.6 is regulated by auto-kinase activity which further phosphorylates ABF-2/AREB1, transcription factor which mediates ABA dependent gene activation (Fujii et al., 2009).

Arabidopsis PP2Cs that are transcriptionally up-regulated by ABA-namely *ABI1*, *ABI2*, and the cold response linked PP2Cs *AtPP2CA* and *AtP2CHA*-act as negative regulators of ABA-responses (Tahtiharju and Palva, 2001; Merlot et al., 2001). *ABI1* and the highly homologous *ABI2* have attracted most attention as partly redundant key regulators of ABA-invoked seed dormancy, stomatal closure and growth inhibition.

The interesting and novel role of PP2C in ABA was elucidated through combined molecular genetic and electrophysiological studies of plants carrying the mutants *abi1*, *abi2* genes. The *abi1* gene and its homologue *abi2*, encode a 2C-type protein phosphatase (Meyer et al., 1994; Leung et al., 1997). Pei et al. (1997) found the activation of the current by ABA was suppressed by the type 1 and 2A protein phosphatase antagonist, okadaic acid, without apparent influence on

gating. Along with these results, PP2C, also dephosphorylates the SnRK2.6, to inactivate its activity (Fujii et al., 2009).

Intracellular free calcium

Calcium is a ubiquitous intracellular secondary messenger, involved in number of pathophysiological and developmental processes. In stomatal guard cells, calcium has a secondary messenger function in the signal transudation of several important stimuli (McAinsh et al., 1997; Leckie et al., 1998). Increase in guard cell Ca^{2+} occurs in response to plant hormones, such as ABA (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1991) or auxins (Irving et al., 1992) and other stimuli including elevated CO_2 (Webb et al., 1996) oxidative stress (McAinsh et al., 1996) and even elevated external calcium (Gilroy et al., 1991). A rise in cytoplasmic Ca^{2+} can induce stomatal closure (Israelsson et al., 2006). ABA activates release of Ca^{2+} from internal stores, but the source and trigger for ABA-induced increase in cytoplasmic Ca^{2+} are uncertain. Such elevations of Ca^{2+} could occur by influx of Ca^{2+} across the plasma membrane and via release of Ca^{2+} from internal stores (Israelsson et al., 2006; Luan, 2009; McAinsh and Pittman, 2009). Elevated guard cell cytosolic Ca^{2+} inhibits the K^+ influx by shifting the gating potential to an impermissibly negative value a situation, which occurs during ABA-induced stomatal closure (Grabov and Blatt, 1999). Further ABA-mediated inactivation of the inward K^+ channels can occur also through phosphatic acid (Jacob et al., 1999; Munnik, 2001; Sang et al., 2001) and G-proteins (Wang et al., 2001).

Reactive oxygen species

Reactive oxygen species (ROS) are essential signaling components during stomatal closure induced by either ABA or MJ (Zhang et al., 2001a, 2001b, 2001c; Suhita et al., 2004). ABA stimulated ROS accumulation activates plasma

membrane calcium channels and leads to stomatal closure (Pei et al., 2000; Murata et al., 2001). In *Vicia faba*, ABA induced ROS production occurred at not only plasma membrane but also in the chloroplast (Zhang et al., 2001b). In the *gca2* mutants of *Arabidopsis*, ABA increased ROS production, but H_2O_2-induced calcium channel activation and stomatal closure were absent in the mutants (Pei et al., 2000). Using *abi1* and *abi2* point mutants with strong reduced phosphatase activities, it was shown that ABA is unable to generate ROS in *abi1* mutants but ABA still induces ROS production in *abi2* mutants (Murata et al., 2001). These data indicate that *ABI1* may act upstream and *ABI2* downstream of ROS production. Protein kinases function between ABA perception and ROS signaling (Mustilli et al., 2002; Suhita et al., 2004). ABA induced ROS production was absent in *ost1* plants, although *ost1* stomata still closed in response to H_2O_2. The notion that *ost1* regulates ROS production directly via the NADPH-oxidase is an attractive hypothesis that has to be validated experimentally.

Nitric oxide

Nitric oxide is ubiquitous, and considered an important secondary messenger in a broad spectrum of pathophysiological and developmental processes in plants (Lamattina et al., 2003; Mur et al., 2006; Hong et al., 2008; Neill et al., 2008). In plants, NO regulated K^+ and Cl^- channels in guard cells through a subset of ABA-evoked signaling pathways (Garcia-Mata et al., 2003). Exogenous addition of NO to both monocot and dicotyledonous epidermis strips was sufficient to induce stomatal closure, through a Ca^{2+}-dependent process (Garcia-Mata and Lamattina, 2001). In *Pisum sativum* and *Vicia faba*, ABA induces an increase of endogenous NO levels. ABA-induced NO production was reported to be sufficient and necessary for ABA induction of stomatal closure (Garcia-Mata and Lamattina, 2002, 2003; Neill et al., 2002a).

Cytosolic pH

Several stimuli, that promote stomatal opening, tend to increase H^+ extrusion from guard cells or protoplasts (Assmann et al., 1985; Edwards et al., 1988) and decrease cytosolic pH (Felle, 1989). Two observations suggested that the "cytosolic pH" as a secondary messenger in ABA signaling: First, exposure to ABA leads to a 0.1-0.3 unit rise in pH of guard cells (Irving et al., 1992; Blatt and Armstrong, 1993); Second, decreasing cytosolic pH by loading with weak acid suppressed out ward K^+ potassium channel in a manner consistent with the action of ABA (Blatt, 1992). The rise in pH was sufficient for activation of outward K^+ channels in ABA (Blatt and Armstrong, 1993).

Molecular analysis of K^+ channels from Solanum and Arabidopsis suggest that one of the sites of H^+ action is either a histidine or aspartic acid residue near the pore in the channel protein (Hoshi, 1995; Hoth et al., 1997). However, pH have also shown to have effect on ABA-mediated control of the K^+ channel via de-phosphorylation of channel protein SnRK2.6 by *ABI1* protein phosphatase (Yoshida et al., 2006).

Phosphoinositides and phosphoinositide kinases

Phosphatidylinositol (PI) metabolism plays a central role in signalling pathways in both animals and higher plants (Drøbak et al., 1999; Stevenson et al., 2000). Phosphoinositides mediate ABA-induced cytosolic Ca^{2+} changes and subsequent stomatal closure (Staxén et al., 1999). Stomatal guard cells contain PI3-phosphate (PI3P) and PI4-phosphate (PI4P), the products of PI3-kinase (PI3K) and PI4-kinase (PI4K) activities. Unlike several distinct PI3K isoforms in animals, only one PI3K type, a PI-specific PI3K related to yeast Vps34p, has been found in plants. Plant PI3K has been suggested to be involved in root nodule development, vesicle trafficking and regulation of transcription (Bunney et al., 2000; Kim et al.,

2001). Guard cells of *Commelina communis* contain PI3P and PI4P, which are suggested to be involved in guard cell signalling (Parmar and Brearley, 1995).

Phospholipases

Phospholipases C and D (PLC and PLD) play an essential part in signal transduction of guard cells. ABA induced stomatal closure was partially inhibited by U73122, an inhibitor PLC (Staxen et al., 1999). However, complete inhibition of ABA induced stomatal closure was achieved by treating stomata with a combination of U73122 and nicotinamide (Jacob et al., 1999; Mac Robbie, 2000) suggesting that both cADPR and PLC signaling systems functioned in ABA signaling. Our previous work from our lab (Kolla et al., 2004) showed that U73122, and 1-butanol (PLD inhibitor) reversed the stomatal closure induced by ABA but not by CO_2. ABA treatment of *Vicia faba* guard cells caused phosphatidic acid levels to transiently increase 2.5 fold (Jacob et al., 1999). Phosphatidic acid promoted stomatal closure and inactivated K^+ currents, while guard cell cytosolic Ca^{2+} did not increase following phosphatidic acid treatment, indicating the importance of PLC and PLD in stomatal response.

Role of Arabidopsis mutants to dissect the guard cell signalling

The wide spectrum of *Arabidopsis* mutants offer an interesting and powerful tool to dissect the signalling components involved in signalling cascades leading to the metabolic functions. In particular, the Arabidopsis mutants, insensitive to ABA: *abi1*, *abi2* have been extensively used to study ABA induced stomatal closure. These *abi1* or *abi2* mutants are gain of functional mutants in type 2C protein phosphatases, which are normally negative regulators of ABA mediated responses (Merlot et al., 2001; Schroeder et al., 2001a). Stomatal closure in response to H_2O_2, but not to ABA, in the abi1/2 mutants provided a clear indication that the action of H_2O_2 is down stream of PP2C (Meinhard and Grill, 2001). Arabidopsis

mutant *ost1* 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, between ABA perception and ROS production (Merlot et al., 2002; Assmann, 2003). The signal from ABA to OST1 was modulated by ABI1 and then proceeds via ROS to regulate cytosolic Ca^{2+} levels and ultimately leads to changes in stomatal aperture. 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. Mutants are also useful to know the crosstalk between the two signaling components. Some of the mutants using in research were mentioned below (Table 1.1).

Future perspectives and need for further work

A thorough understanding of physiological and molecular interactions that occur during stomatal movements requires further studies on genomics, proteomics and metabolomics of guard cells. While considerable information accumulated on the responses of guard cells to ABA these are only limited attempts to examine the molecular basis of the action of other signals, such as CO_2 or light and even the circadian clock on stomatal movements and guard cells.

One of the fascinating progresses is the elucidation of early steps in the pathway from ABA perception to ABA-dependent gene regulation, combined interaction of PYR/PYLs/RCARs-clade-A PP2Cs-SnRK2s-ABFs are the only core components to complete the ABA regulation of gene expression (Fujii et al., 2009). It is possible that other ABA responses such as regulation of ion channels

Table 1.1. Mutants with their impaired function(s) which were used in ABA-signal transduction studies. The mutants are listed in alphabetical order.

Gene	Impaired component	Reference
abcap	ABA hypersensitive loss-of-function mutant	Hugouvieux et al., 2001
abh1	mRNA cap binding protein	Hugouvieux et al., 2001
abi1, abi2	Protein phosphatase 2C	Leung et al., 1994
AtNOS1	Nitric oxide synthase gene	Guo et al., 2003
AtrbohDF	NAD(P)H oxidase	Kwak et al., 2003
det3	Vacuolar H(+)-ATPase	Schumacher et al., 1999
era1	Farnesyltransferase enzyme	Cutler et al., 1996
Gca	Calcium influx currents	Pei et al., 2000
gcr1	G-protein coupled receptors	Pandey and Assmann, 2004
gork1	Outward K^+ currents	Hosy et al., 2003
gpa1	G-protein α subunit	Wang et al., 2001
jar1	Isoleucine conjugating enzyme to jasmonic acid	Staswick et al., 1992
KAT1	Inward K^+ channel	Very and Sentenac, 2002
nia1/2	Nitrate reductase defective	Desikan et al., 2002
nox1	Nitric oxide over production mutant	He et al., 2004
ost1	Serine–threonine protein kinase	Mustilli et al., 2002
rpk1	LRR receptor kinase in the plasma membrane	Hong et al., 1997
tpc1	Ca^{2+}-permeable channel	Peiter et al., 2005

(Roelfsema and Hedrich, 2005) also use components of the PYR/PYLs/RCARs-PP2C-SnRK2 regulatory module but needs further studies. Calcium and reactive oxygen signalling, RNA metabolism and protein degradation are known to be important in regulating ABA sensitivity (Li et al., 2006; Hirayama and Shinozaki, 2007). However, the exact role and the precise source of cytosolic calcium increases in ABA responses is not fully understood. The detailed participation of cyclic nucleotides like cAMP or cGMP and cADPR are yet to be studied in detail (Wu et al., 2003). The intricacies and the orchestration of the many transcription factors involved in guard cell function remain to be fully elucidated. It will be of great interest to determine how these different processes may connect to one or more of the core components to affect ABA responses. Another aspect is the crosstalk of the ABA signalling pathway with other hormonal responses. It would therefore be of great interest to elucidate the participation and interaction of the secondary messengers in the cascades leading to the respective final outputs.

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

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Chapter 2

Scope of the present work, approach and objectives

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Stomatal guard cells are model systems to study the signal transduction in plants, as they respond quickly and reversibly to diverse environmental cues. During the mediation of stomatal closure by ABA, several secondary messengers are involved. However, the exact sequence of action of these secondary messengers, as well as their interactions are not clear. Among the components participating in stomatal closure, our experiments were focused primarily on NO, ROS, cytosolic pH, calcium, calmodulin, PI3K and protein phosphatases. We attempted to study the importance, if any, of these secondary messengers during ABA induced stomatal closure, and their interactions with other signalling molecules were assessed.

Objectives of the present study

- Examine the pattern and dynamics of changes in NO and cytosolic pH in guard cells during stomatal closure by ABA.
- Investigate the sources and interactions of NO and ROS during ABA induced stomatal closure.
- To study the importance of other signalling components, particularly PI3K, Ca^{2+}, CaM and their interactions with NO and ROS during stomatal closure by ABA.
- To understand the role of PP2C during ABA effects, by using Arabidopsis mutants *abi1, abi2* and *aba2*, in comparison with the wild type (Landsberg *erecta*).
- Propose a scheme for signal transduction, integrating the present results and available literature.

Vicia faba, Pisum sativum, Commelina benghalensis and Nicotiana species, besides Arabidopsis are extensively studied in the signal transduction mechanisms and biochemical and bioenergetic features of guard cells. We have chosen *P. sativum* and Arabidopsis for our experiments. The role of ROS, NO and pH during ABA induced stomatal closure was assessed in stomatal guard cells of *P. sativum*. It is quite easy to prepare epidermal strips from pea leaves and use them for monitoring stomatal movement, by a microscope or use an image analysis system to examine the levels of NO, ROS and pH in guard cells with the help of fluorescence probes. Another advantage with *P. sativum* is that both mesophyll and guard cell protoplasts can be isolated easily (Devi et al., 1992). Thus, the leaves of *P. sativum* offer a very good experimental material to study stomatal function at several stages of organization: intact leaves, leaf epidermis, isolated protoplasts and even organelles.

Later on, Arabidopsis plants were used because of the availability of wide spectrum of mutants, as well as well established protocols for isolation of protoplasts, transient expression of recombinant proteins. The role of PP2C and its role in ABA mediated responses including stomatal closure, seed germination, root elongation and ABA mediated gene expression were studied in Arabidopsis.

The importance of NO, ROS, pH, Ca^{2+}, PI3K and calmodulin during ABA induced stomatal closure was established by using suitable modulators as indicated below (Table 2.1). For real time monitoring of NO, ROS and cytosolic pH, we used fluorescence probes specifically such as (i) DAF-2DA for NO (Neill et al., 2002a) (ii) H_2DCFDA for ROS (Murata et al., 2001) (iii) BCECF-AM for pH changes (Irving et al., 1992) in cytosol.

The results were assessed to reach meaningful conclusions and to refine our knowledge of mechanism and the signaling components involved in stomatal closure by ABA. An attempt is made to integrate the observations and propose a model to explain the role and road map of different secondary messengers and the possible sequence of events during ABA induced stomatal closure.

Table 2.1 Modulators that we have used in our study

Secondary messenger	**Positive regulator**	**Negative regulator**
NO	SNP (Sodium nitroprusside)	cPTIO (scavenger) Sodium tungstate (nitrate reductase inhibitor) L-NAME (NOS inhibitor)
ROS	H_2O_2	Catalase (H_2O_2 Scavenger) DPI (NADPH oxidase inhibitor)
pH	Methylamine (cytosolic alkalinizer)	Butyrate (cytosolic acidifier)
Calcium		EGTA (calcium chelator) BAPTA (extracellular calcium chelator) BAPTA-AM (intracellular calcium chelator)
PI3K		WM (PI3K inhibitor) LY294002 (PI3K inhibitor)
Calmodulin (CaM)		W-7 (CaM antagonist) Calmidazolium chloride (CDZ) (CaM antagonist)

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Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant material

Plants of *Pisum sativum* (cv. Arkel) were raised from seeds (Pocha seeds Co. Pvt. Ltd. Pune, India). 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 25^0C until germinated, usually for 3 d. The germinating seeds were then sown in plastic trays filled with soil and farmyard manure (3:1, v/v). The plants were grown in a green house, average day/night temperature of about $30/20^0$C and photoperiod of 12 h and were watered twice daily. The second to fourth completely unfolded leaves were collected from 2 to 3 week-old plants for epidermal bioassays.

The seeds of *Arabidopsis thaliana* (wild type: Landsberg *erecta* or mutants *abi1, abi2, aba2*) were sterilized by using 80% ethanol in 0.1% Triton X-100 and 3% NaOCl. Seeds were then transferred in rows on ½ MS plates (Table 3.1), wrapped with Parafilm, incubated for 2 d at 4^0C in dark to break dormancy. Then plates were transferred to culture room at 25^0C in continuous light. One to two week old seedlings were planted in plastic trays containing 1:1:1 mixture of vermiculite, perlite and soilrite and transferring to growth chambers where the optimal conditions for growth was maintaining [light (125-150 µmol m^{-2} s^{-1}), photoperiod 16/8 h (light/dark), temperature 25^0C] and nutrient solution (Table 3.2) was supplied daily upto three weeks then once in every week.

Bioassays of stomatal closure in epidermal strips

The abaxial (lower) epidermis was peeled off from the leaves (pea or Arabidopsis) and cut into strips of ca. 0.16 cm^2. The epidermal strips were transferred to 3 cm diameter petri dishes containing 3 ml of "incubation medium" (10 mM MES-KOH pH 7.0 and 50 mM KCl) and the epidermal strips were exposed for 3 h to white light 250 and 150 µmol m^{-2} s^{-1} for pea and Arabidopsis respectively. A bank

Table 3.1 The composition of ½ MS Agar for rising the seedlings, germination and root growth assays.

Macronutrients	**(1 Litre)**	**Micronutrients**	**(1 Litre)**
NH_4NO_3	1.65 g	$CoCl_2\,6H_2O$	0.025 mg
KNO_3	1.9 g	KI	0.75mg
KH_2PO_4	0.17 g	$MnSO_4$	10 mg
$MgSO_47H_2O$	0.37 g	$ZnSO_47H_2O$	2 mg
$CaCl_2$	0.44 g	$CuSO_4$	0.025 mg
		H_3BO_4	3 mg
		$FeSO_4\,7H_2O$ (in 0.1 mM Na_2EDTA)	27.8 mg
		Na_2MoO_4	0.25 mg
		H_3BO_4	3 mg
Sucrose 5g/L, MES 1g/L, Agar 10g/L, pH was maintained 5.8 with 1 M KOH and autoclaved.			

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

Macronutrients	**(1 Litre)**	**Micronutrients**	**(1 Litre)**
KNO_3	0.505 g	$FeSO_4\,7H_2O$ (in 50 mM Na_2EDTA)	27.8 mg
KH_2PO_4	0.34 g	$MnSO_4$	10 mg
$MgSO_4$	0.492 g	$ZnSO_4$	2 mg
$Ca(NO_3)_2$	0.47 g	$CuSO_4$	0.025 mg
		H_3BO_4	3 mg
		KI	0.75 mg
		Na_2MO_4	0.25 mg
		$CaCl_2$	0.025 mg

of tungsten lamps, whose light was filtered through water jacket, provided the irradiation with white light. Photon flux was measured with a Li-Cor quantum sensor (Li-Cor Instruments Ltd, Lincoln, NE, USA). The temperature was maintained at 25 ± 1^0C. Test compounds (scavengers or inhibitors) were added to the incubation medium 10 min before the addition of ABA, and the epidermal strips were kept under the same conditions for another 3 h.

The width of the stomatal aperture was measured under a research microscope (Nikon, Eclipse TE 200, Tokyo) with the help of a precalibrated ocular micrometer. Ten apertures were monitored at random in each of three different epidermal strips, from each treatment. The experiments were repeated for 3 different days, making each measurement of stomatal aperture an average of at least 90 stomata (Kolla et al., 2007; Suhita et al., 2004).

Monitoring NO/pH/ROS

The changes in NO/pH/ROS was monitored in guard cells of *Pisum sativum* by probing with 4,5-diaminofluorescein diacetate (DAF-2DA), 2',7'-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Irving et al., 1992; Murata et al., 2001; Neill et al., 2002a).

Paradermal sections of abaxial epidermis for fluorescence studies were prepared by mounting the epidermal sections on glass cover slips with the help of medical adhesive, Telesis V (Premiere Products Inc., Pacaima, California, USA) and sections were allowed to open under light for 3 h in incubation medium. The epidermal strips were loaded with 20 µM BCECF-AM (10 min) or 20 µM DAF-2DA (10 min) in incubation medium containing 0.05% Pluronic F-127 or 20 µM H_2DCFDA (10 min), in dark at 25 ± 1^0C. The strips were rinsed quickly with three changes of incubation medium to wash off the excessive fluorophore. The dye-loaded strips were treated with test compounds as indicated, followed by ABA after 10 min. The strips were then monitored under confocal microscope (Leica,

TCS-SP-2, AOBS 4 channel UV and visible, Heidelberg, Germany) to observe the fluorescence of DAF-2DA or BCECF-AM or H_2DCFDA (Excitation 488 nm, emission 510-540 nm).

In experiments involving time-course monitoring of signalling components in guard cells, the epidermal strips were examined under an inverted fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a monochrome high-resolution digital cooled CD camera (CoolSNAP *cf*, Photometrics, Roper Scientific) that enabled to capture the images with DAF-2DA or BCECF-AM or H_2DCFDA fluorescence (filter: Nikon B-2E/C, excitation 465-495, emission 515-555). The captured images and the relative fluorescence emission of guard cells were analysed by using NIH Image for Windows (Murata et al., 2001; Suhita et al., 2004; Kolla et al., 2007).

Image acquisition and analysis

The levels of the fluorescence in the images acquired through either the epifluorescence microscope or inverted fluorescence 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 analyzing 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.

Preparation of mesophyll protoplast from Arabidopsis leaves

One gram of leaf material from Arabidopsis was digested for 3-4 h with 10 ml of "digestion medium" containing (1 % (w/v) Cellulase Onuzuka R-10, 0.2% (w/v) Macerozyme R-10, 400 mM Mannitol, 8 mM $CaCl_2$, 0.25% (w/v) BSA, 10 mM sodium ascorbate, 1 mM $CaCl_2$, 10 mM MES-KOH; pH 5.6. The digestion medium was filtered and the filtrate washed twice with 10 ml of "washing medium" containing 500 mM mannitol, 5 mM MES/TRIS, pH 5.8-6.0. Protoplasts were re-suspended in 1 ml of "suspension medium" containing 400 mM mannitol, 15 mM $MgCl_2$, 5 mM MES/KOH pH 5.8. The medium was adjusted to have 0.5 to 1.0 x 10^6 protoplasts ml^{-1}. The numbers of protoplasts were counted with a haemocytometer.

Transient expression and reporter assays in mesophyll protoplasts of Arabidopsis

The transformation of protoplasts was based on the principles described by Himmelbach et al. (2002) and Yang et al. (2006), later modified by Moes et al. (2008). The following components were used: *pRD29B::LUC* (promoter of the desiccation-responsive gene RD29B (At5g52300) fused with luciferase, as reporter) and β-glucuronidase (GUS, fused with 35S promoter) for assessing transformation efficiency and normalization. Further, the protoplasts were transformed to express either normal (ABI1, ABI2) or mutant (abi1, abi2) forms of PP2Cs (fused with 35S promoter and GFP).

To 100 µl of protoplast suspension 10-20 µg/30 µl reporter DNA was added, followed by an equal volume of "PEG buffer" containing 40% PEG, 300 mM $CaCl_2$, 0.5 % MES-KOH, pH 5.8. The components were gently mixed by inverting 3-4 times and incubated for 3-5 min. The protoplasts were washed twice with 750 µl of washing media and re-suspended in 100-150 µl of washing media and incubated for expression at 22^0C for 12 h.

To 50 µl of protoplast suspension, 100 µl of "cell lysis and GUS assay reagent" [25 mM Tris-phosphate, pH 7.8, 2 mM Dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid, 10% (v/v) Glycerine, 1% Triton X100, 0.2 mM 4-Methylumbelliferyl-β-D-Glucuronid (MUG)] was added in black micro titer plate and measured GUS activity with micro plate reader ("HTS 7000 Plus Bioassay Reader", Perkin Elmer, excitation-360 nm, emission-465 nm) for 7 min by using software program "HTSsoft", then transferred 100 µl of the same lysate to luminometer tubes, measured the luciferase activity in lumonometer ("flash'n glow", Berthold) with the software "Berthold TubeMaster". Luminescence was measured for 10s as background and 20s for activity, after injection of "luciferase assay reagent" (20 mM Tricine/NaOH pH 7.8, 2.7 mM $MgSO_4$, 0.5 mM EDTA, 33.3 mM DTT, 0.53 mM ATP, (26 mg $(MgCO_3)_4$, 10 mg Coenzyme A, 7.5 mg Luciferin)/50 ml) (Moes et al., 2008).

Seed germination and root elongation assays

Under sterile conditions 100-150 seeds were plated on ½ MS Agar medium and incubated at 4^0C for 2 d in dark to break dormancy. The plates were then transferred to culture room, with a continuous light (60 µE m^{-2} s^{-1}) at 22^0C. After 4 d, seeds were examined under a stereo microscope. Seeds were counted as germinated when the radicles emerged by 1 mm, and germination rate is calculated as percentage of the total number of seeds. For root elongation, five day old seedlings were transferred in a row to MS Agar containing different combinations of treatments and kept in a vertical position at 22^0C in continuous light for 4 d. Root tip position was marked for every 24 h and root lengths were measured with the mm scale under a microscope (Moes et al., 2008).

Replication and statistical analysis

The data presented are the average values (± SE) of results from at least three experiments conducted on different days. Software from Sigma were used for

statistical analysis, student's *t-test* (SigmaPlot for Windows Version 10.0) or one way ANOVA (SigmaStat for Windows Version 3.1).

Solvents, chemicals and materials

Abscisic acid was dissolved in 10 mM MES-KOH pH 7, while wortmannin, LY 294002, W-7, calmidazolium chloride, L-NAME, DPI, H_2DCFDA, BCECF-AM, DAF-2DA were dissolved in DMSO and all others in milli Q water. Most of the chemicals were from Sigma (Sigma Chemical Company, St Louis, MO, USA). Cellulase R-10, Macerozyme were from Sheishin Corporation (Tokyo, Japan), W-7 and other inhibitors were from BIOMOL (Plymouth, PA, USA). H_2DCFDA, DAF-2DA, L-NAME 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

Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid

Chapter 4

Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid

The components of ABA signaling leading to stomatal closure include reactive nitrogen species, i.e. nitric oxide (NO), besides reactive oxygen species (ROS, Hetherington, 2001; Ng et al., 2001; Neill et al., 2003; Zhang et al., 2007). An increase in ROS of guard cells has been reported during stomatal closure induced by methyl jasmonate or bicarbonate (Suhita et al., 2004; Kolla et al., 2007). Further, NO plays an important role during ABA-induced stomatal closure as observed in *Pisum sativum*, *Vicia faba* and Arabidopsis (Desikan et al., 2002; Neill et al., 2002a, 2003; Garcia-Mata and Lamattina, 2003; Yan et al., 2007). The levels of NO in guard cells increase on exposure to bicarbonate too (Kolla and Raghavendra, 2007). Exogenous application of sodium nitroprusside (SNP), a NO donor, increased plant tolerance to drought stress, by restricting stomatal apertures (Garcia-Mata and Lamattina, 2001). However, the mechanism by which ABA induces an increase in guard cell NO levels and its place in the signalling cascade leading to stomatal closure by ABA are not completely clear.

Marked changes in cytosolic pH of plant tissues are observed during responses to a variety of hormones, including ABA or MJ. For e.g. the pH of guard cells increases in presence of ABA or MJ (Irving et al., 1992; Van der Veen et al., 1992; Suhita et al., 2004). Exposure to even H_2O_2 can lead to a rise in intracellular pH as shown in case of *Vicia faba* guard cells (Zhang et al., 2001a). The cytosolic alkalinization preceded ROS production during stomatal closure by ABA or MJ (Suhita et al., 2004). It is yet to be examined, if pH has any role in NO production during ABA effects on guard cells.

This chapter describes the experiments designed to examine the importance and interactions of NO, cytosolic pH and calcium during stomatal responses to ABA in the abaxial epidermis of *Pisum sativum*.

Results

Patterns of NO production and cytosolic pH during ABA induced stomatal closure

The fluorescence probes of DAF-2DA or BCECF-AM enabled us to determine the kinetics of NO or pH changes in guard cells on exposure to ABA respectively. Treatment with ABA caused a marked increase in both DAF-2DA or BCECF-AM fluorescence in guard cells (Fig. 4.4B, D). Fluorescence of DAF-2DA started to increase steeply after 9 min and reached maximum at 18 min (Fig 4.5A). In contrast, the increase in BCECF-AM of guard cells on exposure to ABA was visible by 6 min and reached maximum at 12 min (Fig. 4.5B).

Stomatal closure in relation to modulation of NO or pH

Butyrate (a weak acid), prevented stomatal closure by ABA (Fig. 4.2A), while methylamine (a weak alkalinizing agent), enhanced ABA-induced stomatal closure (Fig. 4.2B). The ABA-induced stomatal closure was prevented completely by cPTIO (Fig. 4.3A).

Figs. 4.6 and 4.7 represent the patterns of increase in fluorescence of DAF-2DA/BCECF-AM with or without ABA, in presence of different modulators. Butyrate prevented the DAF-2DA (Fig. 4.6J) and BCECF-AM fluorescence (Fig. 4.7J) induced by ABA. Butyrate, alone, had no significant effect on either stomatal closure (Fig. 4.2A) or the rise in DAF-2DA/BCECF-AM fluorescence (Fig. 4.6). Methylamine alone induced stomatal closure (Table 4.1), while increasing DAF-2DA (Fig. 4.6E) and BCECF-AM fluorescence (Fig. 4.7E). When incubated with ABA, methylamine further increased both DAF-2DA and BCECF-AM fluorescence (Fig. 4.6K; 4.7K) in guard cells.

Other factors affecting the DAF-2DA/BCECF-AM fluorescence

Table 1 presents comprehensive information on the effects of different modulators on the rise in DAF-2DA/BCECF-AM fluorescence as well as stomatal closure. SNP alone promoted stomatal closure and enhanced to a limited extent the BCECF-AM fluorescence of guard cells (Table 4.1). However, SNP had no further effect on ABA induced BCECF-AM fluorescence (Fig. 4.7H). Similarly, cPTIO did not affect much the BCECF-AM fluorescence (Fig. 4.6I), but restricted quite strongly, the DAF-2DA fluorescence (Fig. 4.7I) by ABA. The presence of SNP, enhanced not only the stomatal closure (Table 1), but also DAF-2DA fluorescence (Fig. 4.6H) in the absence or presence of ABA. cPTIO prevented completely the ABA induced DAF-2DA fluorescence in guard cells (Fig. 4.6I).

Effect of Ca^{2+} chelator on ABA mediated rise in DAF-2DA/BCECF-AM fluorescence

EGTA, a calcium chelator, prevented the stomatal closure by ABA (Fig. 4.3B). When used alone, had no effect on stomatal closure and DAF-2DA/BCECF-AM fluorescence (Fig. 4.6, 7F), but prevented the rise in fluorescence of DAF-2DA/BCECF-AM in presence of ABA (Fig. 4.6, 7L). Apart from the ABA, EGTA also prevented the stomatal closure by 0.1 mM H_2O_2 or SNP (Fig. 4.8A, B).

(Discussion will be continued from page No. 44)

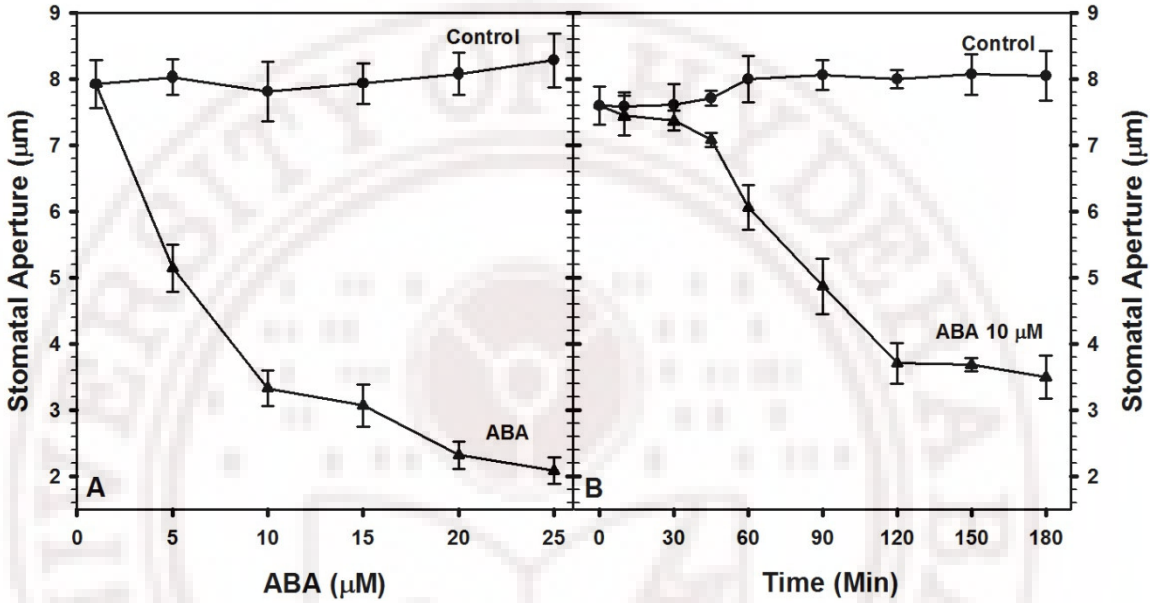

Figure 4.1. Concentration dependent stomatal closure in epidermal strips of *Pisum sativum* by ABA (A) or time dependent stomatal closure by 10 μM ABA (B). Dose dependent assay of stomatal closure revealed that, at a concentration of 10 μM, ABA induced 50% of the stomatal closure and time dependent stomatal closure assay revealed that, stomatal closure starts by 30 minutes and obtained 50% stomatal closure by 120-150 min upon exposure to 10 μM ABA. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

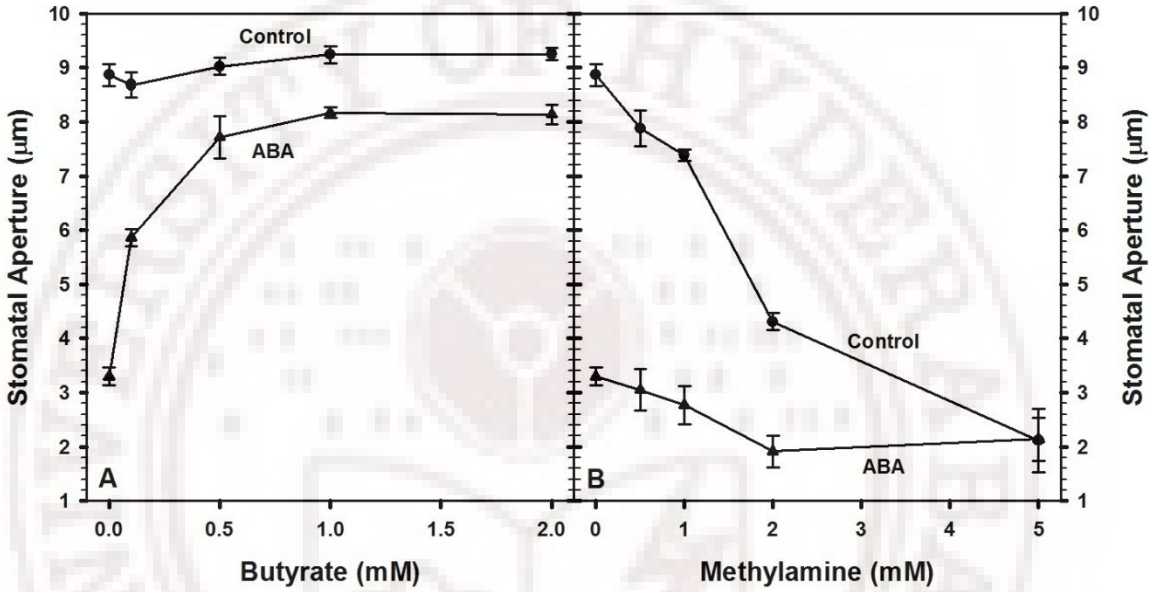

Figure 4.2. Effect of butyrate, a weak acid (A), or methylamine, an alkalinizing agent (B), on stomatal closure induced by 10 μM ABA in epidermal strips of *Pisum sativum*. Butyrate prevented stomatal closure by ABA, while methylamine further enhanced such stomatal closure. Butyrate alone had not much effect, while methylamine promoted stomatal closure, even in the absence of ABA. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

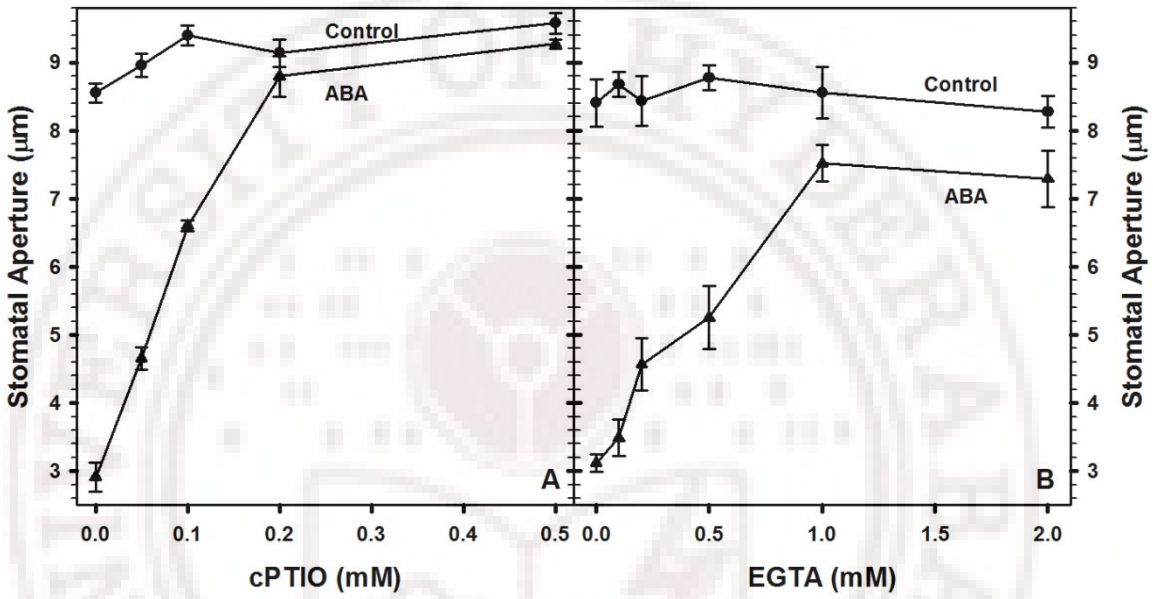

Figure 4.3. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by cPTIO, a NO scavenger (A) or EGTA, Ca^{2+} chelator (B). The presence of 0.2 mM or above, cPTIO, 1mM EGTA prevented the ABA induced stomatal closure. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

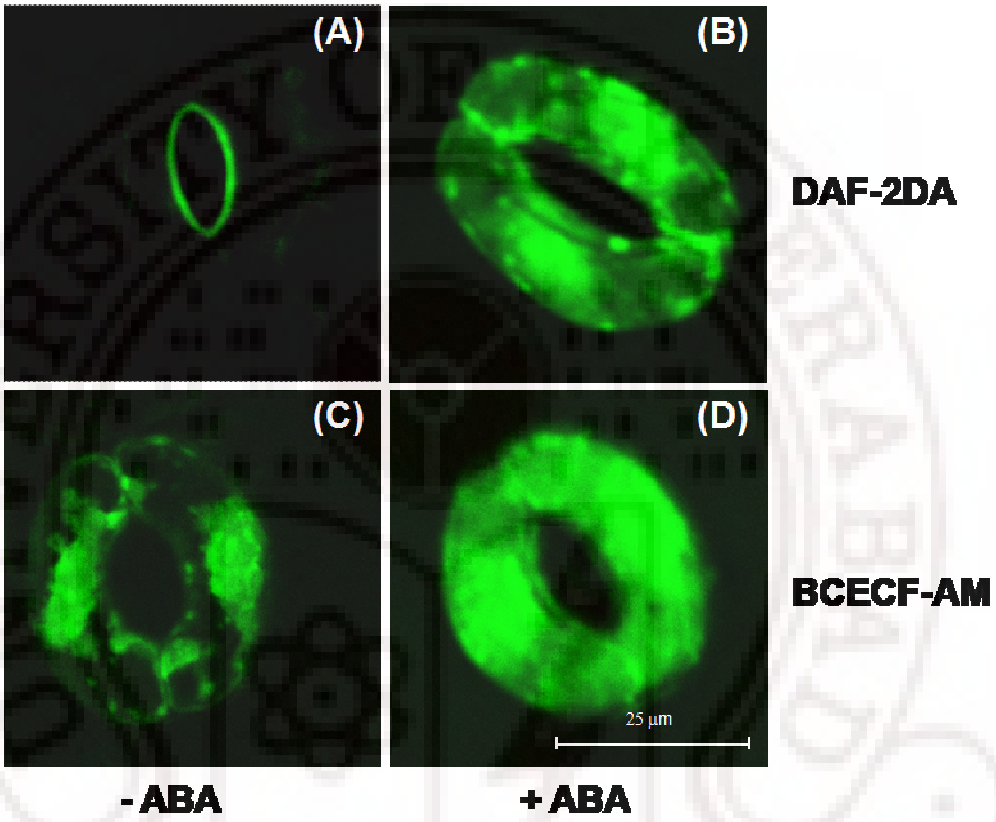

Figure 4.4. Confocal fluorescence images of stomata loaded with DAF-2DA (A, B) or BCECF-AM (C, D). These were taken after 12 min for BCECF-AM and 18 min for DAF-2DA treatment with 10 µM ABA. The panels (A) and (B) are the controls while (C) and (D) are the stomata treated with ABA. Bar = 25 µm.

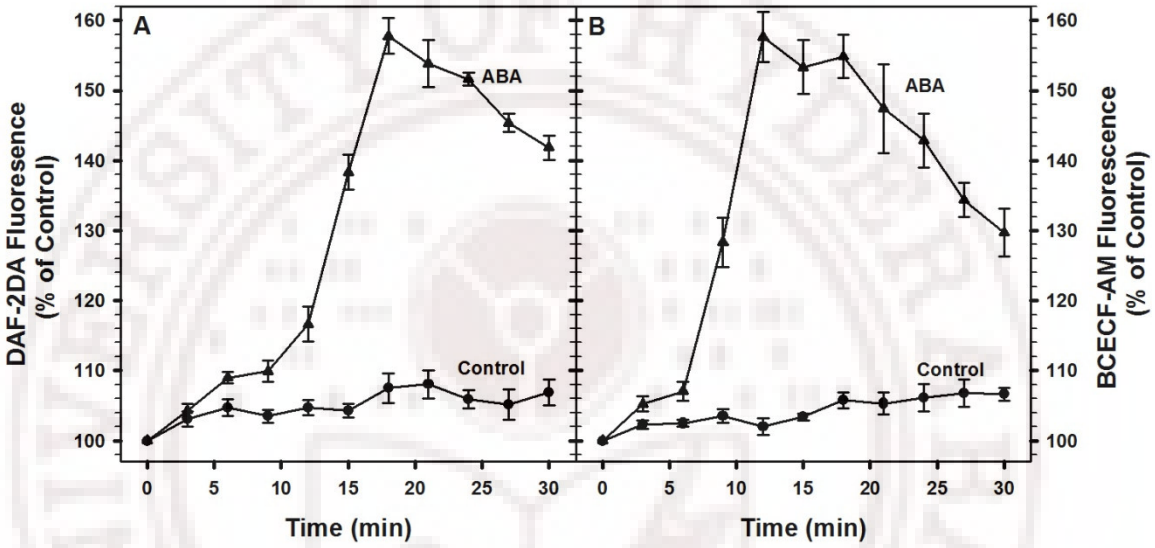

Figure 4.5. Kinetics of increase in NO (A) or pH (B) in epidermal strips of *Pisum sativum* in response 10 μM ABA. The epidermal strips were loaded with either DAF-2DA (for NO) or BCECF-AM (to monitor pH) while incubating with ABA. Nitric oxide production reached maximum at 18 min, after a lag of 9 min where as cytosolic pH reached maximum by 12 min, after a lag period of 6 min. The extent of NO or pH production in the guard cells without ABA is taken as 100%. Further details are described in Materials and Methods. Results are the averages ± SE from at least 3 independent experiments.

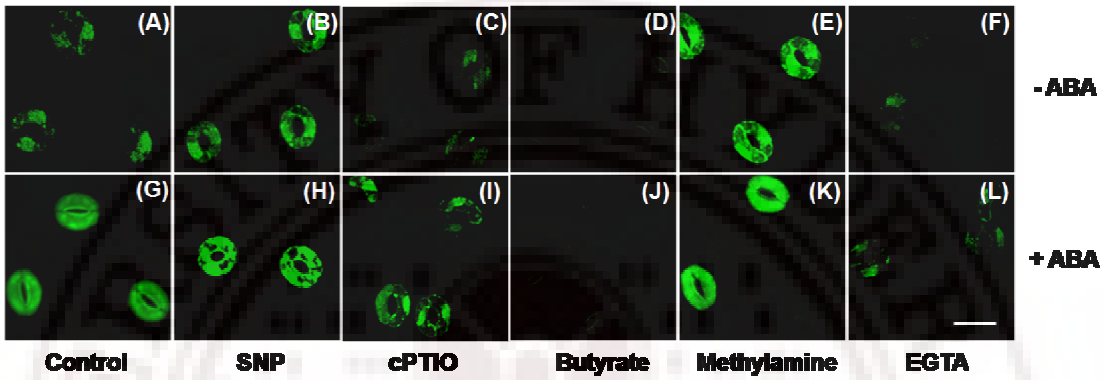

Figure 4.6. Effect of different modulators on 10 µM ABA induced NO production, as indicated by DAF-2DA fluorescence in stomatal guard cells of *Pisum sativum*. The panels A to F are the controls: treated with water (A), 0.1 mM SNP (B) 0.2 mM cPTIO (C), 0.1 mM butyrate (D), 2 mM methylamine (E), 1 mM EGTA (F) in absence of ABA respectively. The panels G to L are epidermal strips treated with ABA, as follows: ABA alone (G), ABA along with 0.1 mM SNP (H), 0.2 mM cPTIO (I), 0.1 mM butyrate (J), 2 mM methylamine (K), 1 mM EGTA (L) in presence of ABA respectively. Confocal fluorescence images were taken at 18 min after addition of 10 µM ABA. Further details are given in Materials and Methods. Bar = 25 µm.

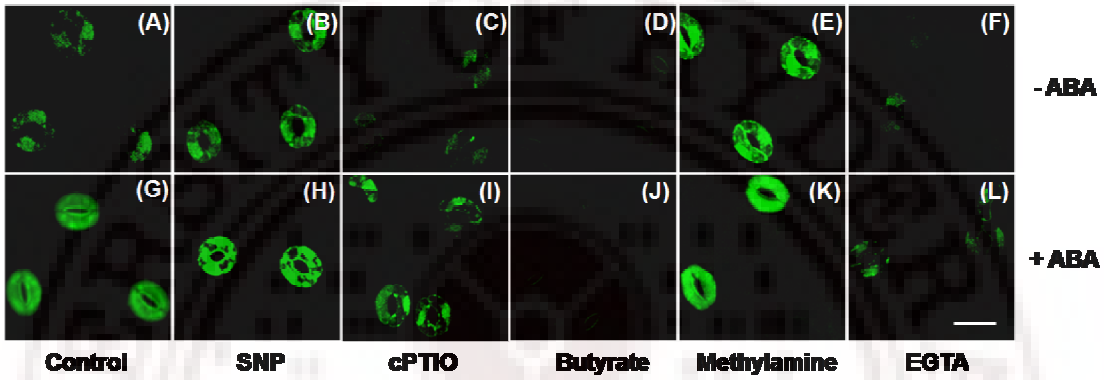

Figure 4.7. Effect of different modulators on 10 µM ABA induced increase in pH, as indicated by BCECF-AM fluorescence in stomatal guard cells of *Pisum sativum*. The panels A to F are the controls: treated with water (A), 0.1 mM SNP (B) 0.2 mM cPTIO (C), 0.1 mM butyrate (D), 2 mM methylamine (E), 1 mM EGTA (F) in absence of ABA respectively. The panels G to L are epidermal strips treated with ABA, as follows: ABA alone (G), ABA along with 0.1 mM SNP (H), 0.2 mM cPTIO (I), 0.1 mM butyrate (J), 2 mM methylamine (K), 1 mM EGTA (L) in presence of ABA respectively. Confocal fluorescence images were taken at 12 min after addition of 10 µM ABA. Further details are given in Materials and Methods. Bar = 25 µm.

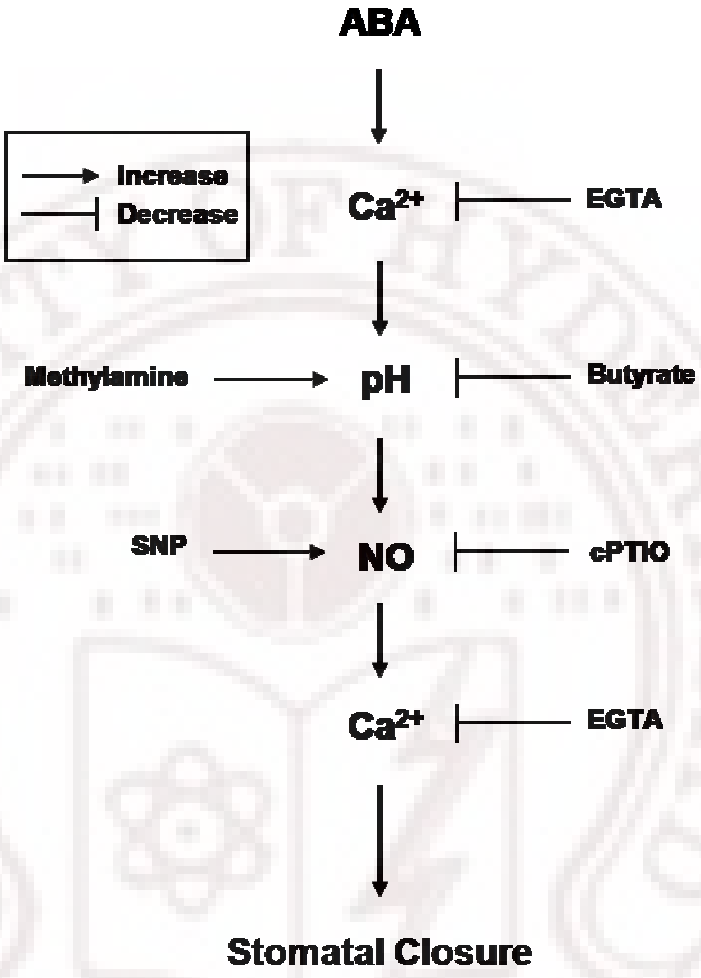

Figure 4.8. Schematic representation of ABA induced stomatal closure. Cytosolic alkalinization is one of the key and early steps leading to the stomatal closure. Exposure to ABA, leads to an increase in cytosolic pH, raises the level of NO and subsequently leads to stomatal closure. Modulation of guard cell pH by butyrate or methylamine affects NO-levels in guard cells, and the extent of stomatal closure. Similarly, modulation of NO levels affects stomatal closure but not the pH-rise. Ca^{2+} appears to be necessary for ABA-induced rise in pH as well as the action of NO. The role of Ca^{2+} upstream of NO is well-known in the literature.

Table 4.1 The effect of pH modulators (butyrate, methylamine) or NO modulators (cPTIO or SNP) and calcium chelator (EGTA) on ABA induced stomatal closure, cytosolic pH changes and NO production in guard cells of *Pisum sativum.*

The extent of fluorescence without ABA and without any effector is taken as 100%. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods. ∗Significant at *P* value < 0.05, compared to the respective treatment without ABA.

	No ABA			**+ 10 μM ABA**		
Treatment	Stomatal Aperture (μm)	BCECF-AM Fluorescence (% Control)	DAF-2DA Fluorescence (% Control)	Stomatal Aperture (μm)	BCECF-AM Fluorescence (% Control)	DAF-2DA Fluorescence (% Control)
None (Control)	8.9 ± 0.2	100 ± 0	100 ± 0	3.3* ± 0.2	157* ± 3	161* ± 4
0.1 mM Butyrate	9.2 ± 0.2	91 ± 2	107 ± 2	6.2* ± 0.1	101 ± 4	111 ± 2
2 mM Methylamine	4.3 ± 0.2	173 ± 3	159 ± 4	1.9* ± 0.3	174 ± 7	166 ± 6
0.2 mM cPTIO	9.1 ± 0.2	108 ± 2	106 ± 2	8.8 ± 0.3	140* ± 5	109 ± 3
0.1 mM SNP	3.9 ± 0.2	122 ± 5	164 ± 8	3.1 ± 0.2	156* ± 3	168 ± 4
1 mM EGTA	9.0 ± 3.9	110 ± 2	105 ± 2	8.6 ± 0.2	108 ± 4	110 ± 3

Discussion

It is well established that NO and cytosolic Ca^{2+} are essential signaling components during ABA-induced stomatal closure (Neill et al., 2002a). The present study demonstrates the importance and interactions of cytosolic alkalinization with NO and Ca^{2+} during ABA induced stomatal closure. The cytosolic alkalinization appears to be necessary and occuring upstream of NO production during ABA-induced stomatal closure.

Cytosolic alkalinization appears to precede NO production in guard cells on exposure to ABA

The pH is an important signaling component during several of plant responses including stomatal movements (Irving et al., 1992; Felle, 2001; Jeremiah et al., 2001). Effectors that cause the cytosolic alkalinization (ABA, MJ) result in stomatal closure (Blatt and Armstrong, 1993; Suhita et al., 2004), while those lowering the cytosolic pH (auxin, fusicoccin) open stomata (Irving et al., 1992). Even during stomatal closure by H_2O_2, cellular alkalinization was an early event (Zhang et al., 2001a). However, Zhang et al. (2001a) did not examine levels of either ROS or NO in guard cells. In our experiments, when guard cells were treated with ABA, there was a marked increase in not only NO-levels but also cytosolic pH (Fig. 4.4B, D), indicating the involvement of NO and cytosolic alkalinization during ABA mediated stomatal closure. The kinetics of increase in NO or pH, monitored by DAF-2DA and BCECF-AM respectively, revealed that ABA induced increase in cytosolic pH had a shorter lag and reached peak faster than that of NO-levels (Fig. 4.5A, B). These results suggest that the action of cytosolic pH could be prior to the NO elevation during stomatal closure by ABA.

Modulation of cytosolic pH and consequence on NO production or stomatal closure

Cytosolic pH can be modulated by weak alkalinizing agents, such as methylamine or NH_4Cl, and weak acids, such as butyric acid or acetic acid (Danthuluri et al., 1990; Van der Veen et al., 1992; David et al., 1998). Our observations on modulation of ABA induced stomatal closure, as well as the NO levels in guard cells by butyrate or methylamine (Fig. 4.6K, L & 7K, L), indicate that change in cytosolic pH is either associated or necessary for NO production during stomatal closure by ABA. Since the NO-molecule is quite active at an alkaline pH of 7.4 (Reiter et al., 2000), NO can be expected to become effective as the pH rises. cPTIO, prevented the ABA induced stomatal closure, but did not prevent the extent of alkalinization (Table 4.1). We therefore suggest that the change in cytosolic pH is upstream of NO production. The production of NO may also have some feedback effect on cytosolic pH as SNP, a NO donor, partially increased the cytosolic pH. This point needs further study.

The cytosolic pH and ROS in guard cells are already known to be important signaling components during the effects of MJ or bicarbonate (Suhita et al., 2004; Kolla et al., 2007). The present results highlight the involvement and interaction of NO, cytosolic pH and cytosolic calcium during the transduction of also ABA signal.

Calcium may act upstream of NO production or cytosolic alkalinization

The increase in cytosolic Ca^{2+} of guard cells is a common signaling component during stomatal closure in response to diverse signals (McAinsh et al., 1997). 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). It is therefore proposed that the signaling components during these events converge at the level of calcium.

The marked prevention of ABA induced stomatal closure and decrease in the levels of NO or rise in cytosolic pH by EGTA (Table 4.1), suggested that

cytosolic Ca^{2+} is necessary to sustain NO levels and rise in cytosolic pH during stomatal closure by ABA. However, a major limitation with these experiments is that EGTA depletes the cellular calcium, thus affecting multiple components and consequently all ABA responses. Garcia-Mata and Lamattina (2007) also have indicated that Ca^{2+}-dependent NO production and stomatal closure by ABA is mediated by Ca^{2+}. We propose that calcium may act upstream of cytosolic pH and NO-production, besides its known action downstream of NO production during stomatal closure by ABA (Neill et al., 2008).

Conclusions

1. ABA induced stomatal closure was associated with an increase in not only NO but also cytosolic pH of guard cells.
2. Real time monitoring with the help of fluorescent dyes indicated that alkalinization of the guard cell preceded NO production.
3. Modulation of cytosolic pH changed the patterns of NO production and stomatal closure.
4. Internal Ca^{2+} appears to be necessary to sustain the rise in cytosolic pH and NO.
5. A schematic representation of possible events occurring during ABA induced stomatal closure is shown in Fig. 4.8.

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Chapter 5

Importance and interactions of ROS with NO during stomatal closure by ABA in epidermal strips of *Pisum sativum*

Chapter 5

Importance and interactions of ROS with NO during stomatal closure by ABA in epidermal strips of *Pisum sativum*

Application of exogenous abscisic acid (ABA) resulted in a rapid generation of ROS in the guard cells of *Vicia faba* (Miao et al., 2000) and Arabidopsis (Pei et al., 2000). In case of pathogen infection too, the challenged plants frequently elevate ROS such as superoxide and hydrogen peroxide (H_2O_2), which in turn can trigger the hypersensitive responses (Torres et al., 2002). Thus, plants appear to purposefully generate ROS as signaling molecule to control various processes including pathogen defense, programmed cell death and stomatal behavior (Delledonne et al., 2001; Kwak et al., 2003; Gechev et al., 2006).

NO is ubiquitous and plays a key role in a broad spectrum of pathophysiological and developmental processes (Lamattina et al., 2003; Mur et al., 2006; Hong et al., 2008; Neill et al., 2008). In plants, NO interacts with other signaling elements such as lipids, cGMP, ion channels, ROS and Ca^{2+} (Desikan et al., 2004; Shapiro, 2005; Courtois et al., 2008). Exogenous addition of NO to both monocot and dicotyledonous epidermal strips induced stomatal closure (García-Mata and Lamattin, 2001). Several recent reports emphasized the key function of NO in the fine-tuned regulation of stomatal closure (García-Mata and Lamattina, 2002; Bright et al., 2006; Neill et al., 2008).

Several enzymes were suggested to be the sources of NO/ROS, such as cell wall peroxidases, amine oxidases, NADPH oxidase and other flavin-containing enzymes (Pei et al., 2000; Neill et al., 2002b; 2002c) for ROS and nitric oxide synthase and nitrate reductase are for NO (Desikan et al., 2002; García-Mata and Lamattina, 2007). In view of the unclear information, further studies are necessary to identify the source and importance of ROS and its interaction with other signaling components particularly NO during stomatal closure by ABA.

The present work is designed to assess the pattern and mechanism of ROS production, its interaction with NO, and further, the experiments were extended to

know the sources of NO and ROS during the stomatal closure by ABA in epidermal strips of *Pisum sativum*.

Results

Kinetics of ROS production by ABA and stomatal closure by H_2O_2/SNP

The levels of ROS in guard cells were monitored by cell permeable fluorophore, H_2DCFDA. ABA induced a marked rise in production of ROS in stomatal guard cells and increase in ROS-levels of guard cells was evident at 5 min (Fig. 5.1) after exposure to ABA, and did not rise much thereafter. Hydrogen peroxide, one of the components of ROS, or sodium nitroprusside (SNP, a nitric oxide donor), induced stomatal closure at a concentration of 0.1 mM (Fig. 5.2A, B).

Effect of NO/ROS modulators on stomatal closure

NO modulators, cPTIO, 2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide, sodium tungstate (inhibitor of nitrate reductase; NR) (Fig. 5.3A), completely and L-NAME (N-nitro-L-Arg-methyl ester; NOS inhibitor) (Fig. 5.3B) partially prevented the extent of stomatal closure by ABA. ROS modulators, catalase (H_2O_2 scavenger) (Fig. 5.4A) or diphenyleneiodonium chloride (DPI, a NAD(P)H oxidase inhibitor), NADPH oxidase inhibitor (Fig. 5.4B) prevented the ABA induced stomatal closure in epidermal strips of *Pisum sativum*.

Modulation of DAF-2DA/H_2DCFDA fluorescence by NO/ROS modulators during ABA-induced stomatal closure

A quantitative evaluation of fluorescence images demonstrated clearly the difference in the patterns of NO/ROS changes in guard cells on exposure to ABA. NO modulators, cPTIO or sodium tungstate completely and L-NAME partially prevented the DAF-2DA fluorescence but could not prevent the extent of H_2DCFDA fluorescence by ABA (Figs. 5.5A, B; Table 5.1). ROS modulators catalase or DPI prevented the DAF-2DA as well as H_2DCFDA fluorescence,

Similarly, These inhibitors alone did not have any direct effect on stomatal closure.

Discussion

The involvement of NO and ROS during stomatal closure was further demonstrated by additional evidences: modulation of NO or ROS levels within cells by either scavenging these molecules or inhibition of production source enzymes and finally real time monitoring of NO/ROS using fluorescent dyes.

Rise and essentiality of ROS during ABA induced stomatal closure

Reactive oxygen species are essential signaling components during stomatal closure induced by not only ABA but also MJ and bicarbonate (Kwak et al., 2003; Suhita et al., 2004; Kolla et al., 2007). The importance of ROS during ABA induced stomatal closure was demonstrated by multiple observations: significant rise in ROS levels in guard cells (Fig. 5.1), prevention of stomatal closure along with a decrease in ROS levels by catalase and DPI during stomatal closure by ABA (Figs. 5.4A, B; Table 5.1). Our results endorse the opinion that common signaling components such as NO, ROS participate during transduction of diverse signals emulating from biotic or abiotic stress, including UV-B or ozone stress (Holley et al., 2003; Fujita et al., 2006).

Kinetics of ROS

The release of ROS in cells can be monitored by real time imaging with epifluorescence microscopy, with the help of H_2DCFDA (Murata et al., 2001). Kinetic studies using H_2DCFDA revealed that ABA induced increase in ROS reached maximum by 5 min (Fig. 5.1). This demonstrated that ROS production

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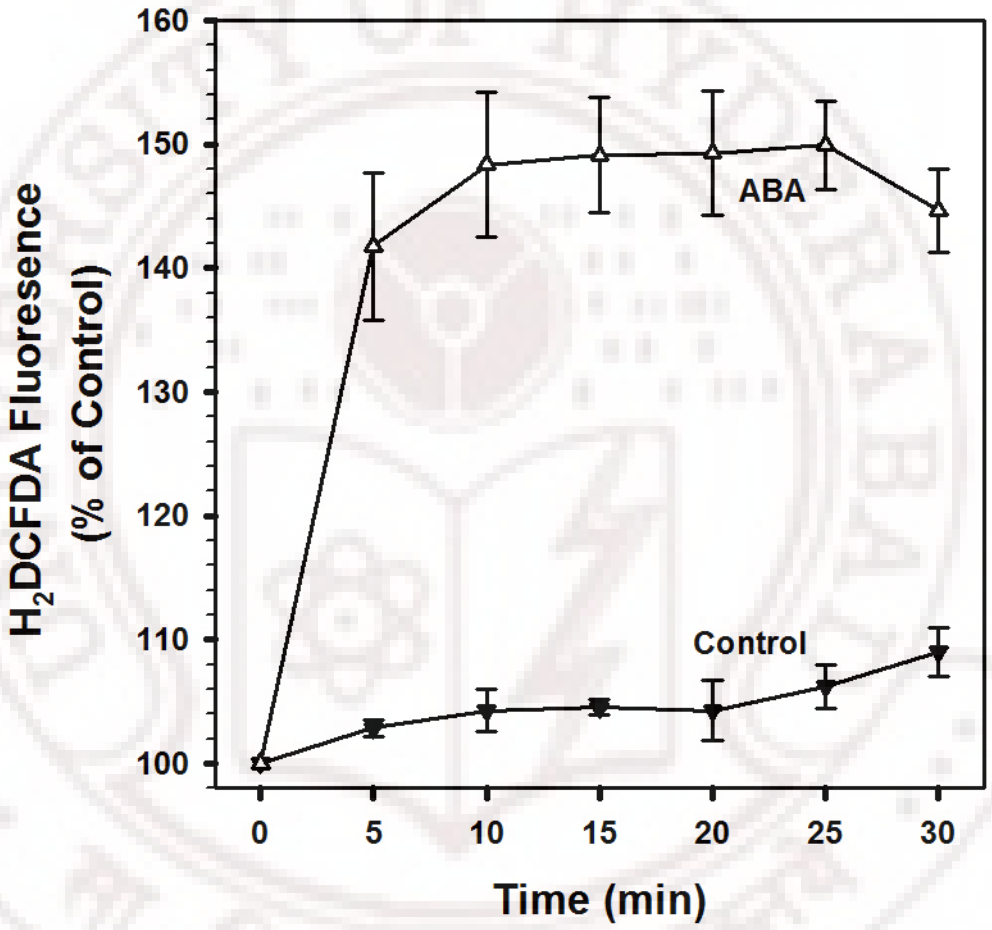

Figure 5.1. Kinetics of increase in ROS of guard cells in response to 10 µM ABA. The epidermal strips were loaded with 20 µM H_2DCFDA for ROS and incubated with or without ABA. The levels of ROS reached maximum by 5 min. The extent of ROS production in the guard cells without ABA is taken as 100%. Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

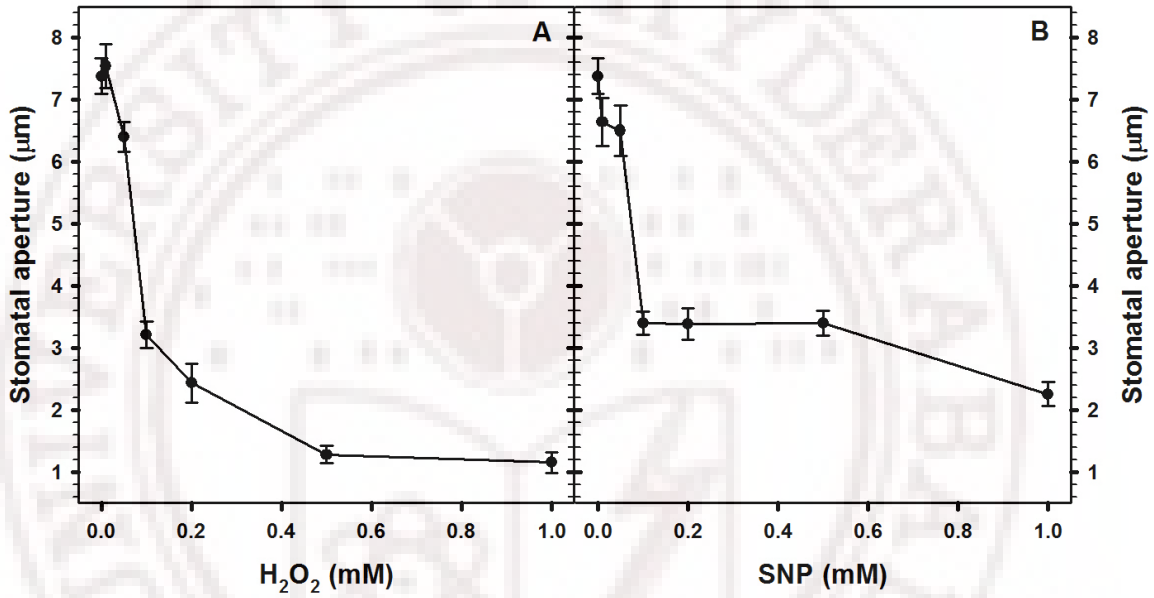

Figure 5.2. Concentration dependent stomatal closure in epidermal strips of *Pisum sativum* by H_2O_2 (A) or SNP (B). Both H_2O_2 and SNP at a concentration of 0.1 mM induced 50% of the stomatal closure. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

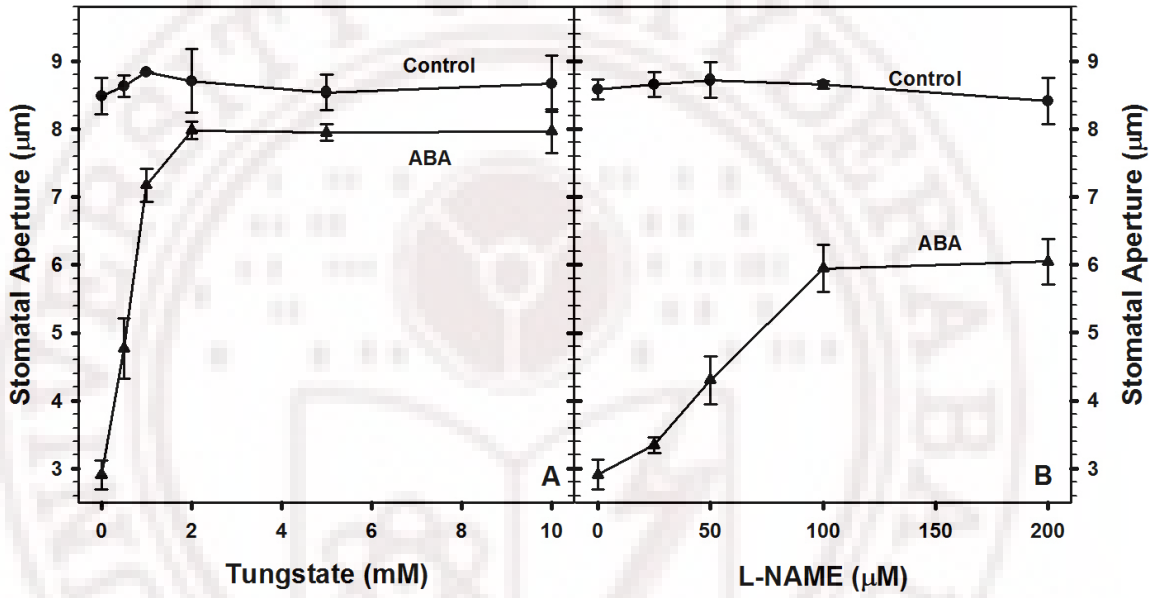

Figure 5.3. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by tungstate, a nitrate reductase inhibitor (A), or L-NAME, a NOS inhibitor (B). Presence of 1 mM or above, tungstate prevented the ABA induced stomatal closure where as L-NAME at a concentration of 0.1 mM prevented the stomatal closure partially. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

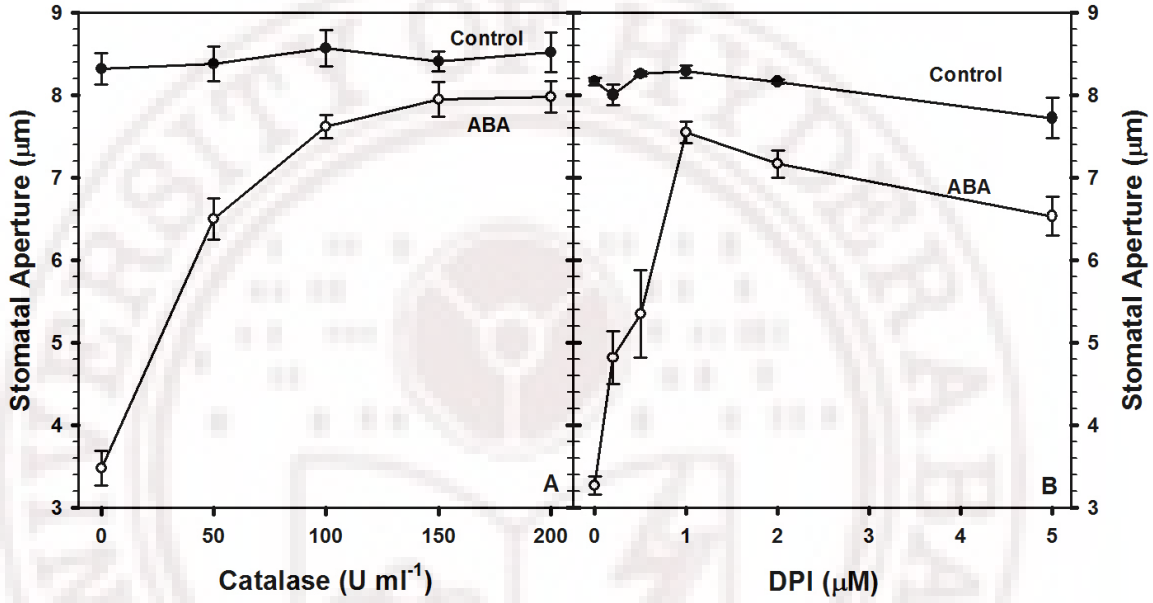

Figure 5.4. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by ROS modulators, Catalase, and H_2O_2 scavenger (A) or DPI, a NADPH oxidase inhibitor (B). Presence of 100 U ml^{-1}, catalase or 1 μM DPI prevented the ABA induced stomatal closure. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

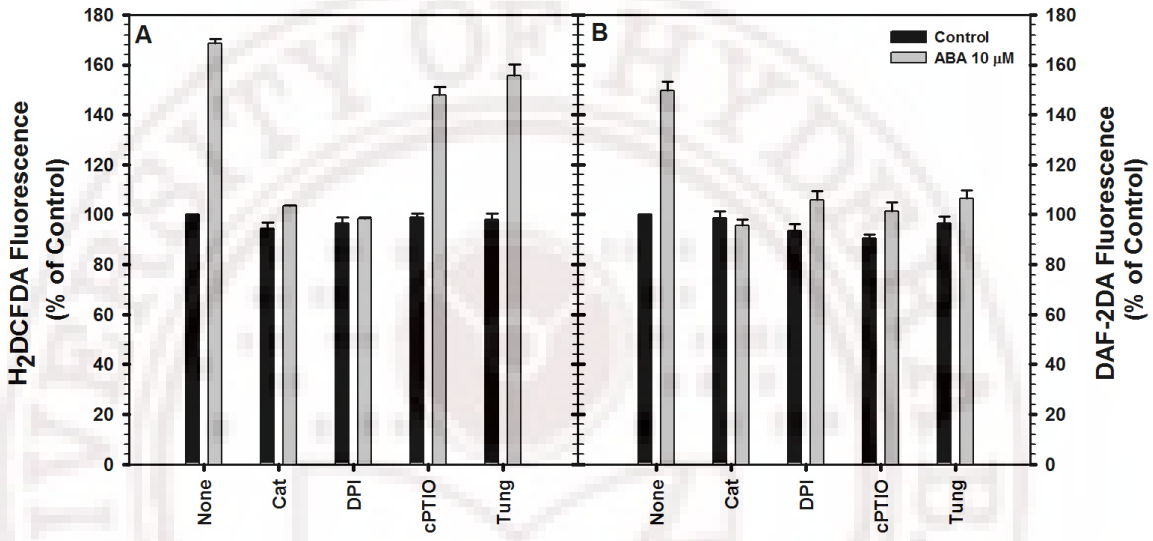

Figure 5.5. The effect of ROS/NO modulators on increase in the levels of ROS (A) or NO production (B) in guard cells of *Pisum sativum* on exposure to ABA, as indicated by the fluorescent probes. Experiments were performed by loading the guard cells with 20 µM H_2DCFDA reflecting the levels of ROS or 20 µM DAF-2DA for NO. The extent of ROS/NO production in the guard cells without ABA is taken as 100%. Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

Table 5.1 The effect of ROS (Catalase, DPI) or NO modulators (cPTIO, tungstate) on ABA induced NO/ROS production in guard cells of *Pisum sativum*. The extent of fluorescence without ABA and without any effector is taken as 100%. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods. ∗Significant at P value < 0.05, compared to the respective treatment without ABA.

	No ABA		**+ 10 μM ABA**	
Treatment	H_2DCFDA Fluorescence (% Control)	DAF-2DA Fluorescence (% Control)	H_2DCFDA Fluorescence (% Control)	DAF-2DA Fluorescence (% Control)
None (Control)	100 ± 0	100 ± 0	168* ± 2	149* ± 4
0.1 mM L-NAME	102 ± 4	103 ± 2	156* ± 3	120* ± 4
2 mM Tungstate	98 ± 2	97 ± 3	155* ± 4	106 ± 4
0.2 mM cPTIO	99 ± 2	90 ± 2	148* ± 3	101 ± 3
200 U ml^{-1} Catalase	94 ± 2	98 ± 3	103 ± 1	96 ± 3
1 μM DPI	96 ± 2	94 ± 3	98 ± 1	106 ± 4

occurred much before the rise in NO and cytosolic pH (Gonugunta et al., 2008) during ABA induced stomatal closure in guard cells of *Pisum sativum*.

Crosstalk of NO and ROS

The importance of ROS for the rise in NO levels of guard cells during stomatal closure by ABA, was confirmed by multiple observations i.e. the ability of catalase or DPI to restrict the ROS as well as NO production in guard cells (Figs. 5.5A, B) and the inability of NO modulators to restrict the ROS levels, but NO (Figs. 5.5A, B; Table 5.1). H_2O_2 production was required for ABA-induced NO generation in guard cells of both *V. faba* and Arabidopsis (Dong et al., 2005; Bright et al., 2006). Similar interactions of ROS and NO were observed during UV-B effects on stomata of broad bean (He et al., 2005). It would be interesting to study further the mechanism of ROS induced production of NO, during ABA effects.

Sources of NO and ROS during stomatal closure by ABA

The source of NO in plants is under continuous debate. The activity and biological function of AtNOS1 in *Arabidopsis* was questioned (Zemojtel et al., 2006). So far, there is no strong evidence to indicate the occurrence of an animal like NOS in plants. While the role of NR in mediating the rise in NO levels is possible, there could be other sources of NO (García-Mata and Lamattina, 2003; del Río et al., 2004). A clear picture may emerge only after further studies in future.

García-Mata and Lamattina (2007) suggested that nitric oxide synthase (NOS) may mediate the production of NO during inhibition of stomatal opening. On the other hand, Desikan et al. (2002) suggested that nitrate reductase (NR) was involved in NO production induced by ABA, based on their studies on the double mutant of *Arabidopsis nia1, nia2*, deficient in NR. The prevention of ABA-induced stomatal closure as well as the rise in NO of guard cells by sodium

tungstate and L-NAME (Fig 5.4A, B; Table 1) indicated that both NR and NOS-like activity could participate during ABA induced NO production.

Although several investigators used DPI as an inhibitor of NAD(P)H oxidase (Murata et al., 2001; Kwak et al., 2006; Beffagna and Lutzu, 2007; Zhang et al., 2007), being a flavoprotein inhibitor, DPI also may affect NOS (Moulton et al., 2000). However, the prevention by DPI of not only stomatal closure (Fig 5.3B) but also the ROS (Table 5.1) production is a strong evidence in favor of the importance of NAD(P)H oxidase. Further experiments are required to confirm the importance of NAD(P)H oxidase and to assess alternative sources for raising the ROS levels in guard cells.

Conclusions

1. ROS is an important secondary messenger, besides NO during ABA induced stomatal closure.
2. NADPH oxidase and nitrate reductase are the possible sources of ROS and NO production respectively as DPI or sodium tungstate prevented the stomatal closure by ABA.
3. Time course experiments with fluorescent probes demonstrated that ROS-production occurred within 5 min.
4. The ability of catalase or DPI to restrict the production of ROS as well as NO, and the inability of NO-modulators (cPTIO and tungstate) to prevent the rise in ROS levels in guard cells, indicated that ROS production was necessary for NO production.
5. Further studies are warranted to understand the mechanism of modulation by ROS of NO production and to establish if there are any interactions of ROS with NO.

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Chapter 6

Role and importance of PI3K, calcium and CaM during ABA induced stomatal closure in abaxial epidermis of *Pisum sativum*

Chapter 6

Role and importance of PI3K, calcium and CaM during ABA induced stomatal closure in abaxial epidermis of *Pisum sativum*

Phosphatidylinositol kinase synthesizes the phosphatidylinositol 3-phosphate [PtdIns(3)P], is a phosphoinositide, present at very low levels in plant cells (Brearley and Hanke, 1992). PtdIns(3)P and PI3K are essential for normal plant growth and development (Welters et al., 1994) such as root nodule formation (Hong and Verma 1994), auxin-induced ROS production and root gravitropism (Joo et al., 2005), Rhizobium infection and root hair curling in *Medicago truncatula* (Peleg-Grossman et al., 2007), increased plasma membrane endocytosis and the intracellular production of ROS in the salt tolerance response (Leshem et al., 2007) and also stomatal movement (Jung et al., 2002; Park et al., 2003). Stomatal guard cells contain PI3-phosphate (PI3P) and PI4-phosphate (PI4P), the products of PI3-kinase (PI3K) and PI4-kinase (PI4K) activities.

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 (Schroeder et al., 2001a).

Calmodulin (CaM) is known to regulate several different enzymes in plant cells. We do not know yet any precise knowledge of the process involved in bringing about or controlling the ionic fluxes mediated by CaM in guard cells. CaM has been found in many plant species. For example, CaM was detected in the medium of suspension-cultured cells from *Angelica dahurica*, carrot and tobacco. Indeed, CaM modulates varied functions, and also accelerates pollen germination and tube growth (Ma and Sun, 1997; Ma et al., 1999; Shang et al., 2001). The experiments reported here strongly suggest that CaM antagonists affect the responses of guard cells to ABA and it can be suggested that calmodulin-

dependent enzymes take part in the efflux of ions that occurs from guard cells treated with ABA (MacRobbie, 2000).

However, the interactions of PI3K or CaM and Ca^{2+} with other secondary messengers that are involved during ABA induced stomatal closure have not yet been studied in detail. The present chapter attempts to examine some of the aspects.

Results

Effect of PI3K inhibitors or CaM antagonists on stomatal closure induced by ABA

PI3K inhibitors, Wortmannin (WM, 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(metho xymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,- h]-2-h]-2-benzopyran-3,6,9-trione) or LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (Fig. 6.1A, B) prevented the ABA induced stomatal closure at 5 μM or 20 μM, respectively. On the other hand, the calmodulin antagonists, calmidazolium chloride (CDZ, 1-[Bis(4-chlorophenyl) methyl]-3-[2- (2,4-dichlorophenyl)-2-(2,4-dichlorobenzyloxy)ethyl]-1H-imidazolium chloride) or W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) also prevented the ABA induced stomatal closure at a concentrations of 2 μM or 5 μM respectively (Fig. 6.2A, B).

Effect of Ca^{2+} modulators on ABA induced stomatal closure

1, 2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA, a impermeable Ca^{2+} specific chelator) prevented ABA induced stomatal closure partially, or 1, 2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM, intra cellular Ca^{2+} specific chelator) completely prevented stomatal closure by ABA at a concentrations of 0.2 or 0.1 mM respectively (Fig. 6.3A, B).

Effect of modulators on ABA induced increase in DAF-2DA/H_2DCFDA fluorescence

WM or W-7 or BAPTA-AM prevented both the DAF-2DA/H_2DCFDA fluorescence induced by ABA during stomatal closure, whereas BAPTA did not prevent the fluorescence enhancement during stomatal closure by ABA (Fig. 6.4).

Effect of WM or W-7 on the stomatal closure by H_2O_2 or SNP

WM did not prevent the stomatal closure by H_2O_2 or SNP, on the other hand W-7 prevented the stomatal closure by H_2O_2 or SNP (Fig. 6.5).

Discussion

Role of calcium during stomatal closure by ABA

Calcium is an important modulator of stomatal movements in guard cells (Mansfield et al., 1990; Assmann, 1993). The marked prevention of ABA induced stomatal closure by BAPTA-AM or BAPTA (Fig 6.3A, B) suggested that the action of ABA required intra- and extra-cellular Ca^{2+}. Such efficacy of BAPTA-AM to prevent the stomatal closure NO/ROS, and BAPTA to prevent the stomatal closure, despite the high levels of NO/ROS in guard cells (Fig. 6.4A, B), demonstrate that intra- and extracellular calcium is required for stomatal closure. It is possible that extracellular Ca^{2+} participates at downstream of ROS and NO production or acts independent of ROS and NO. Action of Ca^{2+} at downstream of ROS or NO was earlier reported during stomatal closure by ABA or MJ or high CO_2 (Suhita et al., 2004; Kolla et al., 2007) and chitosan induced burst of Ca^{2+} transients in soybean cells (Mithöfer et al., 1999).

(Discussion continued from page no. 66)

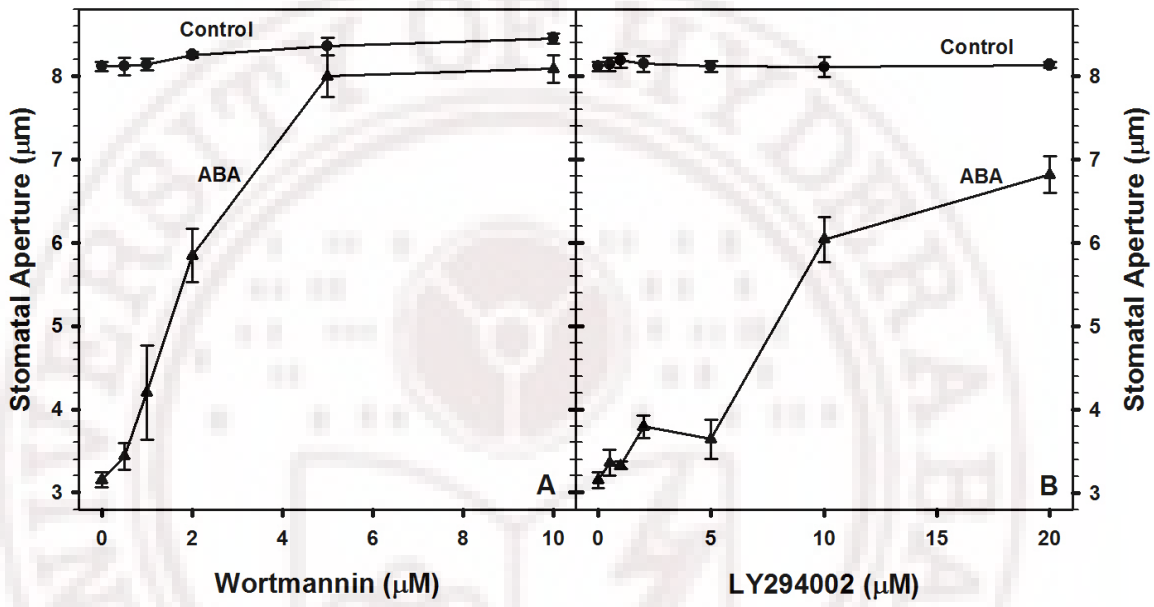

Figure 6.1. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by PI3K inhibitors WM (A) or LY294002 (B). Presence of 5 μM WM or 20 μM LY294002 prevented the ABA induced stomatal closure. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

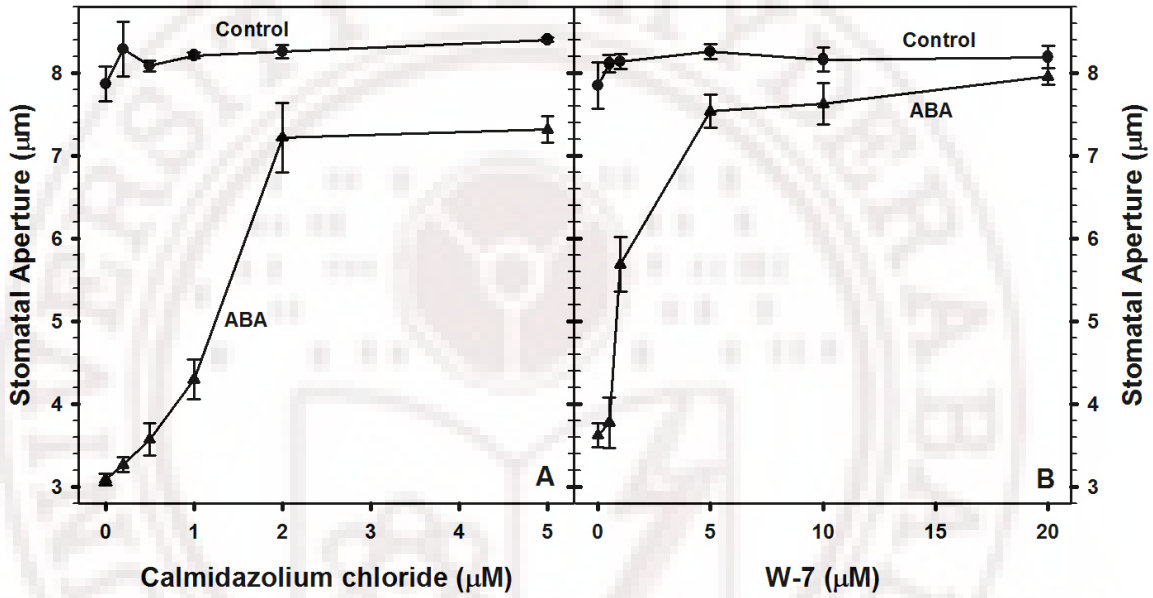

Figure 6.2. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by calmodulin antagonists, Calmidazolium chloride (A) or W-7 (B). Presence of 2 μM CDZ or 5 μM W-7 prevented the ABA induced stomatal closure. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

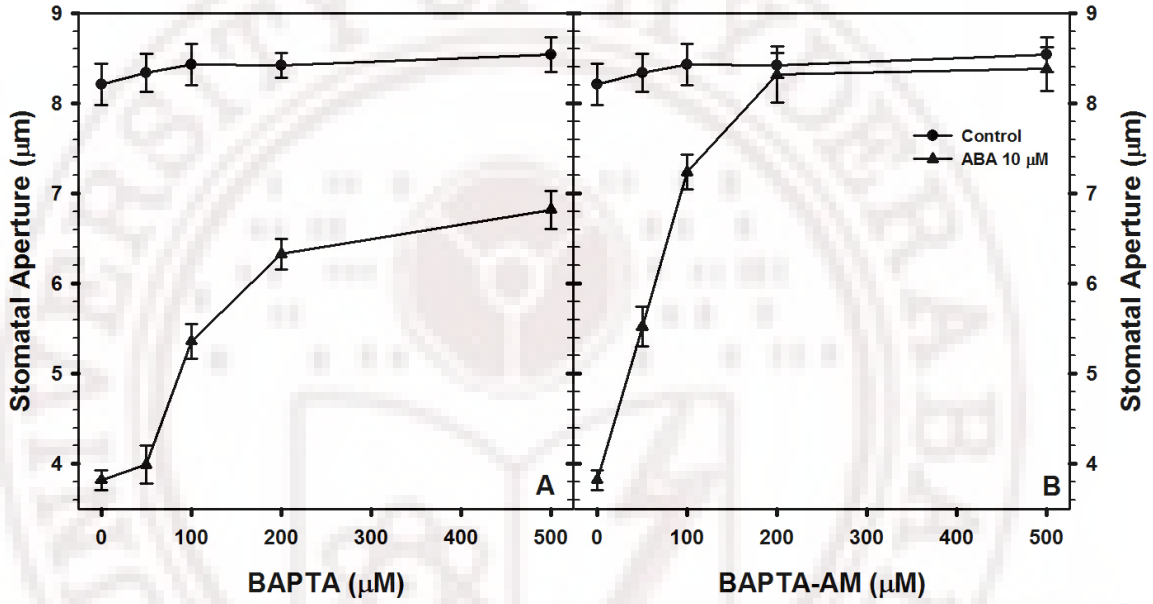

Figure 6.3. Prevention of ABA induced stomatal closure in epidermal strips of *Pisum sativum* by BAPTA, a extracellular calcium chelator (A) or BAPTA-AM a intracellular calcium chelator (B). Presence of 0.2 mM BAPTA partially prevented the ABA induced stomatal closure, while 0.1 mM BAPTA-AM completely prevented. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

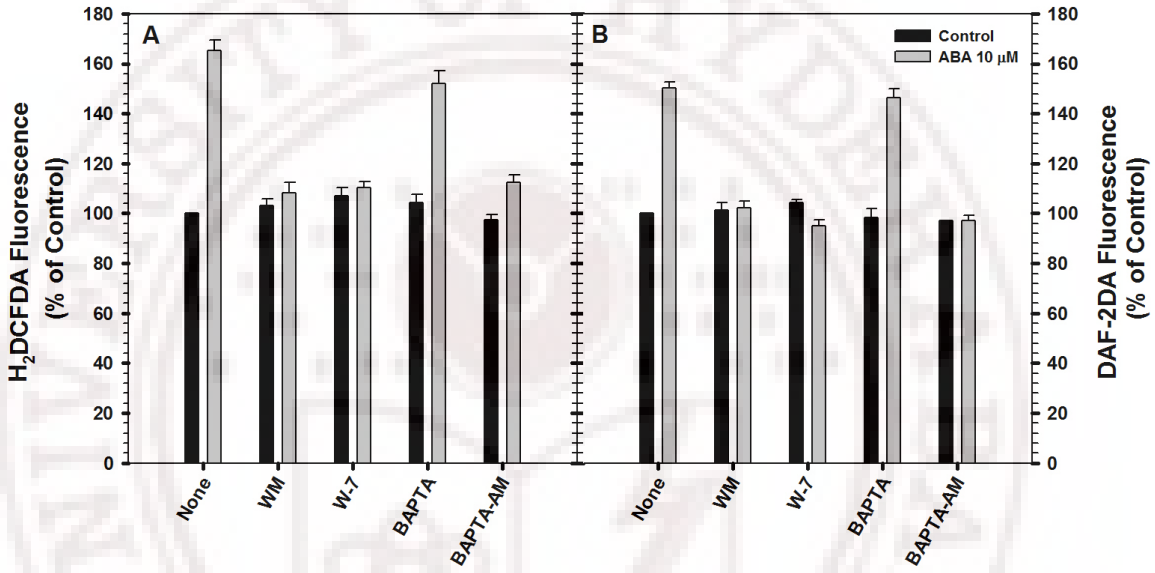

Figure 6.4. The effect of WM, W-7, BAPTA or BAPTA-AM on increase in the levels of ROS (A) or NO production (B) in guard cells of *Pisum sativum* on exposure to ABA, as indicated by the fluorescent probes. Experiments were performed by loading the guard cells with 20 µM H_2DCFDA reflecting the levels of ROS or 20 µM DAF-2DA for NO. The extent of ROS/NO production in the guard cells without ABA is taken as 100%. Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

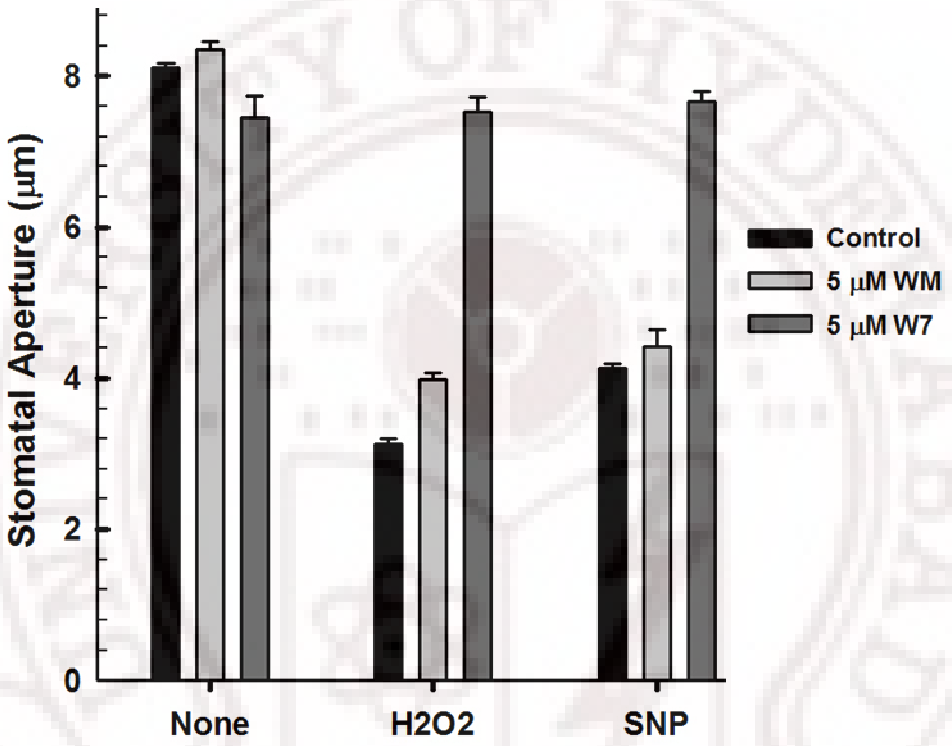

Figure 6.5. The effect of 5 μM WM or 5 μM W-7 on H_2O_2 or SNP induced stomatal closure in guard cells of *Pisum sativum.* WM did not restrict the stomatal closure by H_2O_2 or SNP, whereas W-7 prevented the stomatal closure by H_2O_2 or SNP. Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

An increase in H_2O_2 in guard cells can lead to an increase in the cytosolic free Ca^{2+} concentration (Rentel and Knight, 2004) through the modulation of Ca^{2+} channels (Webb et al., 1996; Pei et al., 2000). Externally applied H_2O_2 also induced stomatal closure in *C. communis* by increasing the cytosolic free Ca^{2+} in guard cells. Elevation of NO also led to a rise in the cytosolic Ca^{2+} (McAinsh et al., 1996; Pei et al., 2000; García-Mata and Lamattina, 2007).

Importance and position of the PI3K in ABA induced stomatal closure

There are reports that PP2C, protein kinase and PI3P could directly or indirectly regulate ROS production in guard cells, particularly during ABA signaling (Murata et al., 2001; Mustilli et al., 2002; Park et al., 2003). PI3K is involved in ABA induced stomatal closure, as WM or LY294002 prevented the stomatal closure (Fig 6.1A, B). PI3K activation occurs at upstream to the NO/ROS production, as ability of WM to prevent the ABA induced stomatal closure, DAF-2DA/H_2DCFDA fluorescence (Fig 6.4A, B), and inability of WM to prevent the stomatal closure by H_2O_2 and SNP (Fig 6.5)

Importance and interaction of Calmodulin with ROS and NO

Involvement of calmodulin (CaM) in ABA induced stomatal closure was demonstrated by the prevention of ABA induced stomatal closure, and ROS/NO production by W-7. On the other hand unlike PI3K, Calmodulin also participates at downstream to the ROS/NO elevations by ABA, as W-7 prevented the H_2O_2/SNP induced stomatal closure. Chen et al. (2004) reported that exogenous application of calmodulin promoted stomatal closure. Our results endorse the results of Chen et al. (2004), to explain the participation of calmodulin in stomatal closure by ABA.

Conclusions

1. PI3K, Ca^{2+} and calmodulin are involved in the cascade leading to stomatal by ABA.
2. PI3K acts at only upstream to the NO/ROS production by ABA, where as calcium and calmodulin are participated at both up- and down streams to the NO/ROS production by ABA.
3. Extra cellular calcium participates at downstream to the NO/ROS where as intracellular calcium elevation participates at upstream to the NO/ROS elevation by ABA.

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Chapter 7

Bifurcation of other ABA responses with stomatal closure at protein phosphatase level

Chapter 7

Bifurcation of other ABA responses with stomatal closure at protein phosphatase level

The modulators by ABA are reflected in several physiological processes, such as seed dormancy and germination, seedling and root development besides the typical stomatal closure (LeNoble et al., 2004; De Smet et al., 2006). Stomatal aperture is regulated through rapid ABA-triggered alteration of ion fluxes in guard cells (Li et al., 2006), most of the ABA-mediated processes emanated involve marked changes in gene expression. Genome-wide expression analyses in Arabidopsis seedlings and guard cells led to the identification of a large number of genes regulated by ABA (Hoth et al., 2002; Seki et al., 2002; Leonhardt et al., 2004; Takahashi et al., 2004; Nemhauser et al., 2006).

ABA-induced transcriptional upregulation has been reported for genes encoding Mg^{2+}-dependent serine/threonine phosphatase type 2C (PP2C) that act as negative regulators of ABA responses in Arabidopsis (Rodriguez, 1998; Saez et al., 2004; Kuhn et al., 2006; Robert et al., 2006; Yoshida et al., 2006a; Nishimura et al., 2007). Among these PP2Cs, ABI1 and its closest structural homologue ABI2, both act as negative regulators with partially overlapping roles in ABA-controlled processes (Merlot et al., 2001). However, the role and downstream elements that are involved in all ABA mediated responses are not completely identified.

The present chapter elucidates the role of PP2Cs and PI3K during stomatal closure by ABA in epidermal strips, in comparison with other three of the ABA mediated responses, i.e., prevention of seed germination, root growth by using wild type and PP2C mutants (*abi1, abi2*) seeds or seedlings, and activation of gene *pRD29B::LUC* expression in protoplasts by transiently overexpressing the ABI1/ABI2/abi1/abi2 as fusion proteins with GFP in ABA biosynthesis deficient mutant (*aba2*) (Moes et al., 2008).

Results

ABA mediated responses in wild type and mutants of Arabidopsis

Presence of ABA, induced stomatal closure at 10 µM (Fig. 7.1), prevented seed germination at 1 µM (Fig. 7.2) and root growth at 3 µM in wild type Arabidopsis. These responses were very low or insignificant in *abi1* and *abi2* mutants (Fig. 7.1, 2, 3). At 3 µM, ABA induced *pRD29B::LUC* expression in protoplasts of *aba2* mutant (Fig. 7.4). When constitutively expressed with 35S promoter, PP2Cs, ABI1 or ABI2 partially and abi1 or abi2 mutant proteins completely prevented ABA mediated *pRD29B::LUC* expression (Fig. 7.4).

Effect of WM on ABA mediated responses

WM at a concentration of 5 µM prevented the ABA induced stomatal closure, while other ABA mediated responses, prevention of seed germination, root growth and ABA mediated *pRD29B::LUC* expression were not affected (Fig. 7.5, 6, 7).

Effect of H_2O_2 on stomata

Abscisic acid or H_2O_2 induced stomatal closure in wild type Arabidopsis, but H_2O_2 only induced stomatal closure in epidermal strips of *abi1* or *abi2*, while ABA did not induced stomatal closure in mutant's *abi1* or *abi2* (Fig. 7.9).

Discussion

Importance of protein phosphatases in ABA responses

Type2 C Protein phosphatases (PP2Cs) are known to participate in number of ABA responses. Impairment of ABA mediated stomatal closure (Fig. 7A), prevention of root growth (Fig. 7B), seed germination (Fig. 7C) in abi1 or *abi2* mutants reaffirms participation of PP2Cs in ABA mediated three mentioned responses. These results also compliment the several findings from

(Discussion continues in page no. 79)

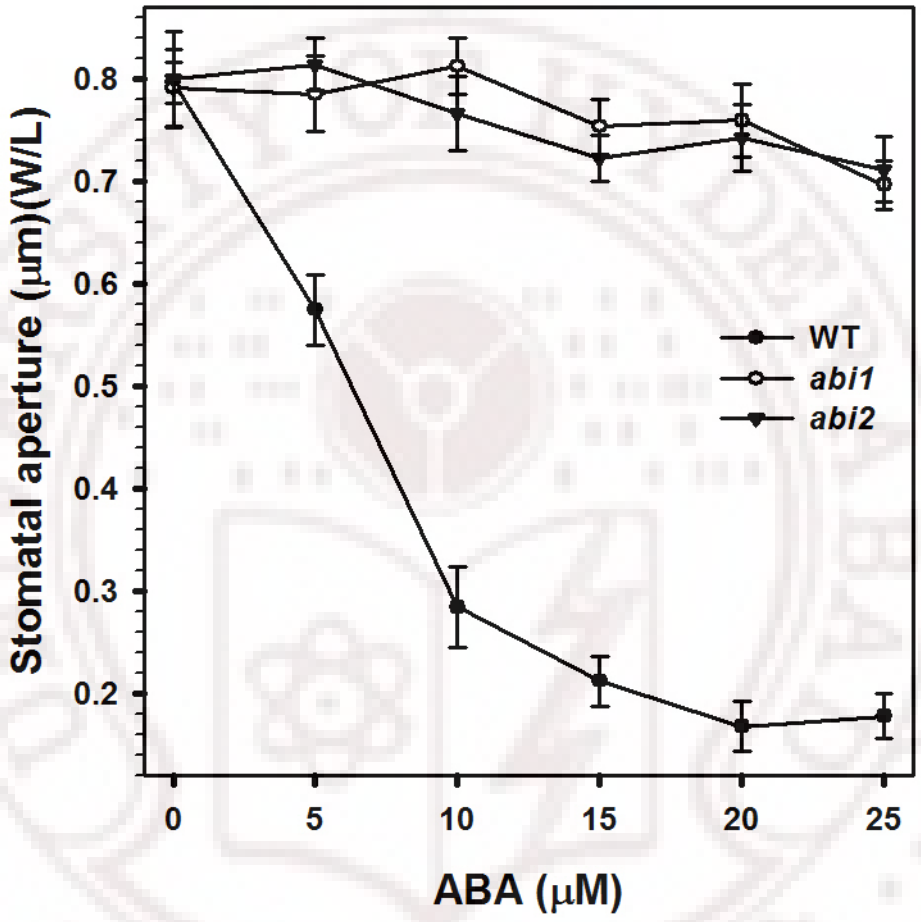

Figure 6.1. Concentration dependent stomatal closure by ABA in wild type or ABA insensitive mutants, *abi1* and *abi2*. ABA induced stomatal closure in wild type at a concentration of 10 μM, while *abi1* or *abi2* are insensitive to ABA mediated induction of stomatal closure in epidermal strips. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

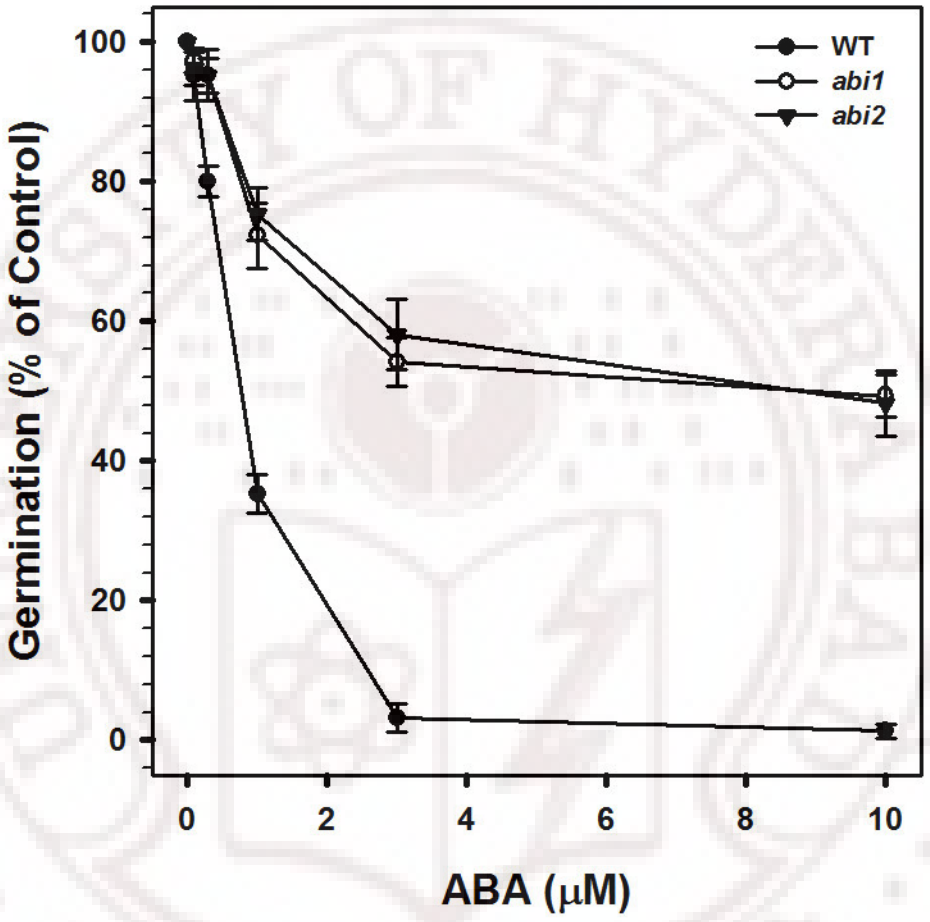

Figure 6.2. Concentration dependent prevention of germination by ABA in wild type or ABA insensitive mutants, *abi1* and *abi2*. ABA prevented germination in wild type at a concentration of 1 μM, while *abi1* or *abi2* are insensitive to ABA mediated prevention of seed germination. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

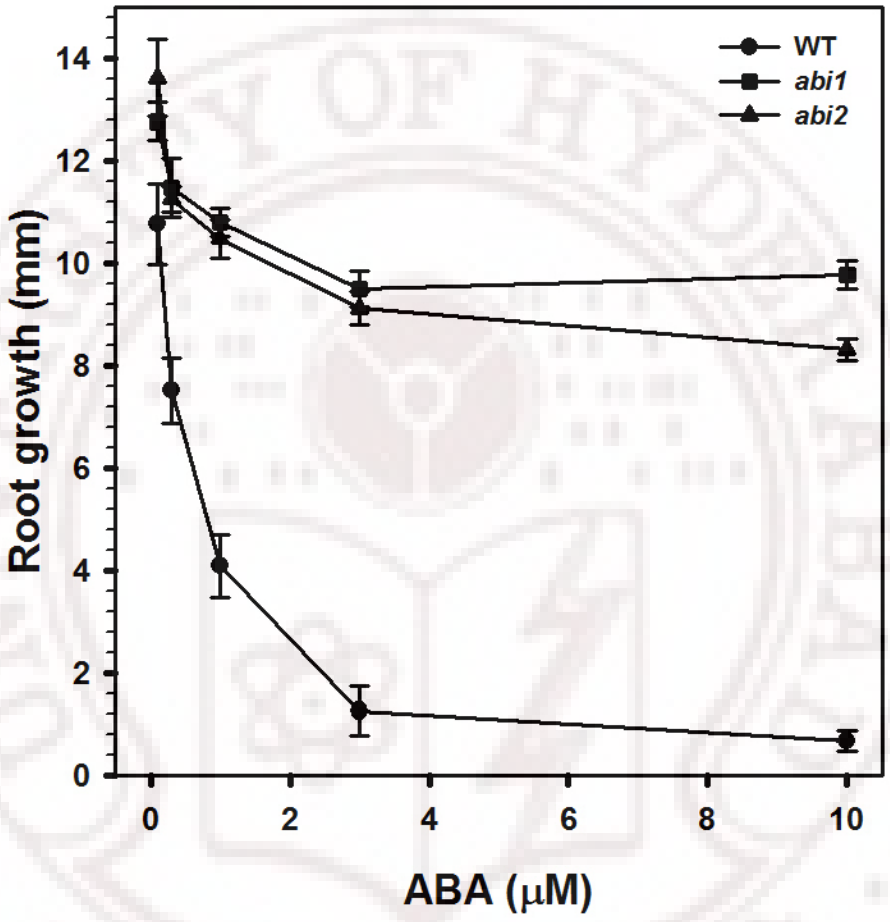

Figure 6.3. Concentration dependent prevention of root growth by ABA in wild type or ABA insensitive mutants, *abi1* and *abi2*. ABA prevented root growth in wild type at a concentration of 3 μM, while *abi1* or *abi2* are insensitive to ABA mediated prevention of root growth. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

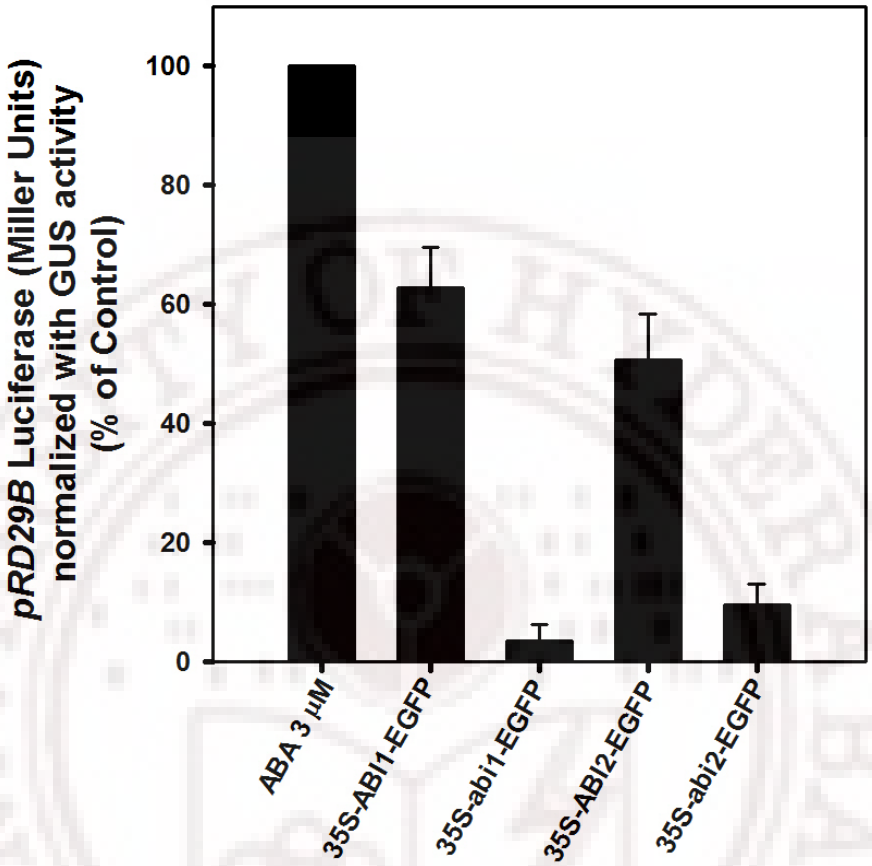

Figure 6.4. Effect of constitutive expressed wild type PP2C's (ABI1 or ABI2) or mutants (abi1 or abi2) on ABA induced *pRD29B::LUC* expression (ABA responsive element promoter fused with luciferase) in protoplasts of ABA biosynthesis mutant *aba2*. Activity of luciferase was normalized with GUS activity which was co expressed within the same protoplasts and expressed as the ratio of relative fluorescence units to light units (RFU/RLU). ABA induced luciferase activity was taken as 100%. ABI1 or ABI2 partially and abi1 or abi2 completely prevented the ABA induced luciferase activity. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods

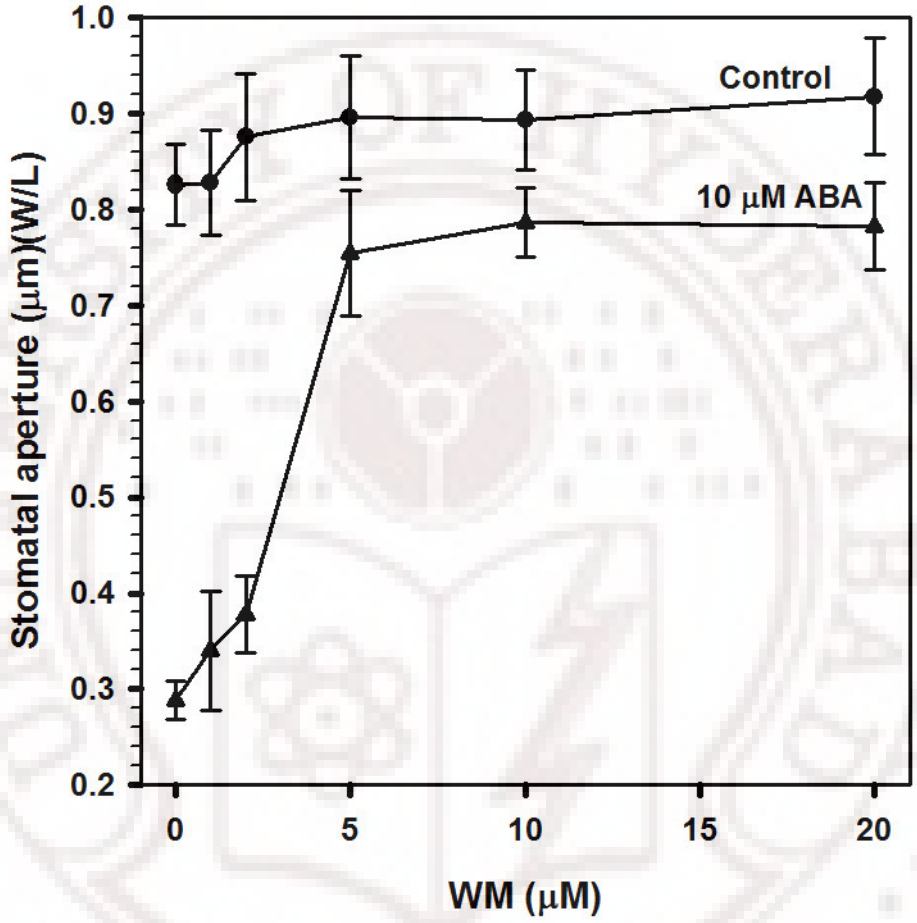

Figure 6.5. The pattern of stomatal responses in presence of WM. At a concentration of 5 μM WM prevented the stomatal closure by 10 μM ABA in epidermal strips of Arabidopsis. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

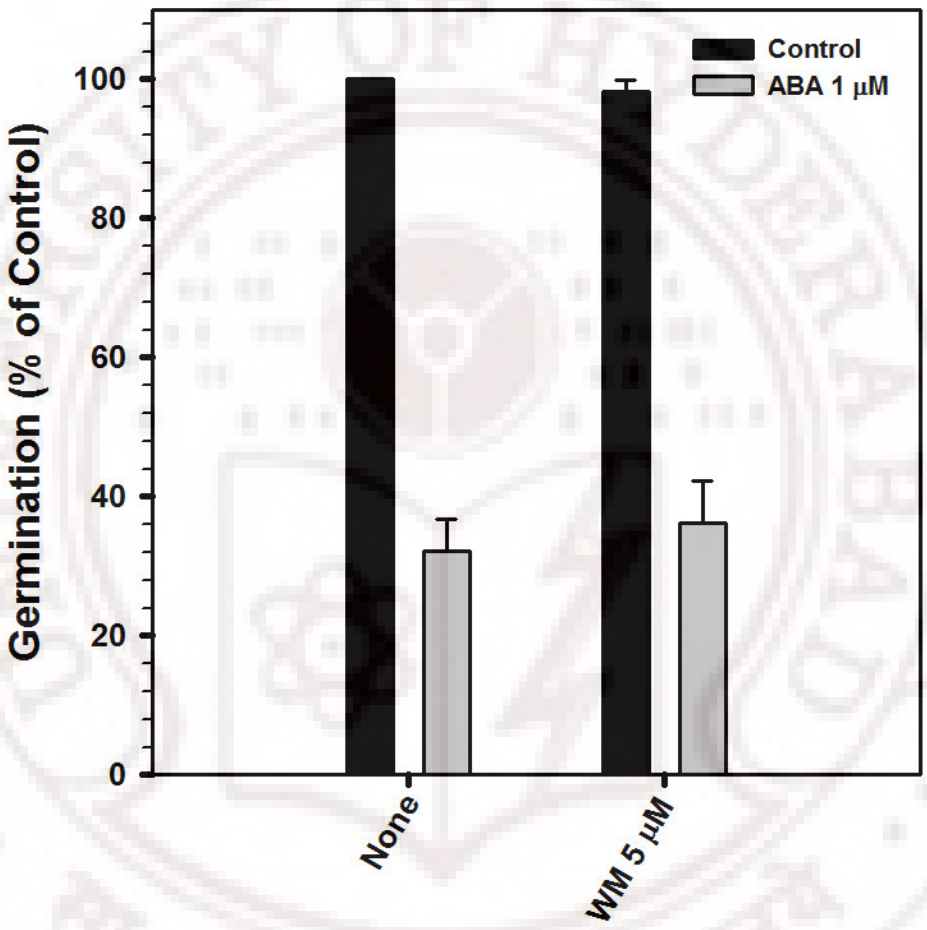

Figure 6.6. Effect of 5 µM WM on 1 µM ABA mediated prevention of germination in wild type Arabidopsis seeds. In absence or presence of 5 µM WM, ABA prevented germination. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

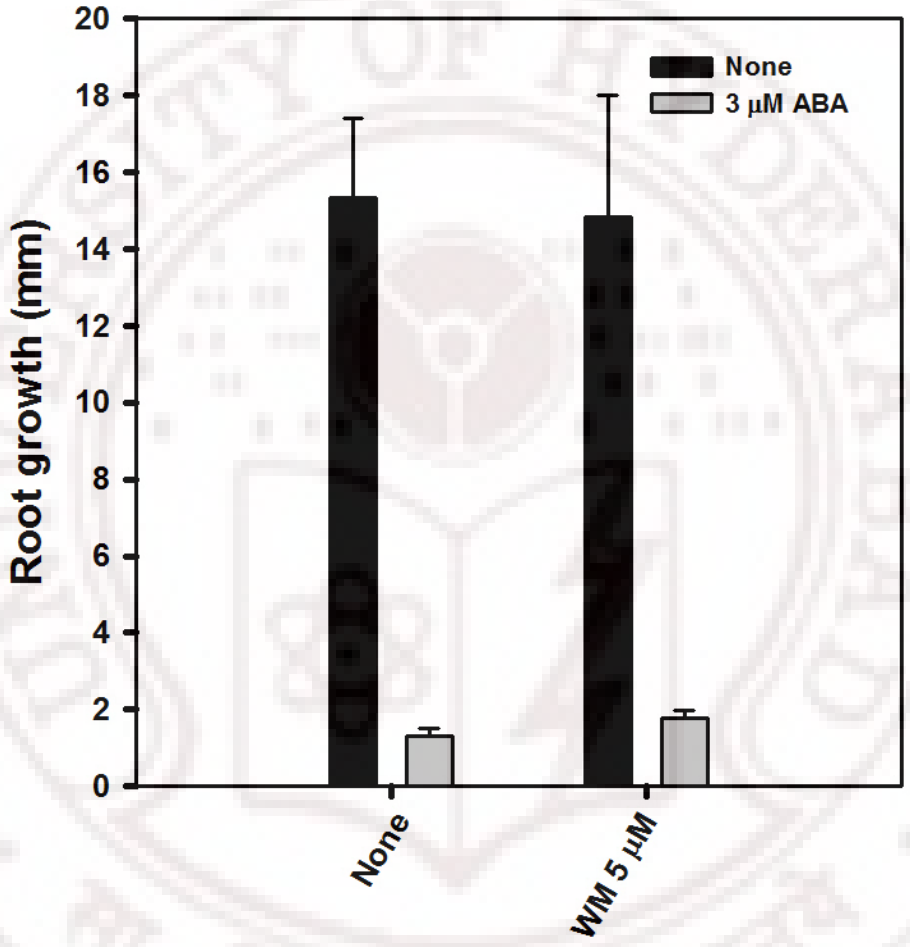

Figure 6.7. Effect of 5 µM WM on 3 µM ABA mediated prevention of root growth. In presence or absence of 5 µM WM, ABA prevented root growth in wild type Arabidopsis. WM alone has no effect on root growth of Arabidopsis. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

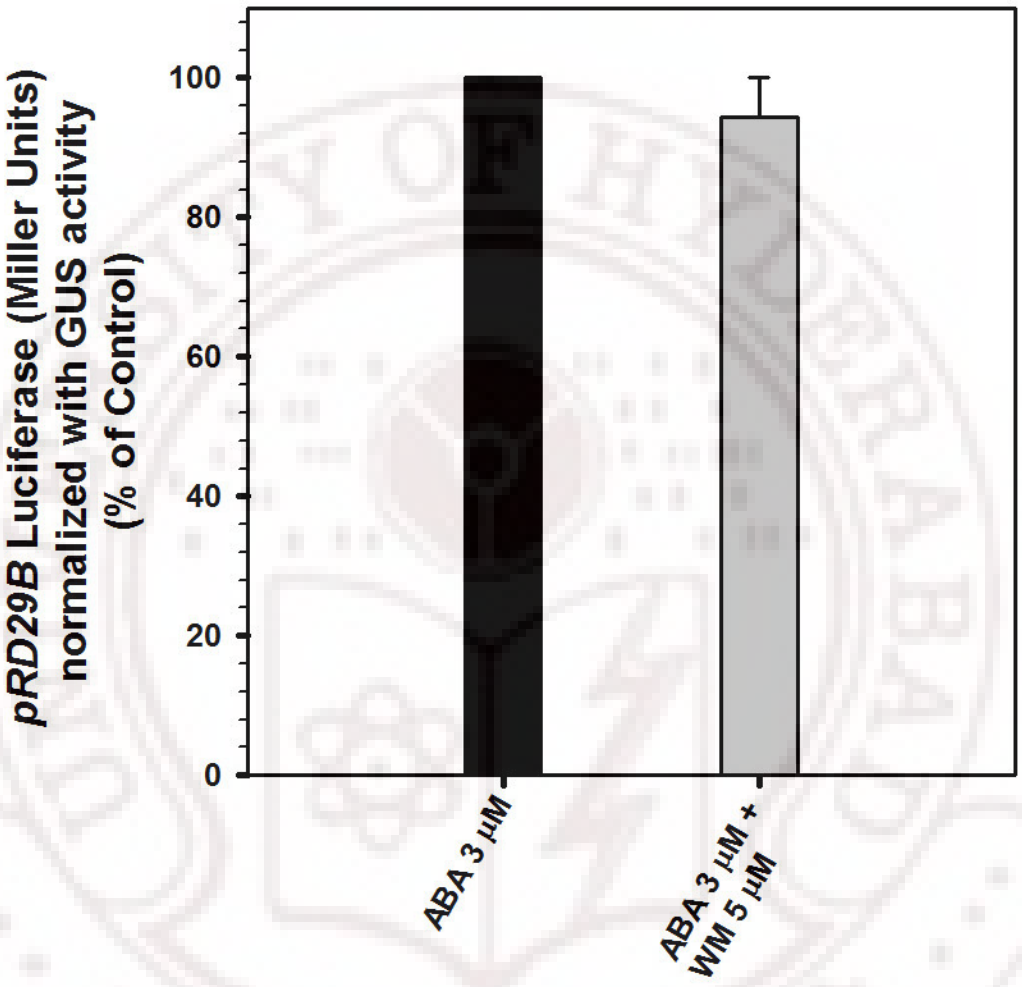

Figure 6.8. Effect of 5 µM WM on ABA induced *pRD29B::LUC* expression in protoplasts of ABA biosynthesis mutant *aba2*. Activity of luciferase was normalized with GUS activity which was co expressed within the same protoplasts and the ABA induced luciferase activity was taken as 100%. ABA induced *pRD29B::LUC* expression in absence or presence of 5 µM WM. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

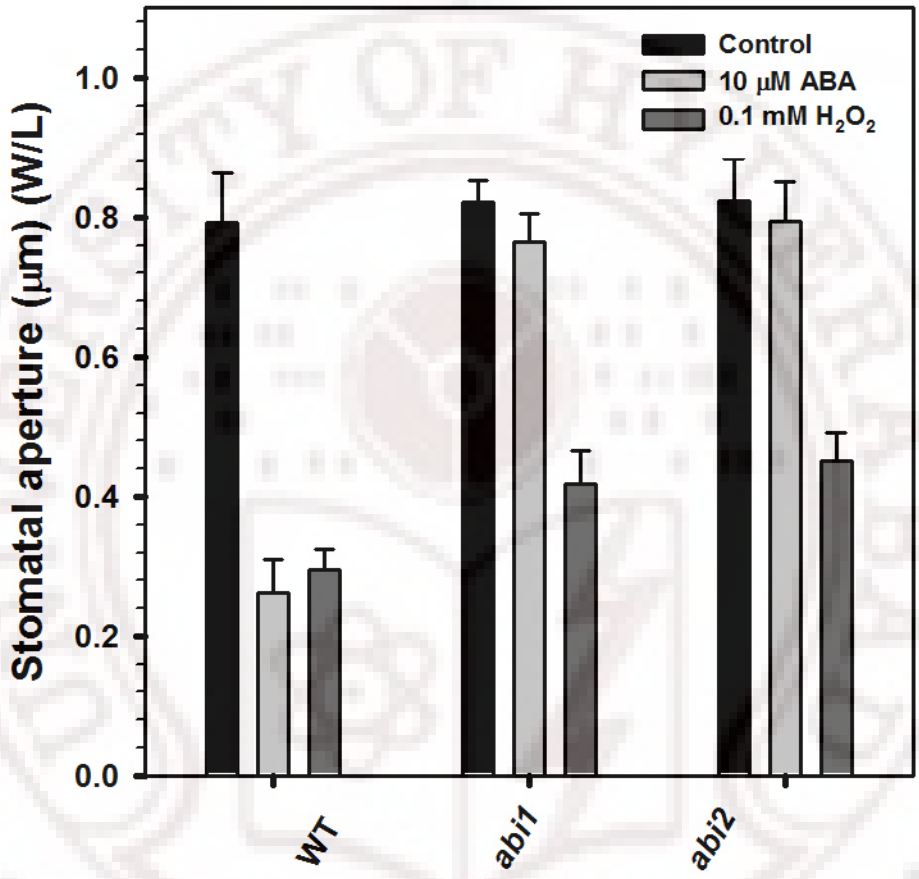

Figure 6.9. Effect of 10 µM ABA or 0.1 H_2O_2 on epidermal strips of wild type and PP2C mutants, *abi1* or *abi2* of Aarabidopsis. ABA or H_2O_2 induced stomatal closure in wildtype but only H_2O_2 induced stomatal closure in *abi1* or *abi2* not ABA. Results are the averages ± SE of 3 to 4 independent experiments. Further details are given in Materials and Methods.

others (Schroeder et al., 2001a; LeNoble et al., 2004; De Smet et al., 2006; Zhang et al., 2007).

Protein phosphatases, ABI1or ABI2 and abi1 or abi2 can block ABA-inducible gene expression when they are ectopically expressed in protoplasts of maize or Arabidopsis (Sheen, 1998; Yang et al., 2006). To monitor the action of these PP2Cs on the ABA induced gene expression, Arabidopsis mutant (*aba2*) protoplasts were transfected with four PP2Cs effector genes ABI1/ABI2/abi1/abi2 along with an ABA-dependent reporter construct (*pRD29B::LUC*). Transiently over expressed, ABI1 or ABI2 partially and abi1 or abi2 completely prevented the ABA responsive pRD29B::LUC expression (Fig. 7.4). Aliquots of the transfected samples were analysed for their ABA response after incubation in the presence and absence of ABA, by normalizing the reporter expression to GUS activity that was generated by co-transfection of a constitutively expressed GUS cassette. In the absence of effectors, ABA exposure of the mesophyll protoplasts resulted in an approximately 20-fold increase of reporter activity. When co-expressed ABI1/ABI2 (40%) partially and abi1/abi2 completely (97%) prevented the ABA induced reporter activity (Fig. 7.4). Suggested that PP2Cs are the important key messengers that mediate all mentioned four ABA responses. Our results complementing the similar results obtained by Wu et al. (2003), their microinjection experiments of ABI1 and abi1 revealed a rescue of ABA-inducible transcription by possibly out-competing the response-inhibitory mutant protein with ABI1 (Wu et al., 2003) and overexpression of ABI1 inhibits ABA-inducible gene transcription in transient transfection analyses via its protein phosphatases activity (Sheen, 1998).

Importance of PI3K in all ABA mediated responses

Although phosphatidylinositol 3 kinase (PI3K) participates in numerous signaling pathways in both animals and plants, the importance of the PI3K in stomatal movements was discovered in recent years (Jung et al., 2002). Ability of WM, a

PI3K inhibitor to prevent the ABA mediated stomatal closure in epidermal strips of wild type Arabidopsis, and inability to prevent the other ABA mediated responses like inhibition of seed germination (Fig. 7.6), prevention of root growth (Fig. 7.7) and ABA induced gene expression (*pRD29B::LUC*) (Fig. 7.8), demonstrating for the first time that bifurcated the signalling cascades leading to different out puts, i.e. ABA mediated inhibition of seed germination, prevention of root growth and ABA induced gene expression (*pRD29B::LUC*) with stomatal closure by ABA (Fig. 7.5) at upstream to the PI3K participation and downstream to the PP2C participation. Reactive oxygen species are the downstream signaling messengers leading to the stomatal closure by ABA in epidermal strips of *P. sativum* (Chapter. 6), so we thought of checking effect of ROS on stomatal closure by ABA, in PP2C mutants (*abi1* or *abi2*). Hydrogen peroxide induced stomatal closure in epidermal trips of abi1 or abi2. Our results re-confirmed that the bifurcation of the ABA responses is true and it bifurcates at downstream to the PP2Cs and upstream to the PI3K, as H_2O_2 induced stomatal closure in epidermal strips of *abi1* or *abi2* but not by ABA (Fig. 7.9).

Conclusions

1. The disruption of ABA mediated responses in ABA insensitive mutants *abi1* and *abi2,* suggested that the PP2Cs were the important secondary messengers mediating all ABA responses.
2. The ability of WM to prevent only the stomatal closure but not the other ABA mediated responses, confirmed that, stomatal closure by ABA has no part in other ABA regulated pathways.
3. These results suggested that ABA mediated pathway leading to stomatal closure bifurcates at downstream to the PP2Cs and upstream to the PI3K with other ABA regulated pathways.

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Chapter 8

General discussion

Chapter 8

General discussion

The present study is an attempt to investigate the importance of selected key signalling components in guard cells and their interaction with the other secondary messengers during ABA induced stomatal closure in epidermal strips of *Pisum sativum.* Further, we have extended our experiments to know the similarities in secondary messengers which participate during stomatal closure by ABA with other ABA mediated responses like prevention of seed germination, root growth and ABA mediated gene expression in Arabidopsis. We used Arabidopsis for this part of the work because availably of mutants, small size of the seeds and easy to measure the stomatal aperture as well as root growth with microscope.

In the first part, the patterns of change in the levels of NO, cytosolic pH, ROS and their interactions with each other, were studied. Then, the sources of NO and ROS generation were evaluated. Then the experiments were extended to know the participation and interaction of PI3K, Ca^{2+}, CaM with NO and ROS during ABA mediated stomatal closure in epidermal strips of *P. sativum*. In the last experiments, the similarities and differences in stomatal closure by ABA with other three ABA mediated responses were assessed by using Arabidopsis wild type and mutants (*abi1*, *abi2,* and *aba2*).

Nitric oxide: during stomatal closure by ABA

The participation of NO during ABA signaling in guard cells received intense attention during the past few years (Lamattina et al., 2003, Desikan et al., 2004; Fan et al., 2004). The release of NO in cells can be monitored by using suitable fluorophore DAF-2DA (Kojima et al., 1998; Foissner et al., 2000; Rodriguez-Serrano et al., 2006). In our experiments, we observed elevated levels of NO during stomatal closure by ABA (Fig. 4.4B), consistent with the results observed in *Pisum sativum*, *Vicia faba* and Arabidopsis (Desikan et al., 2002; Neill et al.,

2002a, 2003; Garcia-Mata and Lamattina, 2003; Yan et al., 2007; Gonugunta et al., 2008). On external application of NO (SNP, NO donor) induced stomatal closure by elevating NO levels in guard cells of *Pisum sativum* (Fig. 4.6B) other instance, increased plant tolerance to drought stress, by restricting stomatal opening in *Vicia faba*, *Salpichroa organifolia* and Tradescantia spp (Garcia-Mata and Lamattina, 2001, 2003).

Abscisic acid induced stomatal closure and NO was significantly prevented in presence of cPTIO, a NO scavenger (Fig. 4.3A; 4.6I), again demonstrating the importance of NO during stomatal closure by ABA. Similar results were also observed in guard cells of Arabidopsis (Desikan et al., 2004)

Several lines of evidences in the present study, demonstrated the involvement of ROS in ABA mediated stomatal closure. For e.g, ABA induced stomatal closure by enhancing ROS production as monitored with ROS specific fluorescence probe H_2DCFDA (Fig. 5.1); externally added H_2O_2 induced stomatal closure (Fig. 5.2A) and catalase (H_2O_2 scavenger) reversed stomatal closure by ABA (Fig. 5.4A). Our results complement the reports on the elevation of ROS observed during stomatal closure by elicitors like oligogalacturonic acid or chitosan in guard cells of *Lycopersicon esculentum* L and *Commelina communis* L (Lee et al., 1999). Chitosan induced NO and ROS also observed during the stomatal closure in epidermal strips of *P. sativum* (Srivastava et al., 2009).

Cytosolic pH change also acts as signalling component to induce various responses. Participation of cytosolic pH during stomatal closure by ABA was also demonstrated by multiple observations; ABA enhanced cytosolic alkalinization during stomatal closure, butyrate a weak acid prevented the stomatal closure by preventing the cytosolic alkalinization, and finally, methylamine, a weak base, induced stomatal closure by enhancing the cytosolic alkalinization (Table 4.1). Our results suggest that the cytosolic alkalinization is a major step in the ABA-triggered signal cascade in guard cells leading to H^+ efflux and stomatal closure (Irving et al., 1992; Blatt, 2000). Cytosolic alkalization of the guard cell cytoplasm

was a common event along with H_2O_2 production in response to ABA or MJ (Pei et al., 2000; Zhang et al., 2001b; Suhita et al., 2004).

Sources of NO/ROS

In plant cells, H_2O_2 can be produced in multiple ways (Park et al., 2003; Apel and Hirt, 2004). Membrane peroxidation occurs during stress conditions leading to a significant production of ROS (Montillet et al., 2004). Organelles containing electron transport systems, such as mitochondria and chloroplasts, can also produce H_2O_2 (Asada, 1999). However, the direct involvement of such processes in signaling is not yet clear. Our results provided convincing evidence that the NADPH oxidase was the possible source of ROS during stomatal closure by ABA, as ROS production in guard cells of pea was prevented not only with catalase but also by DPI, a NADPH oxidase inhibitor (Fig. 5.4A, B; 5.5A). It is now known that, a major source of H_2O_2 in guard cells is the plasma membrane NAD(P)H oxidase (Murata et al., 2001; Kwak et al., 2003; Suhita et al., 2004).

Although NO is well recognized as an important signaling molecule, its source has not yet been clearly identified and is a topic of debate. There are at least two enzymes that could mediate NO production: nitrate reductase (NR) (Desikan et al., 2002, Kaiser et al., 2002, Meyer et al., 2005) and NOS (Guo et al., 2003; del Rio et al., 2004; Crawford, 2006). NO can be generated from L-arginine (Arg) by nitric oxide synthase (NOS), the activity of which is inhibited by Arg analogues such as N-nitro-L-arginine methylester (L-NAME). Although the activity and biological function of AtNOS1 is questioned (Zemojtel et al., 2006), The partial effect of L-NAME on stomatal closure (Fig. 5.3B), as well as NO production during stomatal closure by ABA (Table 5.1), suggests that NOS like activity is involved but is not the sole source of NO production during ABA effects on guard cells.

Partial involvement of NOS, lead us to investigate the role of nitrate reductase (NR). Sodium tungstate (a NR inhibitor), efficiently prevented stomatal

closure (Fig. 5.3A) along with the NO production (Table. 5.1) during stomatal closure by ABA, suggesting, NR was the possible source for the NO production during stomatal closure by ABA. Our findings complement, NR was the source of NO during induction of stomatal closure in response to ABA as stomata of NR-deficient mutants exhibit impaired NO production and stomatal closure (Desikan et al., 2002).

Interactions of NO, ROS and Ca^{2+}

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). Further NO and ROS are essential intermediates in ABA induced stomatal closure in *Pisum sativum* and *Vicia faba* (Zhang et al., 2001b; Garcia-Mata and Lamattina, 2002; Neill et al., 2002a). The production of H_2O_2, cytosolic alkalization and the involvement of calcium have all been shown to be common signaling elements during stomatal closure caused by ABA (Pei et al., 2000; Zhang et al., 2001b; Suhita et al., 2004).

Nitric oxide can interact with other signalling elements such as lipids, cGMP, ion channels, ROS and Ca^{2+} (Desikan et al., 2004; Shapiro, 2005; Courtois et al., 2008). Both cGMP and cADPR were required for NO- and ABA induced stomatal closure (Neill et al., 2002a). Modulation of the activity of ion channels by cGMP in guard cells may be a mechanism by which NO induces stomatal closure. Guard cells utilize NO and H_2O_2 to modulate K^+ or Cl^- channels possibly through protein phosphorylation (Lum et al., 2002; Garcia-Mata and Lamattina, 2003; He et al., 2004; Sokolovski et al., 2005; Bright et al., 2006).

Multiple observations lead us to establish the interactions among the NO/pH/ROS during stomatal closure by ABA. Prevention of stomatal closure and NO production by cPTIO or tungstate and ability of butyrate to prevent the stomatal closure, cytosolic alkalinization and NO production during stomatal closure by ABA (Table 4.1), clearly indicated the importance of cytosolic

alkalinization in elevation of NO levels. Similarly, importance of ROS for NO production during stomatal closure was demonstrated by, ability of catalase or DPI to prevent the stomatal closure, ROS production besides NO production, and ability of cPTIO or tungstate to prevent the NO but not that of ROS. Similar interactions were also observed in guard cells of *Paphiopedilum tonsum, P. sativum*, Arabidopsis with (Irving et al., 1992; Suhita et al., 2004; Gonugunta et al., 2008; Srivastava et al., 2009).

Kinetic studies using DAF-2DA (for NO), H_2DCFDA (for ROS) or BCECF-AM (for pH changes) revealed that ABA induced increase in NO reached maximum by 18 min (Fig. 4.5A), cytosolic alkalinization by 12 min (Fig. 4.5B) and ROS elevation was already by 5 min (Fig. 5.1). This part of the study accompanying with previous part of the results, demonstrate ROS-cytosolic alkalinization-NO are the sequence of signaling components during stomatal closure by ABA. This is similar to the time course observed for the rise in NO, along with accumulation of large amounts of H_2O_2 in soybean suspension cells (Delledonne et al., 2001).

Nevertheless, there have been detailed studies on NO or 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). Further NO and ROS are essential intermediates in ABA induced stomatal closure in *Pisum sativum* and *Vicia faba* (Zhang et al., 2001b; Garcia-Mata and Lamattina, 2002; Neill et al., 2002a). The production of H_2O_2, cytosolic alkalization and the involvement of calcium have all been shown to be common signaling elements during stomatal closure caused by ABA (Pei et al., 2000; Zhang et al., 2001b; Suhita et al., 2004).

Calcium (Ca^{2+}) is another ubiquitous intracellular second messenger, involved in many signal transduction pathways in both plants and animals.

Although, the role of calcium in various signaling pathways was studied by several species (Schroeder and Hagiwara, 1989; McAinsh et al., 1990, 1992; Webb et al., 1996; Mac-Robbie, 2000), studies on signal transduction pathways in guard cells was very limited (McAinsh et al., 1997; Blatt, 2000). The importance of free calcium was demonstrated by EGTA, a divalent cation chelator. EGTA prevented the stomatal closure along with NO production or cytosolic pH elevation by ABA during stomatal closure. Suggesting, the cellular calcium acts at upstream of the NO elevation or cytosolic pH increase (Table 4.1) (Gonugunta et al., 2008). However, a major limitation with these experiments is that EGTA depletes the cellular calcium, thus affecting multiple components and consequently all ABA responses.

We used BAPTA, an extra Ca^{2+} cellular chelator, or BAPTA-AM a cell permeable Ca^{2+} chelator. Both prevented the stomatal closure (Fig. 6.3A, B), but BAPTA-AM prevented only NO/ROS production (Fig. 6.4A, B). Demonstrating, that the both intra- and extra-cellular calcium was required for stomatal closure by ABA, and calcium acts both up- and downstream to NO/ROS production during stomatal closure. Garcia-Mata and Lamattina, (2007) also have indicated that Ca^{2+}-dependent NO production and stomatal closure by ABA is mediated by Ca^{2+}. We propose that calcium may act upstream of cytosolic pH and NO-production, besides its known action downstream of NO production during stomatal closure by ABA (Neill et al., 2008).

Role of PI3K in stomatal closure induced by ABA

It has been established that phosphoinositides play an important role throughout the plant life (Stevenson et al., 2000). Role for Pl3P in abscisic acid-induced reactive oxygen species generation in guard cells was reported earlier (Park et al., 2003). As per the earlier reports, PI3K in guard cells can induce ROS/NO production, which in turn may activate plasma membrane Ca^{2+} channels leading to stomatal closing (Lee et al., 1999; Pei et al., 2000), suggesting a critical role of

PI3K in NO/ROS production in the mediation of stomatal closure. PI3P is a second messenger that increases transiently and elicits an intracellular calcium release in response to various stimuli, including ABA treatment (Jung et al., 2002).

Ability of PI3K inhibitors, WM or LY2940002 (Fig. 6.1A, B) or CaM antagonists (Fig. 6.2A, B) to prevent the stomatal closure by ABA demonstrate the importance of PI3K or CaM during stomatal closure induced by ABA. In our experiments, modulators of PI3K or CaM (WM for PI3K and W-7 for CaM antagonist) were chosen and were checked for their effects on stomatal closure as well as their possible interactions with NO or ROS. The results obtained demonstrated that the participation of PI3K was important in producing the NO/ROS. PI3K appeared to act only up-stream to the NO/ROS elevation by ABA during stomatal closure, as WM prevented the NO/ROS production (Fig. 6.4A, B) but was unable to prevent the SNP/H_2O_2 induced stomatal closure (Fig. 6.5). On the other hand, CaM could act both up- and down-stream to the NO/ROS elevations by ABA during stomatal closure, as W-7 prevented the ABA induced NO/ROS production (Fig. 6.4A, B) along with the SNP/H_2O_2 induced stomatal closure (Fig. 6.5).

Possible limitations in the present study

Doubts have been expressed about the specificity of DAF-2DA, H_2DCFDA to detect NO or ROS (Planchet and Kaiser, 2006). However, with the use of proper controls and scavengers of NO or ROS during these experiments (Table 4.1; 5.1), we are confident that the monitored fluorescence is related to either NO or ROS, as intended. Similarly, one may argue that catalase may not enter the guard cells, but the efficacy of catalase to decrease ROS (Fig. 5.1) and sustain stomatal opening (Fig. 5.4A) was consistent and significant. External catalase was used earlier to demonstrate the importance of ROS in plant tissues (Beffagna and Lutzu, 2007; Zhang et al., 2007) and even guard cells (Lee et al., 1999; Zhang et al., 2001c). Yet these limitations would not affect the broad conclusions drawn in the

present work, namely increase in NO-levels occurred after that of ROS and the major effect of calcium was downstream of NO and ROS, during ABA-induced stomatal closure.

Bifurcation of signaling pathway after PP2C during ABA induced responses

The phytohormone ABA plays a crucial role in seed developmental processes such as maturation, dormancy or germination (Finkelstein et al., 2002), and regulates a wide spectrum of vegetative responses including growth (Cheng et al., 2002; LeNoble et al., 2004; Lin et al., 2007) and stomatal movements (Schroeder et al., 2001b). Further, ABA could integrate signals resulting from drought, high salinity and low temperature (Christmann et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). In this context, a plethora of genes is regulated by ABA (Hoth et al., 2002). During ABA signal transduction, among the protein kinases and protein phosphatases modulated by ABA-dependent gene expression, the most important one is the including the ABI1, type PP2C phosphatases (Christmann et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Yoshida et al., 2006b; Fujii et al., 2007). The involvement of ABI1 and its homologue, ABI2, were checked among the four chosen ABA mediated responses, stomatal closure, prevention of seed germination, root growth, induction of ABA responsive *pRD29B::LUC* gene expression in Arabidopsis.

ABA induced stomatal closure in epidermal strips, prevented seed germination, root growth in wild type Arabidopsis, but this three ABA mediated responses were impaired in *abi1* or *abi2* mutants (Fig. 7.1, 2, 3). The mutant proteins abi1 and abi2 are the results of single amino acid exchange in the catalytic domain, G180D (ABI1) and G168D (ABI2), these mutations confer a dominant ABA-insensitive phenotype in seed germination and seedling development, as well as attenuation of seed dormancy and stomatal closure (Koornneef et al., 1984). When transiently overexpressed, abi1 or abi2 completely prevented the ABA mediated gene *pRD29B::LUC* expression in protoplasts of ABA

biosynthesis mutant *aba2* (Fig. 7.4), while ABI1 or ABI2 partially such ABA-enhanced gene expression. These observations indicated an impairment of ABA mediated gene expression in presence of abi1 or abi2.

PI3K inhibitor, WM prevented the stomatal closure by ABA in *P. sativum* up-stream to the NO/ROS (Chapter 6). Bifurcation of the ABA responses at upstream can be demonstrated by ability of WM to prevent only the ABA mediated stomatal closure (Fig. 7.5) but not other three responses i.e. prevention of seed germination (Fig. 7.6), root growth (Fig. 7.7) and ABA induced gene *pRD29B::LUC* expression (Fig. 7.8). To confirm the above concept, the effect of H_2O_2, on stomatal closure was checked in *abi1* or *abi2* mutants, deficient in PP2C. The result that stomatal closure in epidermal strips of PP2C defective *abi1* or *abi2* mutants, could be induced by H_2O_2 but not by ABA (Fig. 7.9), confirmed that H_2O_2 was indeed an active component, downstream of PP2C.

Summary and conclusions are presented in the next chapter, along with two possible schemes of different signaling components participating in ABA promoted stomatal closure and other developmental processes.

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Chapter 9

Summary and conclusions

Chapter 9

Summary and conclusions

Stomatal guard cells are popular model systems for characterizing signal transduction mechanisms and secondary messengers in plants (Fan et al., 2004; Israelsson et al., 2006). Guard cells respond to plant hormone ABA through several secondary messengers, including type 2C protein phosphatases (PP2C), G-proteins, protein kinases, sucrose non-fermenting 1 related protein kinases 2 (SnRK2s), phospholipases, besides cytosolic pH, reactive oxygen species (ROS), calcium (Ca^{2+}) and nitric oxide (NO) (Bright et al., 2006; Zhang et al., 2007; Neill et al., 2008).

The present work is an attempt to investigate the role of selected key signalling components in guard cells and their interaction with the other secondary messengers during ABA induced stomatal closure in epidermal strips of *Pisum sativum* and *Arabidopsis thaliana*. The initial focus was on the patterns of change in the levels of NO, cytosolic pH, ROS and their interactions with each other. Then, the sources of NO and ROS generation were assessed. Further, the experiments were extended to know the role and importance of PI3K, Ca^{2+}, CaM and its interactions with NO and ROS. A possible scheme of the integration of signaling components during ABA-induced stomatal closure is shown in Fig. 9.1. Finally, the bifurcation of signalling pathway if any, among the four ABA mediated responses, i.e. stomatal closure, prevention of seed germination, root growth and gene expression in protoplasts was assessed by using ABA insensitive mutants (*abi1* and *abi2*) and ABA biosynthesis mutant (*aba2*). Involvement of PP2Cs and PI3K in four ABA mediated responses, stomatal movement, seed germination, root growth and gene expression are illustrated in Fig. 9.2.

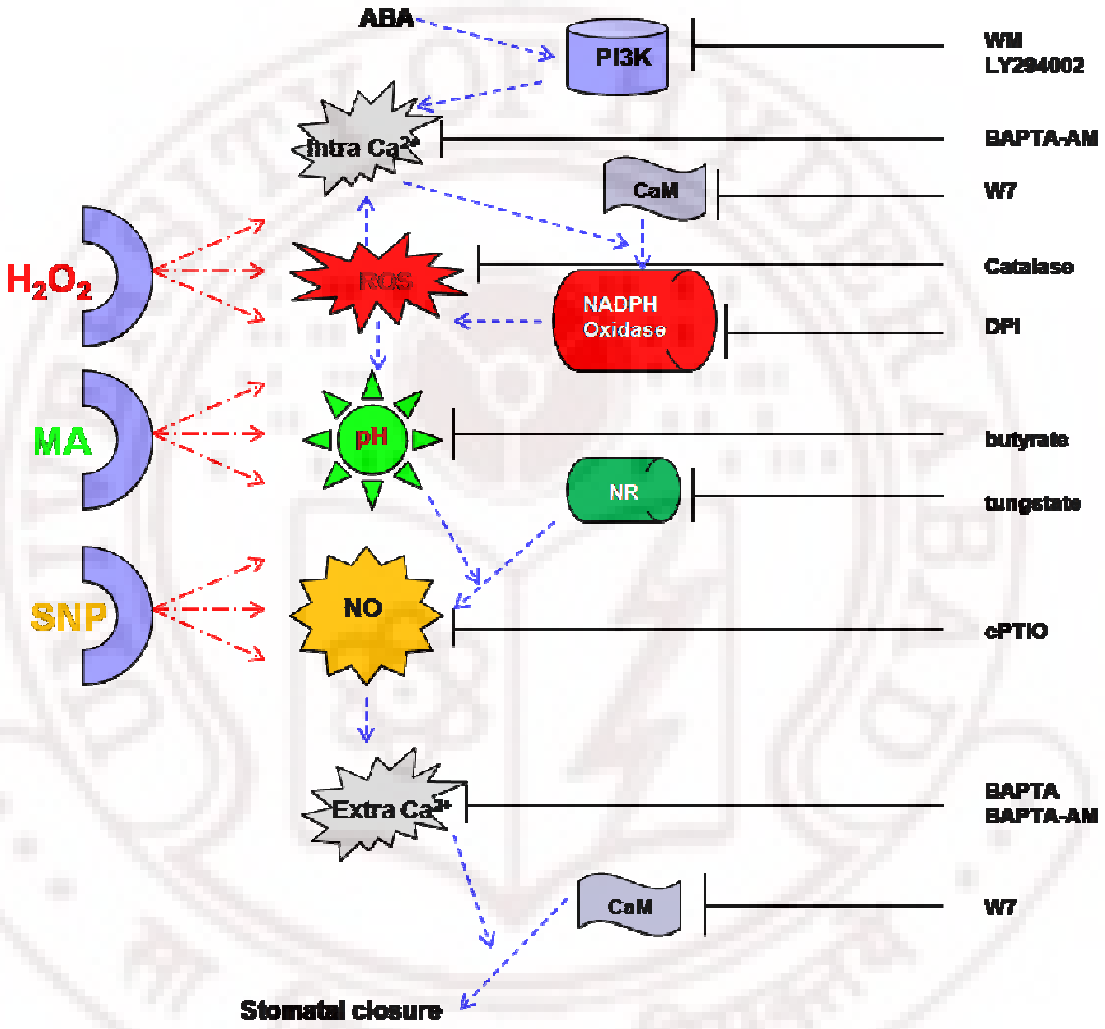

Figure 9.1 Schematic representation of the signaling components leading to the stomatal closure by ABA in epidermal strips of *P. sativum* has shown**.**

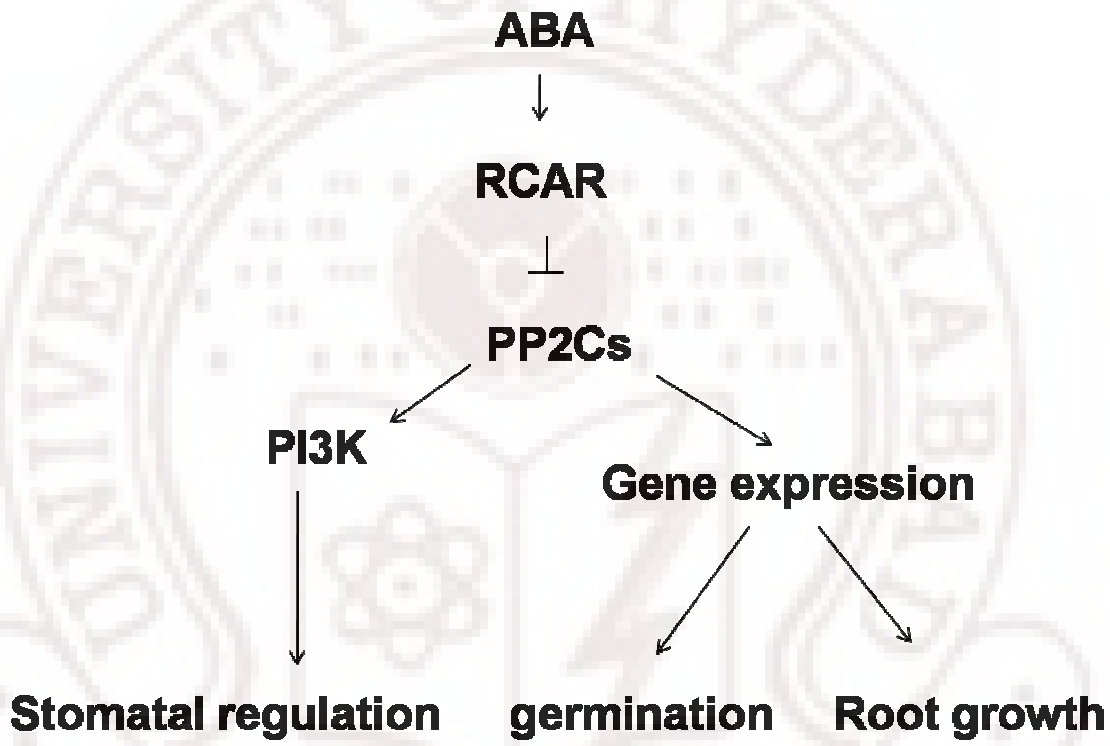

Figure 9.2. Schematic representation of involvement of PP2Cs and PI3K during four ABA mediated responses. Stomatal closure by ABA was WM sensitive but not other ABA mediated responses like gene expression, seed germination and root in seedlings.

Major conclusions

1. ABA induced stomatal closure was associated with an increase in not only NO, ROS but also cytosolic pH of guard cells.
2. Real time monitoring with the help of fluorescent probes indicated that (i) cytosolic alkalinization of the guard cell preceded NO production. (ii) ROS production occurs earlier than the NO production and cytosolic alkalinization by ABA.
3. As a complement, the ability of catalase or DPI to restrict the production of ROS as well as NO, and the inability of NO-modulators (scavenger of inhibitor) to prevent the rise in ROS levels in guard cells, indicated the necessity of ROS elevation for NO production during stomatal closure by ABA.
4. The prevention of ROS production by DPI and NO production by sodium tungstate indicated NADPH oxidase and nitrate reductase were the possible sources for NO and ROS, respectively during ABA induced stomatal closure.
5. Ca^{2+} was necessary to sustain the rise in cytosolic pH and NO as EGTA prevented the both. As a complement, the reduction of NO, ROS and ABA-induced stomatal closure by BAPTA-AM, confirmed that the requirement of intra-cellular Ca^{2+} for stomatal closure which act at upstream of NO/ROS production by ABA. In contrast, the action of BAPTA suggested, that extra-cellular Ca^{2+} acted at downstream of NO/ROS elevation.
6. The restriction by W-7 of NO/ROS production as well as ABA/H_2O_2/SNP induced stomatal closure confirmed that calmodulin acted at up- and downstream of NO/ROS elevation by ABA. In contrast, the restriction by WM of NO/ROS production by ABA, but not the SNP/H_2O_2 induced

stomatal closure confirmed that PI3K acted at only upstream of NO/ROS elevation by ABA

7. The PP2Cs are the negative regulators of all the four ABA responses, included in the present study, i.e., stomatal closure, seed germination, root growth and gene expression. The sensitivity of only stomatal closure to WM indicated that the signalling pathway bifurcated at downstream of PP2C and upstream to the PI3K. The ABA promoted inhibition of seed germination, root growth and gene expression were all WM insensitive and were obviously independent of PI3K action.

Based on the above observations, generalized scheme illustrating the components of signal transduction during ABA induced stomatal closure can be drawn (Fig. 9.1).

The present work open up a few interesting leads which can be pursued in future some of these are:

(i) The regulation by upstream messengers cytosolic pH, NO and ROS production, and the mechanisms of regulation of downstream elements.

(ii) The identities of up-stream elements regulating either NR or NADPH oxidase or both.

(iii) The convergence of CaM, calcium and PI3K in regulating the stomatal closure by ABA.

(iv) The mechanism of ABA regulation bifurcation at PP2C into a PI3K-dependent and PI3K-independent pathway.

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Chapter 10

Literature cited

Chapter 10

Literature cited

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Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid

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ABSTRACT

Abscisic acid (ABA) raised the cytosolic pH and nitric oxide (NO) levels in guard cells while inducing stomatal closure in epidermis of *Pisum sativum*. Butyrate (a weak acid) reduced the cytosolic pH/NO production and prevented stomatal closure by ABA. Methylamine (a weak base) enhanced the cytosolic alkalinization and aggravated stomatal closure by ABA. The rise in guard cell pH because of ABA became noticeable after 6 min and peaked at 12 min, while NO production started at 9 min and peaked at 18 min. These results suggested that NO production was downstream of the rise in cytosolic pH. The ABA-induced increase in NO of guard cells and stomatal closure was prevented by 2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (cPTIO, a NO scavenger) and partially by N-nitro-L-Arg-methyl ester (L-NAME, an inhibitor of NO synthase). In contrast, cPTIO or L-NAME had only a marginal effect on the pH rise induced by ABA. Ethylene glycol tetraacetic acid (EGTA, a calcium chelator) prevented ABA-induced stomatal closure while restricting cytosolic pH rise and NO production. We suggest that during ABA-induced stomatal closure, a rise in cytosolic pH is necessary for NO production. Calcium may act upstream of cytosolic alkalinization and NO production, besides its known function as a downstream component.

Key-words: *Pisum sativum*; abscisic acid; calcium; cytosolic pH; guard cells; nitric oxide.

INTRODUCTION

Gas exchange regulation by stomata is crucial for plant growth and development (Hetherington & Woodward 2003). The stomatal guard cells are able to sense and integrate multiple internal and external stimuli (Assmann & Shimazaki 1999; Schroeder *et al.* 2001). On exposure to drought, stomata close so as to reduce the loss of water via transpiration, and this response is mediated by the phytohormone, abscisic acid (ABA) (Assmann & Shimazaki 1999; Schroeder *et al.* 2001; Roelfsema & Hedrich 2005).

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ABA activates a complex web of signalling components including G-proteins, protein kinases, protein phosphatases, cytosolic pH, reactive oxygen species (ROS), cytosolic calcium and ion channels (Irving, Gehring & Parish 1992; Hamilton *et al.* 2000; Schroeder *et al.* 2001; Wang *et al.* 2001; Bright *et al.* 2006). Additional components of ABA signalling include sphingosine-1-phosphate, phospholipase C and reactive nitrogen species, that is, nitric oxide (NO) (Hetherington 2001; Ng *et al.* 2001; Neill, Desikan & Hancock 2003; Zhang *et al.* 2007). An increase in ROS of guard cells has been reported during stomatal closure induced also by methyl jasmonate (MJ) or bicarbonate (Suhita *et al.* 2004; Kolla, Vavasseur & Raghavendra 2007).

Recent evidence suggests the existence of a crosstalk between NO and some plant hormones (auxins, ethylene, salicylic acid and ABA) during adaptive responses to biotic or abiotic stress (Lamattina *et al.* 2003; Ali *et al.* 2007; Neill *et al.* 2008). For example, NO has been shown to be important during ABA-induced stomatal closure as observed in *Pisum sativum*, *Vicia faba* and *Arabidopsis* (Desikan *et al.* 2002; Neill *et al.* 2002, 2003; Garcia-Mata & Lamattina 2003; Yan *et al.* 2007). The levels of NO in guard cells increase on exposure to bicarbonate too (Kolla & Raghavendra 2007). Exogenous application of sodium nitroprusside (SNP), a NO donor, increased plant tolerance to drought stress by restricting stomatal apertures (Garcia-Mata & Lamattina 2001). However, the mechanism by which ABA or bicarbonate induces an increase in guard cell NO levels is not completely clear.

Marked changes in cytosolic pH of plant tissues are observed during responses to a variety of hormones including ABA or MJ. For example, the pH of guard cells increases in the presence of ABA or MJ (Irving *et al.* 1992; Van der Veen, Heimovaara-Dijkstra & Wang 1992; Suhita *et al.* 2004). Exposure to even H_2O_2 can rise in intracellular pH as shown in the case of *V. faba* guard cells (Zhang *et al.* 2001). Cytosolic alkalinization preceded ROS production during stomatal closure by ABA or MJ (Suhita *et al.* 2004). Whether pH has any role in NO production during ABA effects on guard cells is yet to examined. The present work is an attempt to assess the importance and interactions of cytosolic pH and NO during stomatal responses to ABA in the abaxial epidermal strips of *P. sativum*. The components involved in upstream or downstream of pH and NO during stomatal responses to ABA were also examined.

MATERIALS AND METHODS

Plant material and growth conditions

Plants of *P. sativum* (cv. Arkel) were raised from seeds. The plants were grown outdoors under natural conditions (average day/night temperature 30/20 °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.

Bioassays of stomatal closure in epidermal strips

The abaxial (lower) epidermis was peeled off from the leaves and was cut into strips of ca. 0.16 cm^2. The epidermal strips (ca. 0.16 cm^2) were transferred to 3-cm diameter Petri dishes containing 3 mL of 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 50 mM potassium chloride (KCl), pH 7.0. The epidermal strips were exposed to white light (250 μmol m^{-2} s^{-1}) for 3 h. A bank of tungsten lamps provided the light, filtered through water jacket. The photon flux was measured with a Li-Cor quantum sensor (Li-Cor Instruments Ltd., Lincoln, NE, USA). The temperature was maintained at 25 ± 1 °C. When used, the test compounds (pH modulators, inhibitors or scavengers) were added after the 3 h light period, followed by ABA after 10 min. Incubation of the epidermal strips was then continued for another 3 h in the same light, before measuring stomatal apertures.

The width of stomatal aperture was measured under a research microscope with the help of a precalibrated ocular micrometer. Ten apertures were monitored at random in each of three different epidermal strips from each treatment. The experiments were repeated on three different days, making each measurement of stomatal aperture an average of 90 stomata.

Monitoring NO or pH

NO production in guard cells of *P. sativum* was followed by using 4,5-diaminofluorescein diacetate (DAF-2DA), as previously described (Neill *et al.* 2002) with minor changes. The changes in pH were monitored with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM), as described earlier by Irving *et al.* (1992) with minor modifications.

Epidermal peels were mounted on a microscope slide with medical adhesive Telesis V (Premiere Products, Inc., Pacoima, CA, USA). Stomata were allowed to open by incubating the epidermal strips under 250 μmol m^{-2} s^{-1} white light for 3 h, in a medium of 50 mM KCl and 10 mM MES-KOH, pH 7.0. After allowing stomata to open in light for 3 h, the test compounds were added to the medium. Then, the epidermal strips were loaded with the required dye, 20 μM DAF-2DA (10 min) or 20 μM BCECF-AM (10 min), in incubation medium containing 0.05% Pluronic F-127 in the dark at 25 ± 1 °C. The strips were rinsed quickly with incubation buffer three times (to wash off excessive fluorophore), followed by the addition of ABA.

In experiments involving time-course monitoring of signalling components in guard cells, the epidermal strips were examined under an inverted fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with monochrome high-resolution digital cooled CD camera (Coolsnap fx, Photometrics, Roper Scientific, USA) that enabled to capture the images with DAF-2DA or BCECF-AM fluorescence (excitation filter, 465–495 nm, and emission, 515–555 nm). The captured images and the relative fluorescence emission of guard cells were analysed by using NIH Image for Windows (Murata *et al.* 2001).

In some of the experiments, a confocal microscope (TCS-SP-2, AOBS 4 channel UV and visible; Leica, Heidelberg, Germany) was used to observe the fluorescence of cytosolic pH or NO in the epidermal strips of *P. sativum* (excitation filter, 488 nm, and emission, 515–540 nm).

Solvent effects, replications and statistical analysis

The control sets were added with an equal volume of solvents used for their stocks. Ethanol was the solvent used for ABA, dimethyl sulfoxide for DAF-2DA or BCECF-AM, and milli-Q water for cPTIO, L-NAME, EGTA or SNP. The data presented are the average values (±SE) of results from at least three experiments conducted on different days. The statistical significance of treatments was checked using Student's *t*-test. The data were considered statistically significant when *P* values were below 0.05.

RESULTS

Patterns of cytosolic pH and NO production during ABA-induced stomatal closure

The fluorescence probes of BCECF-AM or DAF-2DA enabled us to determine the kinetics of NO or pH changes in guard cells on exposure to ABA. Treatment with ABA caused a marked increase in both pH and NO levels of guard cells (Fig. 1c,d). The increase in pH of guard cells on exposure to ABA was visible by 6 min and reached its maximum at 12 min (Fig. 2a). In contrast, NO production started to increase steeply after 9 min and reached its maximum at 18 min. Thus, the rise in pH of guard cells appeared to occur earlier to that of NO increase (Fig. 2b). We are not sure of the exact reasons for such a decrease in NO levels (Fig. 2b). The decrease could be due to the scavenging of NO or bleaching of the dye, or both.

Stomatal closure in relation to modulation of pH or NO

Butyrate (a weak acid) prevented stomatal closure by ABA (Fig. 3a), while methylamine (a weak alkalinizing agent) enhanced ABA-induced stomatal closure (Fig. 3b).

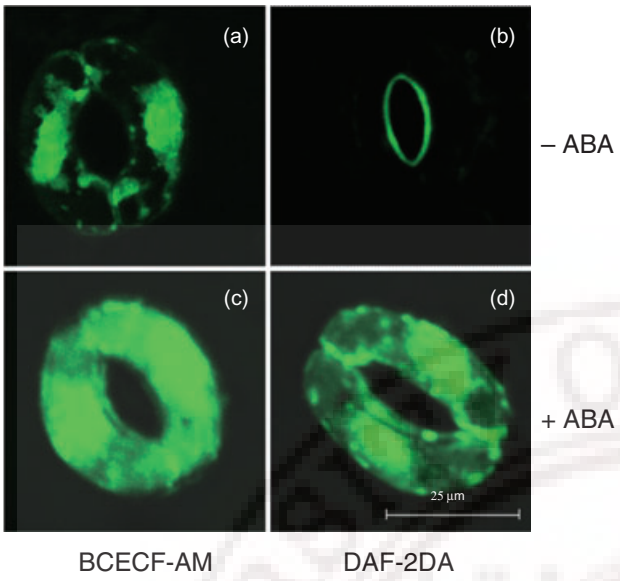

Figure 1. Confocal fluorescence images of stomata stained with 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) (a,c) or 4,5-diaminofluorescein diacetate (DAF-2DA) (b,d). These were taken after 12 min for BCECF-AM and 18 min for DAF-2DA treatment with 10 μM abscisic acid (ABA). (a) and (b) are the controls, while (c) and (d) are the stomata treated with ABA. Bar = 25 μm.

ABA-induced stomatal closure was prevented completely by cPTIO (Fig. 4a), and partially by L-NAME (Fig. 4b).

Figures 5 and 6 represent the patterns of pH increase or NO production with or without ABA, in the presence of different modulators. Butyrate prevented the cytosolic alkalinization (Fig. 5j) and NO production (Fig. 6j) induced by ABA. Butyrate alone had no significant effect on either stomatal closure (Table 1) or the rise in pH/NO (Figs 5c & 6c). Methylamine alone induced stomatal closure (Table 1) while increasing cytosolic alkalinization (Fig. 5d) and NO production (Fig. 6d). When incubated with ABA, methylamine further increased both cytosolic alkalinization (Fig. 5k) and NO production (Fig. 6k).

Other factors affecting the pH rise or NO production

Table 1 presents a comprehensive information on the effects of different modulators on the rise in pH/NO as well as on stomatal closure. SNP alone promoted stomatal closure and enhanced, to a limited extent, the pH of guard cells (Table 1). However, SNP had no further effect on ABA-induced cytosolic alkalinization (Fig. 5b). Similarly, cPTIO or L-NAME did not affect much the cytosolic alkalinization (Fig. 5l,m), but restricted quite strongly the NO production (Fig. 6l,m) by ABA.

The presence of SNP enhanced not only stomatal closure (Table 1) but also NO production (Fig. 6i) in the absence or presence of ABA. cPTIO prevented completely the ABA-induced NO production in guard cells (Fig. 6e,l), whereas L-NAME restricted stomatal closure (Table 1) or NO production only partially (Fig. 2f,m).

Role of calcium in ABA-mediated alkalinization and NO production

EGTA, a calcium chelator, prevented stomatal closure (Table 1) and cytosolic alkalinization (Fig. 5m) as well as NO production (Fig. 6m) induced by ABA. When used alone, EGTA had no significant effect on cytosolic alkalinization (Fig. 5g) or NO production (Fig. 6g).

DISCUSSION

It is well established that ROS, NO and cytosolic calcium are all essential signalling components during

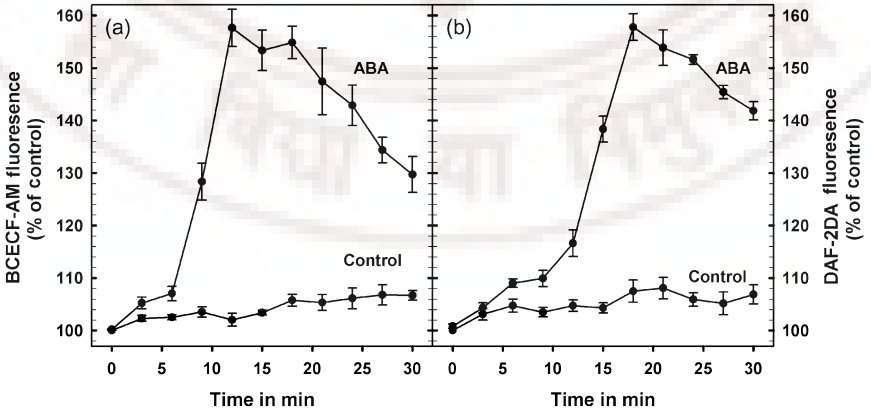

Figure 2. Kinetics of increase in pH (a) or nitric oxide (NO) (b) in epidermal strips of *Pisum sativum* in response 10 μM abscisic acid (ABA). Epidermal strips were loaded with either 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) (to monitor pH) or 4,5-diaminofluorescein diacetate (DAF-2DA) (for NO) while incubating with ABA. Cytosolic pH reached its maximum by 12 min, after a lag period of 6 min, whereas NO production reached its maximum at 18 min, after a lag of 9 min. The extent of NO or pH production in guard cells without ABA is taken as 100%. Further details are described in the Materials and Methods section. Results are the averages ± SE from at least three independent experiments.

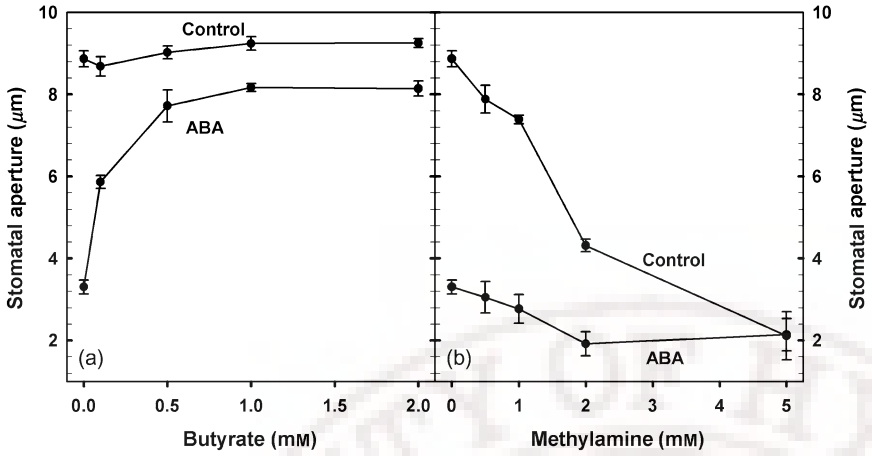

Figure 3. Effect of butyrate, a weak acid (a), or methylamine, an alkalinizing agent (b), on stomatal closure induced by 10 μM abscisic acid (ABA) in epidermal strips of *Pisum sativum*. Butyrate prevented stomatal closure by ABA, while methylamine further enhanced such stomatal closure. Butyrate alone did not have much effect, while methylamine promoted stomatal closure, even in the absence of ABA. Results are the averages ± SE of three to four independent experiments. Further details are given in the Materials and Methods section.

ABA-induced stomatal closure (Neill *et al.* 2002). The present study demonstrates the importance and interactions of cytosolic pH with NO and calcium during ABA-induced stomatal closure. The pH rise appears to be necessary and occurring upstream of NO production during ABA-induced stomatal closure.

Cytosolic alkalinization appears to precede NO production in guard cells after exposure to ABA

The pH is an important signalling component during several of plant responses including stomatal movements (Irving *et al.* 1992; Felle 2001; Jeremiah *et al.* 2001). Effectors that raise the cytosolic pH (ABA and MJ) result in stomatal closure (Blatt & Armstrong 1993; Suhita *et al.* 2004), while those that lower the cytosolic pH (auxin, fusicoccin) open stomata (Irving *et al.* 1992). Even during stomatal closure by H_2O_2, cellular alkalinization was an early event (Zhang *et al.* 2001). However, Zhang *et al.* (2001) did not examine the levels of either ROS or NO in guard cells. In our experiments, when guard cells were treated with ABA, there was a marked increase not only in NO levels but also in cytosolic pH (Fig. 1), indicating the importance of pH. The kinetics of increase in NO or pH, monitored by DAF-2DA and BCECF-AM, respectively, revealed that ABA-induced increase in cytosolic pH had a shorter lag and reached the peak faster than that of NO levels (Fig. 2a,b). These results suggest that the action of cytosolic pH could be upstream of NO during stomatal closure by ABA.

Modulation of cytosolic pH and consequence on NO production or stomatal closure

Cytosolic pH can be modulated by weak alkalinizing agents, such as methylamine or NH_4Cl, and weak acids, such as butyric acid or acetic acid (Danthuluri, Kim & Brock 1990; Van der Veen *et al.* 1992; David, Colin & Anthony 1998). Our observations on modulation of ABA-induced stomatal closure, as well as NO levels in guard cells by butyrate or methylamine (Figs 3a,b & 6j,k), indicate that the change in cytosolic pH is either associated or necessary for NO production during stomatal closure by ABA. Because the NO molecule is quite active at an alkaline pH of 7.4 (Reiter, Teng & Beckman 2000), NO can be expected to become effective as the pH rises. cPTIO or L-NAME prevented ABA-induced stomatal closure, but did not

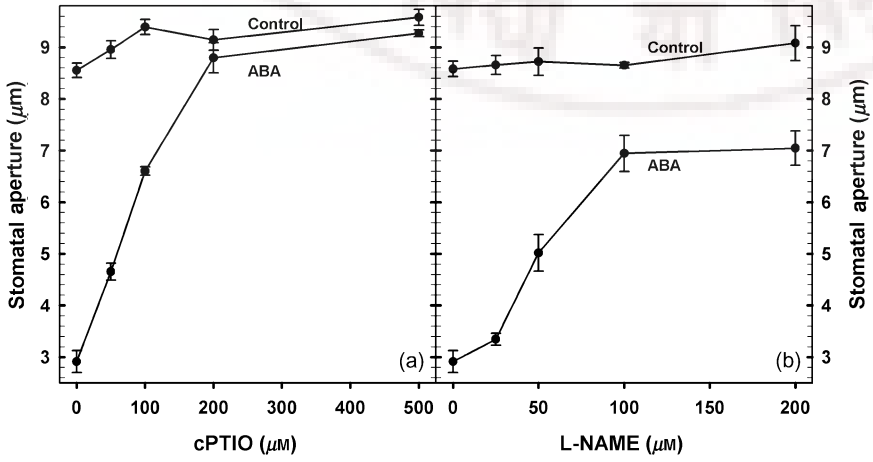

Figure 4. Prevention of abscisic acid (ABA)-induced stomatal closure in epidermal strips of *Pisum sativum* by either cPTIO, a nitric oxide (NO) scavenger (a), or L-NAME, an inhibitor of nitric oxide synthase (NOS) (b). The presence of 0.2 mM or above cPTIO prevented ABA-induced stomatal closure almost completely. L-NAME prevented only to a partial extent of ABA-induced stomatal closure. Results are the averages ± SE of three to four independent experiments. Further details are given in the Materials and Methods section.

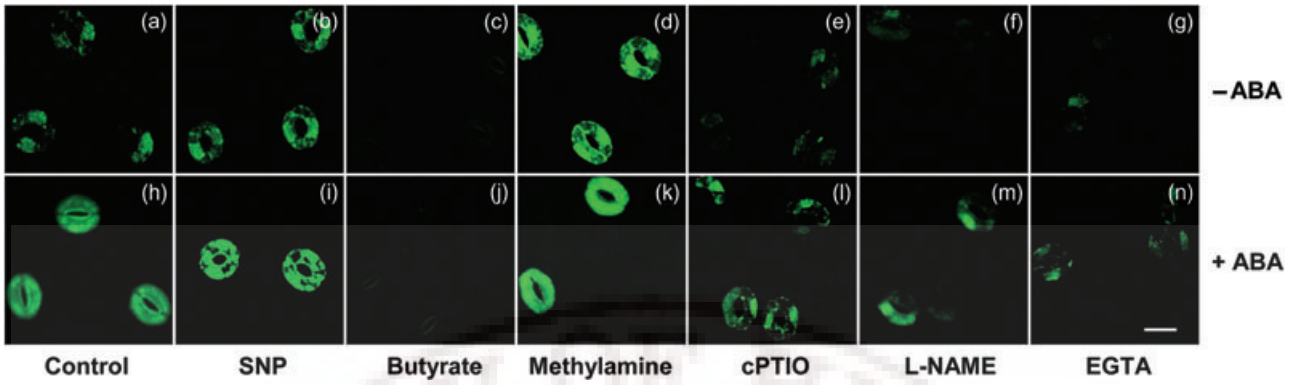

Figure 5. Effect of different modulators on 10 μM abscisic acid (ABA)-induced increase in pH, as indicated by 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) fluorescence in stomatal guard cells of *Pisum sativum*. (a) to (g) are the controls: treated with water (a), 0.1 mM sodium nitroprusside (SNP) (b), 0.1 mM butyrate (c), 2 mM methylamine (d), 0.2 mM cPTIO (e), 0.1 mM L-NAME (f) and 1 mM EGTA (g) in the absence of ABA, respectively. (h) to (n) are epidermal strips incubated with ABA alone (h), ABA along with 0.1 mM SNP (i), 0.1 mM butyrate (j), 2 mM methylamine (k), 0.2 mM cPTIO (l), 0.1 mM L-NAME (m) and 1 mM EGTA (n) in the presence of ABA, respectively. Confocal fluorescence images were taken at 12 min after addition of 10 μM ABA. Further details are given in the Materials and Methods section. Bar = 25 μm.

prevent the extent of alkalinization (Table 1). We therefore suggest that the change in cytosolic pH is upstream of NO production. The production of NO may have some feedback effect on cytosolic pH as SNP, a NO donor, partially increased the cytosolic pH. This point needs further study.

Importance and interactions of pH and NO during ABA signalling

We have earlier shown that cytosolic pH and ROS in guard cells are important signalling components during the effects of MJ or bicarbonate (Suhita *et al.* 2004; Kolla *et al.* 2007). The present results highlight the involvement and interaction of NO, cytosolic pH and cytosolic calcium during the transduction of ABA signal also.

NO levels can be modulated by using cPTIO (a scavenger of NO) and L-NAME [an inhibitor of nitric oxide synthase (NOS)] (Garcia-Mata & Lamattina 2002; Neill *et al.* 2002; Guo, Okamoto & Crawford 2003; Crawford & Guo 2005). Although the activity and biological function of AtNOS1 is questioned (Zemojtel *et al.* 2006), the restriction by L-NAME of ABA-induced stomatal closure (Fig. 4b) suggests that NOS-like activity is involved. However, the partial effect of L-NAME on stomatal closure (Fig. 4b), as well as NO production due to ABA (Fig. 6f,m), suggests that the NOS-like activity is not the sole source of NO during ABA effects on guard cells.

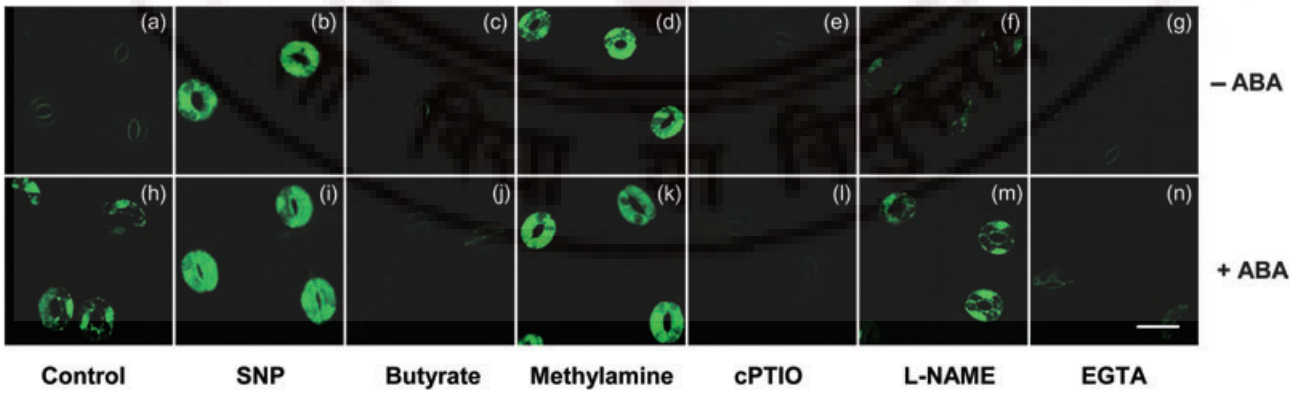

Figure 6. Effect of different modulators on 10 μM abscisic acid (ABA)-induced nitric oxide (NO) production, as indicated by 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence in stomatal guard cells of *Pisum sativum*. (a) to (g) are the controls: treated with water (a), 0.1 mM sodium nitroprusside (SNP) (b), 0.1 mM butyrate (c), 2 mM methylamine (d), 0.2 mM cPTIO (e), 0.1 mM L-NAME (f) and 1 mM EGTA (g) in the absence of ABA, respectively. (h) to (n) are epidermal strips treated with ABA, as follows: ABA alone (h), ABA along with 0.1 mM SNP (i), 0.1 mM butyrate (j), 2 mM methylamine (k), 0.2 mM cPTIO (l), 0.1 mM L-NAME (m) and 1 mM EGTA (n) in the presence of ABA, respectively. Confocal fluorescence images were taken at 18 min after addition of 10 μM ABA. Further details are given in the Materials and Methods section. Bar = 25 μm.

Table 1. The effect of pH modulators (butyrate and methylamine) or NO modulators (cPTIO, L-NAME or SNP) and calcium chelator (EGTA) on ABA-induced stomatal closure, cytosolic pH changes and NO production in guard cells of *Pisum sativum*

Treatment	−ABA 10 μM			+ABA 10 μM		
	Stomatal aperture (μm)	BCECF-AM fluorescence (% control)	DAF-2DA fluorescence (% control)	Stomatal aperture (μm)	BCECF-AM fluorescence (% control)	DAF-2DA fluorescence (% control)
None (control)	8.9 ± 0.2	100 ± 0	100 ± 0	3.3* ± 0.2	157* ± 3	161* ± 4
0.1 mM butyrate	9.2 ± 0.2	91 ± 2	107 ± 2	6.2* ± 0.1	101 ± 4	111 ± 2
2 mM methylamine	4.3 ± 0.2	173 ± 3	159 ± 4	1.9* ± 0.3	174 ± 7	166 ± 6
0.2 mM cPTIO	9.1 ± 0.2	108 ± 2	106 ± 2	8.8 ± 0.3	140* ± 5	109 ± 3
0.1 mM L-NAME	8.7 ± 0.1	108 ± 5	103 ± 2	6.9 ± 0.3	139* ± 5	120 ± 4
1 mM EGTA	9.0 ± 3.9	110 ± 2	105 ± 2	8.6 ± 0.2	108 ± 4	110 ± 3
0.1 mM SNP	3.9 ± 0.2	122 ± 5	164 ± 8	3.1 ± 0.2	156* ± 3	168 ± 4

*Significant at P value < 0.05 compared with the respective treatment without ABA.
The extent of fluorescence without ABA and without any effector is taken as 100%. Results are the averages ± SE of three to four independent experiments. Further details are given in the Materials and Methods section.
NO, nitric oxide; SNP, sodium nitroprusside; ABA, abscisic acid; BCECF-AM, 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester; DAF-2DA, 4,5-diaminofluorescein diacetate.

Calcium may act upstream of cytosolic pH or NO production

The increase in cytosolic Ca^{2+} of guard cells is a common signalling component during stomatal closure in response to diverse signals (McAinsh, Brownlee & Hetherington 1997). 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). It is therefore proposed that the signalling components during these events converge at the level of calcium.

The marked prevention of ABA-induced stomatal closure and decrease in the levels of pH/NO by EGTA (Table 1) suggested that cytosolic Ca^{2+} is necessary to sustain cytosolic pH increase and NO production during stomatal closure by ABA. However, a major limitation with these experiments is that EGTA depletes the cellular calcium, thus affecting multiple components and consequently all ABA responses. Garcia-Mata & Lamattina (2007) also have indicated that Ca^{2+}-dependent NO production and stomatal closure by ABA is mediated by Ca^{2+}. We propose that calcium may act upstream of cytosolic pH and NO production, besides its known action downstream of

Figure 7. Schematic representation of abscisic acid (ABA)-induced stomatal closure. Cytosolic alkalinization is one of the key and early steps leading to stomatal closure. Exposure to ABA leads to an increase in cytosolic pH, raises the level of nitric oxide (NO), and subsequently leads to stomatal closure. Modulation of guard cell pH by butyrate or methylamine affects NO levels in guard cells and the extent of stomatal closure. Similarly, modulation of NO levels affects stomatal closure but not the pH rise. Ca^{2+} appears to be necessary for ABA-induced rise in pH as well as the action of NO. The role of Ca^{2+} upstream of NO is well known in the literature.

NO production during stomatal closure by ABA (Neill *et al.* 2008).

CONCLUDING REMARKS

ABA-induced stomatal closure was associated with an increase not only in NO but also in cytosolic pH of guard cells. Real-time monitoring with the help of fluorescent dyes indicated that alkalinization of guard cell preceded NO production. Modulation of cytosolic pH changed the patterns of NO production and stomatal closure. Internal Ca^{2+} appears to be necessary to sustain the rise in cytosolic pH and NO. A schematic representation of possible events occurring during ABA-induced stomatal closure is shown in Fig. 7. The interrelationship and interaction of cytosolic calcium, cytosolic pH and NO appear to be quite intriguing and need further examination.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*

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Abstract The effects of chitosan (β-1,4 linked glucosamine, a fungal elicitor), on the patterns of stomatal movement and signaling components were studied. cPTIO (NO scavenger), sodium tungstate (nitrate reductase inhibitor) or L-NAME (NO synthase inhibitor) restricted the chitosan induced stomatal closure, demonstrating that NO is an essential factor. Similarly, catalase (H_2O_2 scavenger) or DPI [NAD(P)H oxidase inhibitor] and BAPTA-AM or BAPTA (calcium chelators) prevented chitosan induced stomatal closure, suggesting that reactive oxygen species (ROS) and calcium were involved during such response. Monitoring the NO and ROS production in guard cells by fluorescent probes (DAF-2DA and H_2DCFDA) indicated that on exposure to chitosan, the levels of NO rose after only 10 min, while those of ROS increased already by 5 min. cPTIO or sodium tungstate or L-NAME prevented the rise in NO levels but did not restrict the ROS production. In contrast, catalase or DPI restricted the chitosan-induced production of both ROS and NO in guard cells. The calcium chelators, BAPTA-AM or BAPTA, did not have a significant effect on the chitosan induced rise in NO or ROS. We propose that the production of NO is an important signaling component and participates downstream of ROS production. The effects of chitosan strike a marked similarity with those of ABA or MJ on guard cells and indicate the convergence of their signal transduction pathways leading to stomatal closure.

Keywords Chitosan · Nitric oxide · Pea · ROS · Signal transduction · Stomata

Abbreviations

ABA	Abscisic acid
BAPTA	1,2-bis(*o*-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
BAPTA-AM	1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester
cPTIO	2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide
DAF-2DA	4,5-Diaminofluorescein diacetate
DPI	Diphenyleneiodonium chloride
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
L-NAME	*N*-nitro-L-Arg-methyl ester
MES	2-(*N*-morpholino) ethanesulphonic acid
MJ	Methyl jasmonate
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
ROS	Reactive oxygen species
SNP	Sodium nitroprusside

Introduction

Stomata are essential components of leaves, as they not only control rates of CO_2 uptake and water loss, but also respond quickly to several environmental and internal factors. Further, stomata can play an active role in limiting pathogen invasion as a part of the plant innate immune system (Melotto et al. 2008). Although some pathogens can force entry though closed stomata, many can infect plants only when the stomata are open. Effecting stomatal closure

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can therefore limit the penetration of pathogens, thereby conferring resistance to plants.

Stomatal guard cells are popular model systems for characterizing signal transduction mechanisms and secondary messengers in plants (Fan et al. 2004; Israelsson et al. 2006). Guard cells respond to plant hormones such as abscisic acid (ABA), methyl jasmonate (MJ) or auxin, through several secondary messengers including reactive oxygen species (ROS), nitric oxide (NO), G-proteins, calcium and protein kinases/protein phosphatases (Assmann and Shimazaki 1999; Zeiger 2000; Schroeder et al. 2001; Israelsson et al. 2006; Neill et al. 2008). In case of pathogen infection too, plants activate a variety of defense mechanisms within a few minutes through a signaling cascade. The challenged plants frequently elevate ROS such as superoxide and hydrogen peroxide (H_2O_2), which in turn can trigger the hypersensitive responses (Torres et al. 2006). Plants are equipped with mechanisms to combat increased ROS levels during biotic and abiotic stress conditions. However, plants appear to purposefully generate ROS as signaling molecule to control various processes including pathogen defense, programmed cell death and stomatal behavior (Delledonne et al. 2001; Gechev et al. 2006; Kwak et al. 2006).

Nitric oxide is ubiquitous and plays a key role in a broad spectrum of pathophysiological and developmental processes (Lamattina et al. 2003; Mur et al. 2006; Hong et al. 2008; Neill et al. 2008). In plants, NO interacts with other signaling elements such as lipids, cGMP, ion channels, ROS and Ca^{2+} (Desikan et al. 2004; Shapiro 2005; Courtois et al. 2008). Exogenous addition of NO to both monocot and dicotyledonous epidermal strips induced stomatal closure (García-Mata and Lamattina 2001). Several recent reports emphasize the key function of NO in the fine-tuned regulation of stomatal closure (García-Mata and Lamattina 2002; Bright et al. 2006; Neill et al. 2008).

Elicitors are chemical or biological molecules from various sources that mimic pathogen attack and induce marked physiological changes of the target living organism (Zhao et al. 2005). Cell wall fragments of plants or pathogens can serve as elicitors in many plant species. Exposure of plants to either elicitors or pathogens trigger an array of defense reactions, including the accumulation of defensive secondary metabolites such as phytoalexins (Zhao et al. 2005). The early responses of plant tissues to elicitors are typical of signal transduction: from elicitor perception to defense reactions. For example, elevation in cytosolic Ca^{2+} (Mithöfer et al. 1999; Blume et al. 2000) and production of ROS or NO are common in plant tissues exposed to elicitors during plant pathogen interactions (García-Brugger et al. 2006; Mur et al. 2006).

Unlike vast literature on the responses of guard cells to hormones such as ABA, reports on the effects of elicitors are quite limited. Chitosan is a deacylated derivative of chitin, a major component of fungal cell wall. Although the effects of chitosan on plant tissues has attracted attention (Amborabe et al. 2008), studies on stomata are very few. Lee et al. (1999) reported that two elicitors, namely chitosan and oligogalacturonic acid induced an increase in calcium and ROS production in guard cells of tomato, while Klüsener et al. (2002), observed marked changes in Ca^{2+} transients of guard cells by chitosan.

The present work is an attempt to investigate whether the key signaling components in guard cells can respond to elicitors. The effects of chitosan (a non-species specific elicitor) on stomatal movements were examined in *Pisum sativum* epidermal strips, in comparison to the effects of ABA. The primary focus was on the pattern and relationship of NO-production and stomatal closure induced by chitosan. Experiments were therefore carried out to monitor the NO and ROS levels in guard cells during stomatal closure on exposure to chitosan. Further, the levels of NO and ROS were modulated and the consequence on chitosan induced stomatal closure was assessed.

Materials and methods

Plant material

Plants of *Pisum sativum* (cv. Arkel) were raised from seeds (Pocha seeds Co. Pvt. Ltd, Pune, India). The plants were grown in a green house (average day/night temperature of about 30/20°C and photoperiod of 12 h) and were watered daily. The second to fourth completely unfolded leaves were harvested from 2 to 3 week-old plants, for the experiments. Medium molecular weight chitosan was from Sigma (St Louis, MO, USA), 4,5 diaminofluorescein diacetate (DAF-2DA) and diphenyleneiodonium chloride (DPI) were from Molecular Probes (Eugene, OR, USA), catalase from Roche Chemicals (Basel, Switzerland), and all other chemicals were from Sigma.

Stomatal closure in epidermal strips

The abaxial (lower) epidermis was peeled off from the leaves and cut into strips of ca. 0.16 cm^2. The epidermal strips were transferred to 3-cm diameter Petri dishes, containing 3 ml of 10 mM Mes-KOH pH 7.0 and 50 mM KCl. The epidermal strips were exposed for 3 h to white light (250 µmol m^{-2} s^{-1}), provided by a bank of tungsten lamps and filtered through water jacket. The photon flux was measured with a Li-Cor quantum sensor (Li-Cor Instruments Ltd, Lincoln, NE, USA). The temperature was maintained at 25 ± 1°C. When used, the test compounds (inhibitors or scavengers) were added after the 3 h light period, followed

by chitosan after 10 min. Incubation of epidermal strips was then continued for another 3 h in same light, before measuring the stomatal apertures.

The width of stomatal aperture was measured under a research microscope with the help of a precalibrated ocular micrometer. Ten apertures were monitored at random in each of three different epidermal strips, from each treatment. The experiments were repeated on three different days, making each measurement of stomatal aperture an average of at least 90 stomata.

Monitoring NO or ROS

Nitric oxide production in guard cells of *Pisum sativum* was examined by using DAF-2DA, as described by Neill et al. (2002), with minor modifications. The changes in ROS were monitored with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), based on the procedure of Murata et al. (2001). Further details are described in our earlier articles (Kolla and Raghavendra 2007; Kolla et al. 2007).

The epidermal strips were mounted on a microscope slide with silicone adhesive (Telesis V, Premiere Products Inc., Pacoima, CA, USA). Stomata were allowed to open by incubating the epidermal strips under 250 μmol m^{-2} s^{-1} white light for 3 h, in a medium of 50 mM KCl, 10 mM Mes-KOH, pH 7.0. The epidermal strips were then loaded with the required dye: 40 μM DAF-2DA (20 min) or 30 μM H_2DCFDA (20 min), at 25 ± 1°C. The strips were rinsed quickly with three changes of incubation buffer to wash off the excessive fluorophore. The dye-loaded strips were kept in the incubation medium, the test compounds were added, as indicated, followed by chitosan/ABA after 10 min. The strips were then monitored under confocal microscope (Leica, TCS-SP-2, AOBS 4 channel UV and visible, Heidelberg, Germany) to observe the fluorescence of DAF-2DA or H_2DCFDA (excitation 488 nm, emission 510–530 nm).

In experiments involving time-course monitoring of signaling components in guard cells, the epidermal strips were examined under an inverted fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a monochrome high-resolution digital cooled CD camera (CoolSNAP *cf*, Photometrics, Roper Scientific) that enabled to capture the images with DAF-2DA or H_2DCFDA fluorescence (filter: excitation 465–495, emission 515–555). The captured images and the relative fluorescence emission of guard cells were analyzed by using NIH Image for Windows (Murata et al. 2001).

Solvent effects, replications and statistical analysis

The control sets were added with an equal volume of solvents used for their stocks. Ethanol was the solvent used for ABA, dimethylsulfoxide for DAF-2DA or H_2DCFDA and milli-Q water for others. Stocks of chitosan were made in 0.1 M glacial acetic acid and dilutions in the buffered incubation medium.

The data presented are the average values (±SE) of results from at least three experiments conducted on different days. For comparisons and statistical analysis, one way ANOVA was used. Mean values denoted with different letters differed significantly at $P < 0.05$.

Results

Dose dependent stomatal closure by chitosan

Chitosan, a fungal elicitor, induced a dose-dependent stomatal closure, as is the case with ABA, a plant hormone. Chitosan caused about 35% decrease in stomatal closure at a concentration of 5 μg ml^{-1} (Fig. 1a), while >40% stomatal closure occurred in presence of 10 μM ABA (Fig. 1b). Maximum stomatal closure occurred at 20 μg ml^{-1} chitosan or 20 μM ABA.

Elevation of NO and ROS levels in guard cells and stomatal closure induced by chitosan

The levels of NO and ROS in guard cells were monitored by cell permeable fluorophores, DAF-2DA and H_2DCFDA, respectively. Chitosan at 5 μg ml^{-1} induced a marked rise in production of NO and ROS in stomatal guard cells. The increase in NO-levels of guard cells was not evident at 5 min (Fig. 2b) and could be seen only at 20 min (Fig. 2c) after exposure to chitosan. In contrast, the increase in ROS was visible already by 5 min (Fig. 2g) and did not rise much thereafter (Fig. 2h).

A quantitative evaluation of fluorescence images demonstrated clearly the difference in the patterns of NO and ROS changes in guard cells on exposure to chitosan. The NO production in guard cells exhibited a lag period up to 10 min and reached a maximum by 20 min (Fig. 3a), whereas most of the increase in ROS occurred by 5 min (Fig. 3b). Stomata started to close after 30 min, in case of both chitosan and ABA (Fig. 4). Maximum closure occurred by about 2 h after exposure to chitosan or ABA.

Effect of modulators of NO and ROS on chitosan-induced stomatal closure

Modulators of NO as well as ROS affected the chitosan induced stomatal closure. cPTIO (2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide; NO scavenger) or sodium tungstate (inhibitor of NR) or L-NAME (*N*-nitro-L-Arg-methyl ester; NOS inhibitor) prevented the stomatal

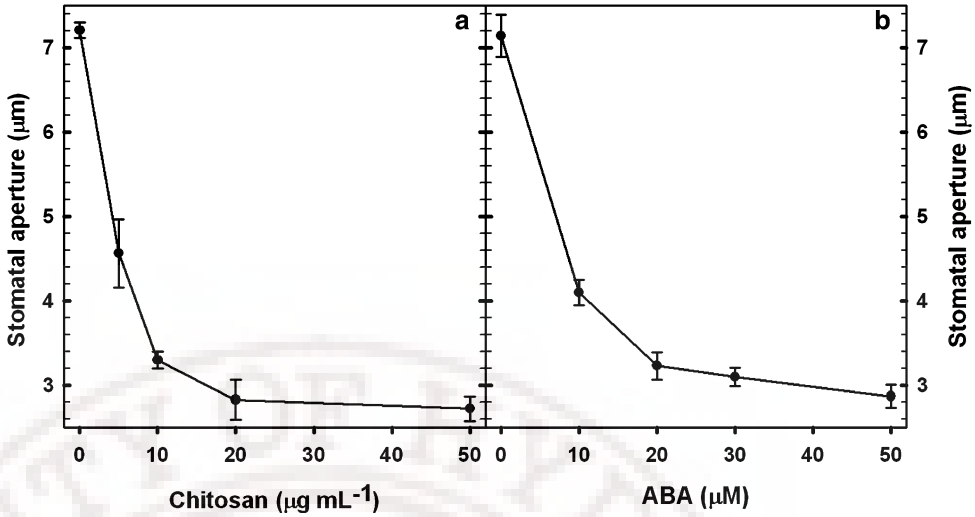


Fig. 1 Concentration dependent stomatal closure in epidermal strips of *Pisum sativum* by chitosan (**a**) or ABA (**b**). Results are the average ± SE of three to four independent experiments. Further details are given in "Materials and methods"

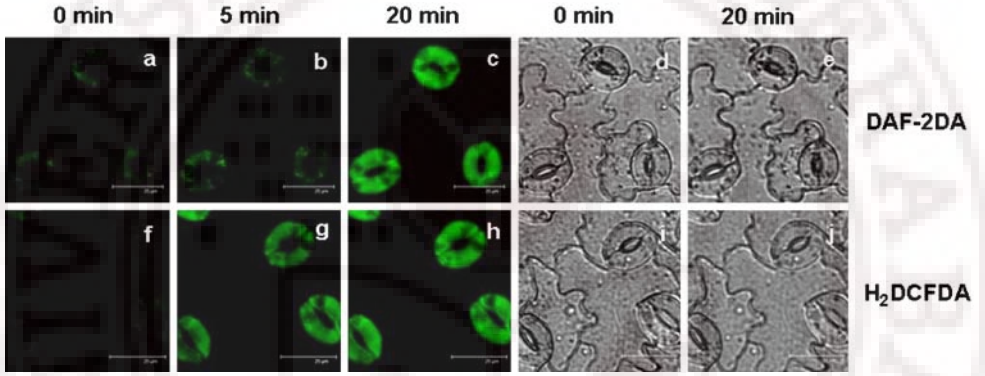


Fig. 2 Increase in the levels of NO or ROS in guard cells of *Pisum sativum* on exposure to chitosan, as indicated by the fluorescent probes. **a–c** Fluorescence images of guard cells loaded with 40 μM DAF-2DA reflecting the levels of NO. **f–h** Changes in ROS as indicated by 30 μM H_2DCFDA. **a**, **f** Images at the beginning of experiment. **b, g** Images at 5 min after treatment with 5 μg ml^{-1} chitosan. **c**, **h** Images at 20 min after treatment. Bright field images of stomata at 0 (**d**, **i**) and 20 min (**e**, **j**) after exposure to chitosan. Further details are given in "Materials and methods." *Bar* 25 μm

closure induced by chitosan (Table 1). These inhibitors alone did not have any direct effect on stomatal closure. Similarly, catalase (H_2O_2 scavenger) or diphenyleneiodonium chloride [DPI, a NAD(P)H oxidase inhibitor] also prevented the chitosan induced stomatal closure (Table 1).

Effects of NO, ROS and Ca^{2+} modulators on NO or ROS production

Different NO and ROS modulators as well as calcium chelators were applied to study their effects on NO and ROS levels in guard cells (Figs. 5, 6). cPTIO or sodium tungstate or L-NAME alone had no effect but restricted the rise in NO induced by chitosan (Fig. 5l–n). These compounds did not prevent the ROS production (Fig. 6l–n). In contrast, catalase or DPI prevented the NO (Fig. 5o, p) as well as ROS production (Fig. 6o, p) during chitosan induced stomatal closure. Calcium chelators, BAPTA-AM (chelator of internal calcium within the cell) or BAPTA (chelator of external calcium) prevented the chitosan induced stomatal closure (Table 1) but NO and ROS levels remained high (Figs. 5q, r, 6q, r).

Discussion

Rise and essentiality of NO during chitosan induced stomatal closure

Nitric oxide, ROS and calcium are essential signaling components during stomatal closure induced by not only ABA but also MJ and bicarbonate (MacRobbie 2000; Neill et al. 2002; Suhita et al. 2004; Kwak et al. 2006; Kolla et al. 2007). The present study highlights that stomatal closure by a fungal elicitor such as chitosan also is mediated by increase in levels of NO besides ROS. The importance of NO during chitosan induced stomatal closure was demonstrated by multiple observations: significant rise in NO

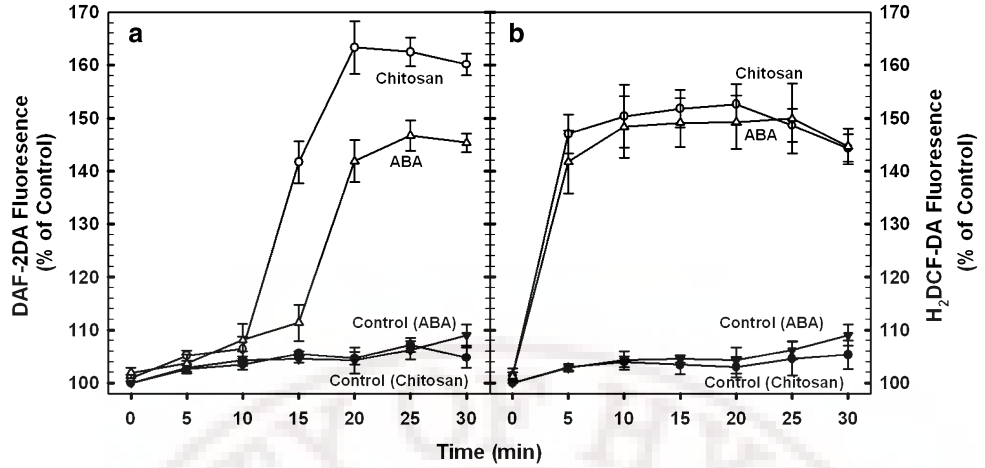
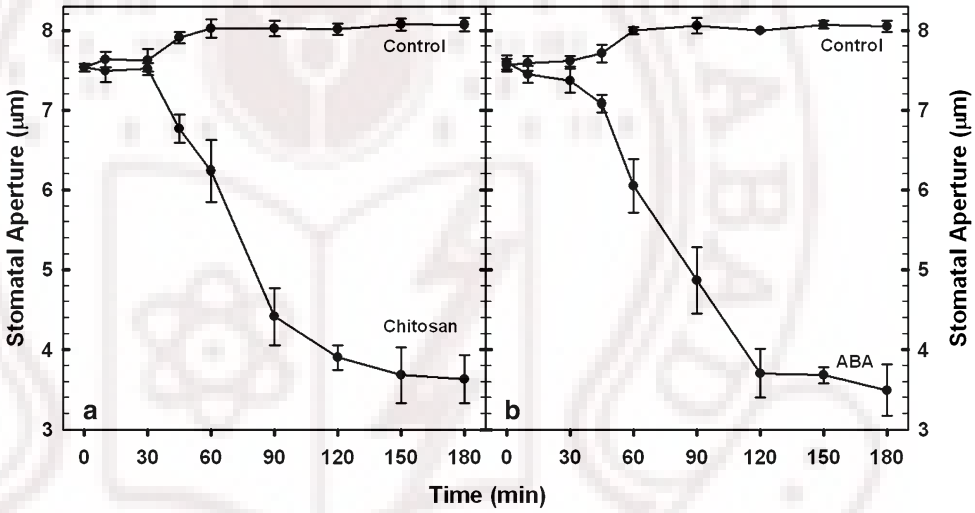


Fig. 3 Kinetics of increase in NO (**a**) or ROS (**b**) of guard cells in response to 5 μg ml^{-1} chitosan or 10 μM ABA. The epidermal strips were loaded with 40 μM DAF-2DA to monitor NO or 30 μM H_2DCFDA for ROS and incubated with or without chitosan. The levels of NO reached maximum at 20 min and those of ROS by about 5 min. The extent of NO or ROS production in the guard cells without chitosan is taken as 100%. Results are the average ± SE from at least three to four independent experiments. Further details are given in "Materials and methods"

Fig. 4 Kinetics of stomatal closure by 5 μg ml^{-1} chitosan (**a**) or 10 μM ABA (**b**) in abaxial epidermis of *Pisum sativum*. The patterns may be compared to those of NO and ROS in Fig. 3. Results are the average ± SE of three to four independent experiments. Further details are given in "Materials and methods"



levels in guard cells (Figs. 2, 5), prevention of stomatal closure along with a decrease in NO levels by cPTIO or sodium tungstate or L-NAME (Fig. 5l–n; Table 1) and initiation of stomatal closure after the rise in NO/ROS (Figs. 3, 4). Thus, the effect of chitosan on guard cells were quite similar to that of ABA (García-Mata and Lamattina 2002; Bright et al. 2006). Our results endorse the opinion that common signaling components such as NO, ROS or Ca^{2+}, participate during transduction of diverse signals emulating from biotic or abiotic stress, including UV-B or ozone stress (Holley et al. 2003; Fujita et al. 2006).

Chitosan raised the levels of ROS and calcium in guard cells during stomatal closure in tomato and *Commelina* (Lee et al. 1999). The marked enhancement in the levels of both NO and ROS by chitosan even at 5 μg ml^{-1} (Fig. 2), emphasized that chitosan mediated stomatal closure required both NO and ROS. The participation of both ROS and NO have earlier been observed in processes such as stomatal movement and antiviral resistance (Lee et al. 1999; Zhao et al. 2007).

Kinetics of fluorescence changes: ROS precedes NO

The release of NO in cells can be monitored by real time imaging with epifluorescence microscopy, with the help of DAF-2DA (Kojima et al. 1998; Foissner et al. 2000). Kinetic studies using DAF-2DA revealed that chitosan induced increase in NO reached maximum by 20 min (Fig. 3a), compared to 5 min required for ROS elevation (Fig. 3b). This demonstrated that NO production occurred much after the rise in ROS during chitosan induced stomatal closure in guard cells of *Pisum sativum*. The importance of ROS for the rise in NO levels of guard cells was further confirmed by the ability of catalase or DPI to restrict the

Table 1 The effect of NO or ROS modulators on chitosan induced stomatal closure and the production of NO or ROS in guard cells of *Pisum sativum*

Modulator	No chitosan			5 μg ml^{-1} Chitosan		
	Stomatal aperture (μm)	DAF-2DA fluorescence (% control)	H_2DCFDA fluorescence (% control)	Stomatal aperture (μm)	DAF-2DA fluorescence (% control)	H_2DCFDA fluorescence (% control)
None (control)	$7.5^{a} \pm 0.1$	$100^{b} \pm 0$	$100^{c} \pm 0$	$3.3^{ad} \pm 0.1$	$160^{be} \pm 5$	$155^{cf} \pm 6$
0.2 mM cPTIO	$7.6^{a} \pm 0.1$	$95^{b} \pm 4$	$107^{c} \pm 4$	$7.3^{a} \pm 0.1$	$97^{b} \pm 4$	$143^{cf} \pm 7$
0.1 mM Sodium tungstate	$7.7^{a} \pm 0.1$	$103^{b} \pm 1$	$107^{c} \pm 2$	$6.3^{a} \pm 0.5$	$111^{b} \pm 2$	$154^{cf} \pm 3$
0.1 mM L-NAME	$6.9^{a} \pm 0.1$	$97^{b} \pm 5$	$107^{c} \pm 4$	$6.8^{a} \pm 0.2$	$116^{b} \pm 5$	$147^{cf} \pm 7$
100 U ml^{-1} Catalase	$8.0^{a} \pm 0.1$	$99^{b} \pm 6$	$99^{c} \pm 3$	$7.5^{a} \pm 0.1$	$109^{b} \pm 6$	$109^{c} \pm 2$
5 μM DPI	$7.4^{a} \pm 0.1$	$97^{b} \pm 3$	$97^{c} \pm 4$	$7.3^{a} \pm 0.1$	$109^{b} \pm 2$	$108^{c} \pm 2$
10 μM BAPTA-AM	$7.8^{a} \pm 0.1$	$102^{b} \pm 3$	$100^{c} \pm 2$	$6.2^{a} \pm 0.8$	$140^{be} \pm 4$	$138^{cf} \pm 2$
20 μM BAPTA	$7.5^{a} \pm 0.2$	$106^{b} \pm 2$	$102^{c} \pm 2$	$7.0^{a} \pm 0.1$	$133^{be} \pm 3$	$138^{cf} \pm 2$

The levels of NO and ROS are monitored by the fluorescence of DAF-2DA and H_2DCFDA, respectively. The values are represented as % of control (no chitosan and no modulator). Results are the average ± SE of three to four independent experiments. For comparisons between different treatments, one way ANOVA was used. Mean values denoted with different letters differed significantly at $P < 0.05$ according to one-way ANOVA

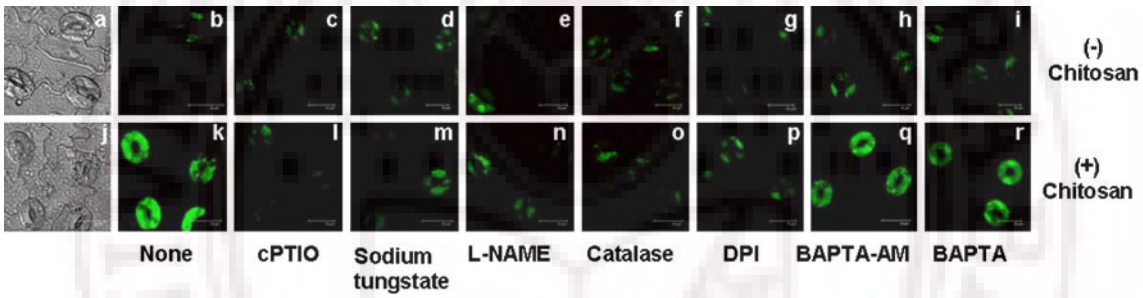


Fig. 5 The effect of NO and ROS modulators on the extent of NO production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe DAF-2DA. **b–i** Guard cells which are not exposed to chitosan. **k–r** Guard cells exposed to 5 μg ml^{-1} chitosan. **b**, **k** No modulators added. Treated with 0.2 mM cPTIO (**c**, **l**), 0.1 mM sodium tungstate (**d**, **m**), 0.1 mM L-NAME (**e**, **n**), 100 U ml^{-1} catalase (**f**, **o**), 5 μM DPI (**g**, **p**), 10 μM BAPTA-AM (**h**, **q**) and 20 μM BAPTA (**i**, **r**). **a**, **j** Bright field images of stomata without (control) or with chitosan, respectively. Images were taken 20 min after the addition of chitosan. Further details are given in "Materials and methods." *Bar* 25 μm

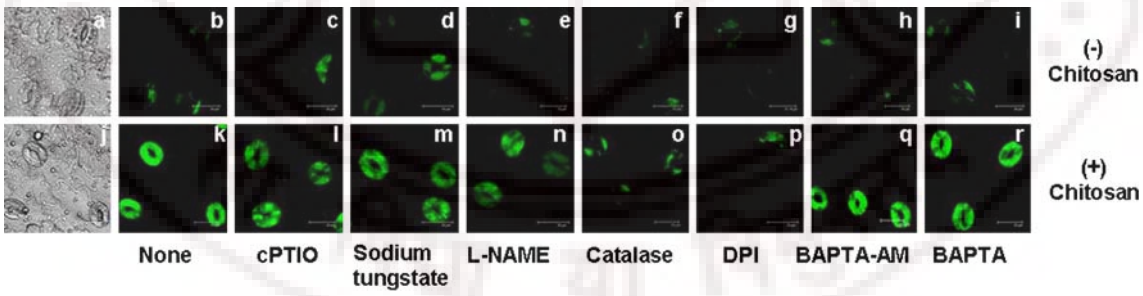


Fig. 6 The effect of NO/ROS modulators on the extent of ROS production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe H_2DCFDA. **b–i** Guard cells which are not exposed to chitosan. **k–r** Guard cells exposed to 5 μg ml^{-1} chitosan. **b**, **k** No modulators added. Treated with 0.2 mM cPTIO (**c**, **l**), 0.1 mM sodium tungstate (**d**, **m**), 0.1 mM L-NAME (**e**, **n**), 100 U ml^{-1} catalase (**f**, **o**), 5 μM DPI (**g**, **p**), 10 μM BAPTA-AM (**h**, **q**) and 20 μM BAPTA (**i**, **r**). **a**, **j** Bright field images of stomata without (control) or with chitosan, respectively. Images were taken 20 min after the addition of chitosan. Further details are given in "Materials and methods." *Bar* 25 μm

ROS as well as NO production in guard cells (Figs. 5o, p, 6o, p) and the inability of NO modulators to restrict the ROS levels (Fig. 6l–n; Table 1), but NO (Fig. 5l–n; Table 1). H_2O_2 production was required for ABA-induced NO generation in guard cells of both *V. faba* and *Arabidopsis* (Dong et al. 2005; Bright et al. 2006). Similar interactions of NO and ROS were observed during UV-B effects on stomata of broad bean (He et al. 2005). It would be interesting to study further the mechanism of ROS induced production of NO, during chitosan effects.

Sources and interactions of NO and ROS

García-Mata and Lamattina (2007) suggested that nitric oxide synthase (NOS) may mediate the production of NO during inhibition of stomatal opening. On the other hand, Desikan et al. (2002) suggested that nitrate reductase (NR) was involved in NO production induced by ABA, based on their studies on the double mutant of *Arabidopsis nia1, nia2*, deficient in NR. The prevention of chitosan-induced stomatal closure as well as the rise in NO of guard cells by not only sodium tungstate but also L-NAME (Table 1) indicated that both NR and NOS-like activity could participate during chitosan induced NO production.

The source of NO in plants is under continuous debate. The activity and biological function of AtNOS1 in *Arabidopsis* was questioned (Zemojtel et al. 2006). So far, there is no strong evidence to indicate the occurrence of an animal like NOS in plants. While the role of NR in mediating the rise in NO levels is possible, there could be other sources of NO (García-Mata and Lamattina 2003; del Río et al. 2004).

Although several investigators used DPI as an inhibitor of NAD(P)H oxidase (Murata et al. 2001; Kwak et al. 2006; Beffagna and Lutzu 2007; Zhang et al. 2007), being a flavoprotein inhibitor, DPI may also affect NOS (Moulton et al. 2000). However, the prevention by DPI of not only stomatal closure (Table 1) but also the ROS (Fig. 6p) production is a strong evidence in favor of the importance of NAD(P)H oxidase. Such importance of NAD(P)H oxidase during chitosan induced stomatal closure is quite similar to the case of ABA signaling (Murata et al. 2001). Further experiments are required to confirm the importance of NAD(P)H oxidase and to assess alternative sources for raising the ROS levels in guard cells.

Role of calcium in stomatal closure by chitosan

Calcium is an important modulator of stomatal movements in guard cells (Mansfield et al. 1990; Assmann 1993). Externally applied H_2O_2 induced stomatal closure in *C. communis* by increasing the cytosolic free Ca^{2+} in guard cells. Elevation of NO also led to a rise in the cytosolic Ca^{2+} (McAinsh et al. 1996; Pei et al. 2000; García-Mata and Lamattina 2007). The marked prevention of chitosan induced stomatal closure by BAPTA-AM or BAPTA (Table 1) suggested that the action of chitosan required Ca^{2+}. Since both BAPTA and BAPTA-AM were effective, the external calcium appeared to be important.

Efficacy of BAPTA-AM or BAPTA in preventing the stomatal closure, despite the high levels of NO/ROS in guard cells (Table 1), demonstrates that calcium is required for stomatal closure, irrespective of the rise in NO/ROS. It is possible that Ca^{2+} participates at downstream of NO and ROS production or acts independent of NO and ROS. Action of Ca^{2+} at downstream of NO or ROS was earlier reported during stomatal closure by ABA or MJ or high CO_2 (Suhita et al. 2004; Kolla et al. 2007) and chitosan induced burst of Ca^{2+} transients in soybean cells (Mithöfer et al. 1999). The relationship between the NO production and calcium in guard cells during chitosan induced stomatal closure needs further examination.

Possible limitations of present work

Doubts have been expressed about the specificity of DAF-2DA to detect NO (Planchet and Kaiser 2006). However, with the use of proper controls and scavengers of NO or ROS during these experiments (Table 1; Figs. 5, 6), we are confident that the monitored fluorescence is related to either NO or ROS, as intended. Similarly, one may argue that catalase may not enter the guard cells, but the efficacy of catalase to decrease ROS (Fig. 6) and sustain stomatal opening (Table 1) was consistent and significant. External catalase was used earlier to demonstrate the importance of ROS in plant tissues (Beffagna and Lutzu 2007; Zhang et al. 2007) and even guard cells (Lee et al. 1999; Zhang et al. 2001). Yet these limitations would not affect the broad conclusions drawn in the present work, namely increase in NO-levels occurred after that of ROS and the major effect of calcium was downstream of NO and ROS, during chitosan-induced stomatal closure.

Concluding remarks

The present work demonstrates that NO is an important secondary messenger, besides ROS and calcium during chitosan induced stomatal closure. Time course experiments with fluorescent probes showed that NO-production occurred after that of ROS. The ability of catalase or DPI to restrict the production of ROS as well as NO, and the inability of NO-modulators to prevent the rise in ROS levels but NO in guard cells, indicated that ROS production was necessary for NO production. The ability of BAPTA-AM and BAPTA to prevent the chitosan-induced stomatal closure, despite the high rise in NO/ROS of guard cells by chitosan, confirmed that calcium is required for closure. Calcium may act either downstream of NO and ROS or independent of NO/ROS. Further studies are warranted to understand the mechanism of modulation by ROS of NO production and to establish the interactions, if any, of NO with ROS.

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Article Addendum

Cytosolic alkalinization is a common and early messenger preceding the production of ROS and NO during stomatal closure by variable signals, including abscisic acid, methyl jasmonate and chitosan

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Key words: abscisic acid, methyl jasmonate, chitosan, cytosolic pH, reactive oxygen species, H_2O_2, nitric oxide, cytosolic calcium

Stomata are unique that they sense and respond to several internal and external stimuli, by modulating signaling components in guard cells. The levels of reactive oxygen species (ROS), nitric oxide (NO) and cytosolic calcium (Ca^{2+}) increase significantly during stomatal closure by not only plant hormones [such as abscisic acid (ABA) or methyl jasmonate (MJ)] but also elicitors (such as chitosan). We observed that cytosolic alkalinization preceded the production of ROS as well as NO during ABA induced stomatal closure. We therefore propose that besides ROS and NO, the cytosolic pH is an important secondary messenger during stomatal closure by ABA or MJ. We also noticed that there is either a cross talk or feedback regulation by cytosolic Ca^{2+} and ROS (mostly H_2O_2). Further experiments on the interactions between cytosolic pH, ROS, NO and Ca^{2+} would yield interesting results.

Introduction

Dynamic regulation of stomatal aperture in leaves is essential for optimizing the balance between transpirational water loss and CO_2 entry into intracellular spaces required for photosynthesis. Such balance is achieved by the ability of two guard cells, which flank stomata, to sense and integrate multiple internal and external stimuli.[1,2] Stomatal opening is promoted by light, low CO_2, fusicoccin (FC) and hormones including indoleacetic acid (IAA) and cytokinins. In contrast, stomatal closure is induced by high CO_2, darkness, low humidity and hormones such as abscisic acid (ABA) or methyl jasmonate (MJ). Among the many factors that induce stomatal closure, the effects of ABA received maximum.[1-3] Several of the secondary messengers are common during the transduction of these signals, notably cytosolic free Ca^{2+}, reactive oxygen species (ROS), nitric oxide (NO) and G-proteins, which have been extensively studied. Besides the above, ABA modulates several other signaling components in guard cells, such as cytosolic pH, protein kinases, protein phosphatases, phospholipases and phosphatidylinositol kinases during stomatal closure.[4-9]

ROS and NO act as secondary messengers in not only guard cells but also other plant tissues, while mediating developmental and physiological processes such as programmed cell death, root development, hypersensitive responses and adaptation to stress conditions.[9-12] In guard cells of several species (Arabidopsis, Vicia, tomato, Commelina and pea) production of ROS and NO occurs in response to ABA, MJ, bicarbonate or even chitosan/oligogalacturonic acid.[6,7,12-16] The involvement of ROS and NO during stomatal closure was further demonstrated by additional evidences: modulation of ROS or NO levels within cells by either scavenging these molecules or inhibition of source enzymes and finally real time monitoring of ROS/NO by using fluorescent dyes.

Calcium (Ca^{2+}) is another ubiquitous intracellular second messenger, involved in many signal transduction pathways in both plants and animals. The cytosolic Ca^{2+} concentration is modulated in response to many physiological stimuli and is delicately balanced by 'Ca^{2+} stores', like vacuoles, endoplasmic reticulum, mitochondria, nucleus, chloroplast and cell wall.[17] For example, when proteinaceous elicitors were used as signals, the Ca^{2+} patterns were clearly different in the cytosol and the nucleus.[18] Upon treatment with cryptogein, a polypeptidic elicitor, a substantial but transient increase in cytosolic Ca^{2+} took place, peaking 5 min post-treatment, and was followed by a sustained cytosolic Ca^{2+} elevation which lasted for at least 2 h.[19]

The pH inside a cell tends to be quite stable and may vary only by a small fraction of a unit, but even with such small change, pH can mediate and exert strong physiological and biochemical responses. For example, application of ABA to plant cells raises the pH of cytosol by approximately 0.2–0.4 units within minutes. Cytoplasmic alkalinization is a major step in the ABA-triggered

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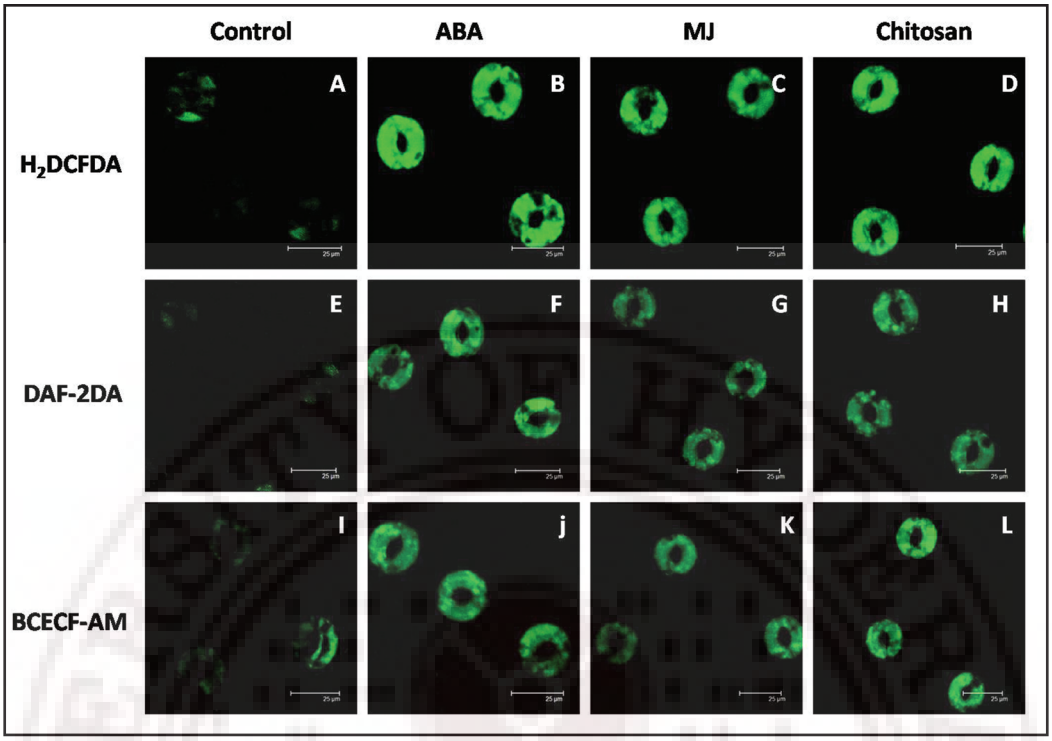

Figure 1. Confocal images showing elevation of ROS (indicated by the fluorescence of H_2DCFDA), NO (DAF-2DA) or cytosolic pH (BCECF-AM) by 10 µM ABA or 20 µM MJ or 5 µg ml^{-1} chitosan in guard cells of *Pisum sativum*. The (B–D, F–H and J–L) are the epidermal strips treated with 10 µl ethanol containing 10 µM ABA, 20 µM MJ and 5 µg ml^{-1} chitosan, respectively. (A, E and I) are the epidermal strips treated with 10 µl ethanol, as controls. (A–D) represents the patterns of ROS levels, and the (E–H and I–L) represent the images of NO and pH, respectively. Confocal fluorescence images were taken at 18 min after addition of individual effectors. Experimental details are further described.[14,26] Bar = 25 µm.

signal cascade in guard cells leading to H^+ efflux and stomatal closure.[4,20] Such intracellular pH alterations play an important role in a variety of processes including, plant defense, coleoptile or root hair growth, nodulation, elicitation[21-25] and response to hormones such as ABA and MJ.[6,26]

We have been studying the role of not only ROS or NO, but also cytosolic pH as signaling components. We characterized the temporal sequence of changes in the level of pH, ROS and NO in guard cells on exposure to ABA or MJ. Our experiments were based on three approaches: (i) Bioassay of stomatal closure by ABA or MJ in presence of pharmacological compounds capable of modulating the different secondary messengers; (ii) Modulation of the secondary messengers by promoters, scavengers; and finally (iii) Direct monitoring of ROS, NO or cytosolic pH by fluorescent dyes. In some of the experiments mutants deficient in NADPH oxidase or insensitive to ABA or MJ were also used.

While examining the pattern and mechanisms of stomatal closure by plant hormones (ABA, MJ), a fungal elicitor (chitosan) and bicarbonate (simulating high CO_2),[6,7,13,14,27] we found that ROS or NO are important signaling components during stomatal closure by these different factors. Further cytoplasmic alkalinization is an early and common component during stomatal closure induced by not only ABA or MJ but also chitosan (Fig. 1).

Change in pH of Guard Cells on Exposure to ABA or MJ is an Early Event

Changes in pH of guard cells have been observed on exposure to hormones such as ABA/MJ or fungal toxin such as FC or even an elicitor such as chitosan. In epidermal strips of *Pisum sativum* or an orchid, *Paphiopedilum tonsum*, application of ABA or weak alkalinizing agents, such as benzylamine or methylamine, enhanced the cytosolic pH and promoted stomatal closure.[8,26] FC, IAA or a weak acid butyrate, decreased the cytosolic pH and promoted stomatal opening.[4,26] Thus, stomatal opening was accompanied by decrease in cytosolic pH, whereas stomatal closure was preceded by cytosolic alkalinization in the guard cells.

Irving group reported that acidification of guard cell cytosol by kinetin, IAA or FC preceded stomatal opening, whereas alkalinization of guard cell cytosol occurred prior to stomatal closure in response to ABA. These results strongly suggested that cytosolic pH was a key factor in the regulation of guard cell movement. However there is an ambiguity, whether enhanced cytosolic pH or cytosolic alkalinization leads to production of H_2O_2. Zhang group suggested that application of H_2O_2 to the guard cells lead to increase in cytosolic pH, which further decreased the stomatal aperture. Our results confirmed that stomatal closure was preceded by the modulation of pH in guard cells.[6,26] Direct real time monitoring of ROS, NO or pH, by fluorescence probes revealed that the cytosolic alkalinization occurs much before the rise in ROS or

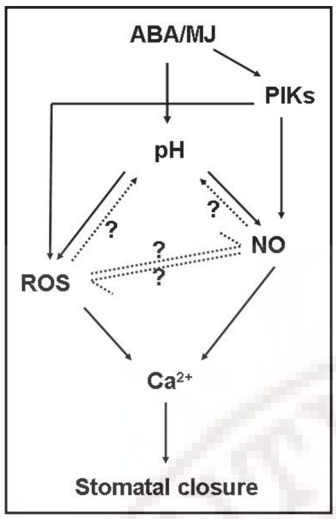

Figure 2. Schematic representation of the signaling cascade, leading to the stomatal closure by ABA or MJ. The rise in cytosolic pH leads to the elevation of the ROS as well as in NO in guard cells. Both ROS and NO lead to rise in cytosolic Ca^{2+} and subsequent stomatal closure. A feedback regulation by ROS and NO on pH appears to operate. These interactions between pH, ROS and NO need further detailed examination. The sequence of changes for which the evidences are either ambiguous or lacking, are indicated by dotted arrows, while the well-established events are represented by solid arrows.

NO during stomatal closure by ABA or MJ. The pH rise appears to be necessary and occurring downstream of the ROS production during ABA-induced stomatal closure. The modulation of cytosolic pH changed the patterns of NO production and stomatal closure but not the ROS production. Similarly, the NO modulators such as cPTIO (NO scavenger) and L-NAME (inhibitor of NO-production) did not affect the cytosolic pH changes,[26] we are of the opinion that cytosolic pH change is necessary and occurs upstream of the NO elevation in guard cells.

Studies on temporal kinetics of changes in pH, ROS and NO can help in identifying the exact sequence of events. In *Paphiopedilum tonsum*, the modulation of pH by IAA, FC or kinetin (pH decrease) or ABA (pH increase) required 5 to 10 min.[4] However, we noticed that it took 18 minutes to attain maximum cytosolic pH by application of 10 μM ABA. As these reports are quite limited in number, further experiments would be necessary to examine the kinetics of pH changes in guard cells during stomatal closure as well as stomatal opening.

Consequences of pH Modulation on Signaling Components and Stomatal Closure

The weak acids cross the membrane in the uncharged form and dissociate in the cytosol, thereby decreasing the pH.[25] Weak acids such as butyrate and propionic acid can acidify plant cells and cause significant changes in pH.[28] Such acidification can hyperpolarize the membrane. However, lowering cytosolic pH was associated with an increase in cytosolic Ca^{2+} and inactivation of inward-rectifying K^+ channels.[20,25] We showed that acidification of guard cells by butyrate restricted the stomatal closure by ABA and alkalinization by methylamine enhanced the stomatal closure.[26]

Depolarization could be achieved by Ca^{2+} influx facilitated by activated Ca^{2+} channels[2] and/or by cytosolic alkalinization, which would reduce the activity of the proton pump. Anionic channels conducting chloride and malate are activated by depolarization, elevated cytosolic Ca^{2+} and this would lead to loss of anions and further depolarization.[2,29] Thus, increases in both cytosolic pH and Ca^{2+} would have a synergistic effect on the depolarization of plasma membrane in guard cells.

Procaine, a weak base, has also been used to alkalinize plant cells.[25,30] Procaine increases rapidly cytosolic pH by 0.1 to 0.4 units within 5 min. However, no increase in cytosolic Ca^{2+} was observed in guard or epidermal cells in response to procaine.[4] On the contrary, a slight decrease in cytosolic Ca^{2+} of *Sinapis alba* root hairs was seen on exposure to procaine. Some of these anomalies have to be reexamined, so as to establish, if the pH rise in guard cells is a causal factor or an associated event during stomatal opening/closure.

Intriguing Effects of Ca^{2+}: Possible Dual Role and Increase during Even Opening

Rise in cytosolic free Ca^{2+} is a common event during stomatal closure caused by ABA or H_2O_2 and even fungal elicitors such as chitosan/oligogalacturonic acid. Such action of Ca^{2+} upstream of changes in ROS or NO levels was observed by several workers.[15,26] The rise in cytosolic pH could be a trigger for the rise in cytoplasmic Ca^{2+}, but this needs experimental validation. The increase in cytosolic Ca^{2+} can be caused by the simulation of Ca^{2+} influx across the plasma membrane and/or release from internal sources, which include endoplasmic reticulum, vacuole and mitochondria. Cytosolic Ca^{2+} signatures have been postulated to act as the second messengers in both stomatal opening and closure in response to biotic and abiotic stress conditions.[17]

However it is yet to be ascertained if change in pH can modulate internal Ca^{2+} or Ca^{2+} in turn affects the cytosolic pH. Also, the changes in cytosolic Ca^{2+} of guard cell protoplasts after ABA treatment were quite variable.[3,17] Since stomatal closure occurred, despite the ambiguous, observations on Ca^{2+} changes, it was suggested that a Ca^{2+}-independent mechanism might operate during the ABA-induced closure of stomata.[3] Further, factors which can induce stomatal opening, such as IAA, FC and kinetin also enhanced cytosolic Ca^{2+}.[2] Experiments need to be designed to establish clearly, if Ca^{2+} can play a dual role: upstream and downstream of ROS/NO production.

Future Perspective

Besides the direct influence of pH on ROS or NO levels, it is possible that these components exert interactive effects. Since the NO-molecule is quite active at an alkaline pH of 7.4,[31] NO can be expected to become very effective as the pH rises. The combination of ROS and NO result in peroxynitrite radicals, which can affect the cell.[32] Thus, the effects of ROS or NO may be enhanced at alkaline pH, besides the interactions of ROS or NO between them. It is not clear that, if the change in cytosolic pH is necessary for NO production or is an associated event during stomatal closure by different stimuli. A schematic representation of possible events

occurring during ABA induced stomatal closure as can be agreed at present is shown in Figure 2. The scheme can change with future work on the interactions of pH, ROS and NO, as indicated by broken lines. The interrelationships and interactions of cytosolic Ca^{2+}, ROS, cytosolic pH and NO need therefore a detailed examination (Fig. 2). Further interactions of these secondary messengers with G-proteins, phospholipases and phosphatidylinositol kinases are all of great interest.

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ABA PERCEPTION AND SIGNALLING

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Since the discovery of abscisic acid (ABA) as a leaf abscission- and seed dormancy-promoting sesquiterpenoid in the 1960s, our understanding of the action of the phytohormone ABA has come a long way. Recent breakthroughs in the field of ABA signalling now unfold a unique hormone perception mechanism and the core pathway in the control of ABA-dependent gene expression and ion channel regulation.

Responses of ABA

Higher plants are sessile organisms that have evolved a high plasticity for adaptation to environmental challenges. Pathogens and abiotic stress such as drought and salt stress severely impact plant performance and productivity. The phytohormone ABA serves as an endogenous messenger in biotic and abiotic stress responses of plants [1-6]. Drought and high salinity result in strong increases of plant ABA levels, accompanied by a major change in gene expression and in adaptive physiological responses [7-11]. How environmental cues are perceived and integrated into alterations of physiologically active ABA levels is still largely a conundrum. A limiting water supply leads to an immediate hydraulic signal in plants that triggers ABA biosynthesis over long distances [8] while high humidity activates an ABA catabolising P450 enzyme within minutes [12].

ABA is not only a stress signal but is also required to fine-tune growth and development under non-stress conditions. The physiological processes controlled include the regulation of growth, stomatal aperture and hydraulic conductivity as well as seed dormancy [13-15]. Stomatal closing is mediated by ABA-triggered changes of ion fluxes in guard cells [16-18]. Alteration of ABA sensitivity in a non-herbaceous plant revealed additional, less known functions [19]. ABA positively affected leaf size and bud dormancy of poplar, and negatively influenced the size of guard cells and internode length. Leaf size is regulated in concert with ethylene by a negative feedback of ABA on ethylene generation [20]. ABA also acts together with other phytohormones

such as bassinosteroids, gibberellic acid and auxin in regulating plant growth and development [20-23].

An overwhelming number of signalling components that affect ABA-dependent stomatal closing and seed germination have been identified by forward and reverse genetic approaches [1,24-26]. The crosstalk between different phytohormone signalling pathways has, however, frequently precluded a clear differentiation between primary and secondary ABA signalling components. The identification of a unique class of ABA receptors has now fundamentally changed this situation and laid the foundation for assembling the core signalling pathway.

Pyrabactin Resistance 1* and *Regulatory Component of ABA Receptor

High affinity ABA-binding proteins of Arabidopsis have recently been identified by two research groups [27,28]. Sean Cutler's group characterized the synthetic chemical pyrabactin as a selective ABA agonist and identified Arabidopsis mutants insensivitve to this growth regulator. His group cloned the *Pyrabactin Resistance 1 (PYR1)* locus and characterized PYR1 and several PYR1-related homologues of Arabidopsis (PYLs) as ABA-dependent inhibitors of Mg^{2+}- and Mn^{2+}-dependent serine/threonine phosphatases type 2C (PP2Cs). Prototypes of these PP2Cs are ABI1 and its close homologue ABI2, which globally repress ABA responses and which have emerged as a hub in the network of ABA signal transduction [29,30].

In a yeast-two-hybrid screen for regulators of ABI1 and ABI2, Ma *et al.* [28] identified the *Regulatory Component of ABA Receptor 1* (*RCAR1*), identical to PYL9 (Fig. 1A), as an ABI1- and ABI2-interacting protein. RCAR1 expression enhanced ABA-dependent gene expression several fold and antagonized the action of ABI1 and ABI2. RCAR1 emerged as a structural homologue of both potential phytohormone-

binding proteins Bet V 1 from birch, proposed to bind brassinosteroids [31], and a cytokinin-binding protein of mung bean [32]. While RCAR1 did not bind brassinosteroids or cytokinins, binding studies with (*S*)-ABA (Fig. 1B) yielded a dissociation constant of 0.7 µM for the physiologically active ABA by isothermal calorimetry. *In vitro* analysis of purified RCAR1 and ABI2 revealed a selective and rapid inhibition of the protein phosphatase activity by (*S*)-ABA with a dissociation constant of 0.06 µM ABA, much lower than the value for RCAR alone. The stereoisomers (*R*)-ABA and *trans*-ABA (Fig. 1B) were more than 1000-fold less active in mediating ABI1 and ABI2 inhibition.

Combinatorial interaction and PP2C regulation

The RCARs/PYR1/PYLs belong to the Bet V 1 superfamily of Arabidopsis and comprise a protein family with 14 members, which can be grouped into 3 subfamilies (Fig. 1A). Members of all three subclades regulate ABI1, ABI2, or HAB1 in dependence of ABA. Analysis of RCAR1, 3, 8, 11, and 12 [28,33-35] and of RCAR6, 9, and 10 revealed an ABA-dependent inactivation of ABI1, ABI2, and/or the homologue of ABI1 (HAB1). These and additional RCAR members physically interact with ABI1 [36]. The findings indicate that all RCAR family members are ABA-binding proteins and that RCAR proteins can interact and regulate the target PP2Cs in a combinatorial manner. There are approximately 80 PP2Cs in Arabidopsis [37] and six of the nine PP2Cs in clade A including ABI1, ABI2, HAB1, and HAB2, have been identified as negative regulators of ABA responses [38-44]. At this stage it is not clear whether all PP2Cs linked to ABA responses are regulated by RCARs, or whether all RCAR members can regulate the same PP2C. If both are true, more than 80 combinations (6 times 14) would be possible. These RCAR/PP2C complexes probably address different

downstream signalling components and allow for the adjustment of ABA-signalling to strongly variable ABA levels.

The transcript levels of different RCARs and PP2Cs vary throughout development and in response to environmental challenge [28,33-35]. Different expression patterns of individual RCARs and PP2Cs are expected to reduce the numbers of combinatorial interactions in plant cells. Expression and interaction analysis using multicolour tags [45] for different RCARs and PP2Cs might shed light on this issue. In general, transcript levels of RCARs are downregulated under stress conditions while the abundance of PP2C transcripts is increased [34]. A concomitant change in RCAR and PP2C protein levels would result in an ABA-desensitization of the plants under abiotic stress, thus providing a mechanism for adjustment of ABA-signalling to strongly increased ABA levels.

Crystal structures

X-ray diffractionation studies of PYR1 (RCAR11) in a complex with ABA [46,47] and trimeric complexes of ABA/ABI1/RCAR12 (PYL1) [48,49] as well as ABA/HAB1/RCAR13 (PYL2) [50] have elucidated the site of ABA-binding and the steric mode of inhibition of protein phosphatase activity. The RCAR provides a cavity in the center, encaged by seven ß-sheets and two α-helical domains, which is similarly found in Bet V1 and related proteins and which functions as a ligand binding site. RCAR proteins thus have an open ligand-binding pocket that is closed upon ABA binding by conformational change of two ß-sheets engulfing the ABA molecule (Fig. 1B), reminiscent of a gate/latch mechanism [50]. The ABA-induced conformational change facilitates the docking of RCAR to the catalytic site of the PP2C, thereby blocking substrate access to the phosphatase. A conserved tryptophan residue of the PP2C is involved in ABA binding by contacting ABA via a bound water molecule (Fig. 1B). The

occupation of the PP2C active site by RCAR in the trimeric receptor complex provides an explanation for the non-competitive inhibition of ABI1 and ABI2 mediated by ABA [28,34].

Receptors or coreceptors?

The question arises as to whether the ABA-binding RCARs are ABA receptors or co-receptors. The interaction between RCAR and PP2C generates the high affinity ABA binding site. The affinity of RCAR1 [28], RCAR3 [34], and RCAR8 [35] did not considerably differ, with K_ds for (*S*)-ABA of 0.7, 1.0, and 1.1 µM, respectively. By contrast, *(S)*-ABA binding to heteromeric receptor complexes revealed more than tenfold lower K_ds of 64 nM for ABI2/RCAR1 and 38 nM for a truncated HAB1/RCAR8 [28,35]. Similarly, RCAR3 revealed half-maximal inhibition of ABI1 and ABI2 in the range of 15 to 40 nanomolar [34]. Three observations are consistent with a co-receptor function of RCAR and PP2C. First, the heteromeric complex generates the high affinity binding site for ABA that is of relevance at physiological ABA levels. Secondly, the PP2C interacts with the RCAR-bound ABA molecule. Third, although the heteromeric receptor complex is also formed in the absence of ABA, the ligand promotes the assembly of or stabilises the holo-receptor, the functional heteromeric receptor complex. The latter conclusion is based on the ABA-stimulated protein interaction of HAB1 and some RCARs in yeast [33], by the stabilisation of PP2C inhibition in the presence of high ABA levels [28,34], and by co-immunoprecipitation studies [36]. Thus, experimental evidence clearly supports a co-receptor function for both RCARs/PYR1/PYLs and PP2Cs.

Other ABA-binding proteins

The identities of ABA receptors have until recently remained either elusive or contested. Reported ABA receptors include plastidic ABAR/CHLH/GUN5 [51,52], and

plasma membrane-localized GCR2 [53] and GTG1/GTG2 [54] (Table 1). Analysis of ABA-binding to these proteins employed radiolabelled assays, which are prone to artifacts [55,56]. Hence, validation of the results by a more robust ABA binding assay, such as ABA titration analysis by isothermal calorimetry, is required to clarify ABA-binding function. The identified components affect ABA responses and are thus likely to be involved in the network of hormonal responses. At this stage, however, it is unclear how the presumed ABA-binding proteins feed in into the molecular events governing the main ABA responses, i.e. regulation of germination, stomatal aperture and growth, all of which are controlled by RCAR/PYR1/PYL-PP2C complexes.

ABA signalling to ion channels

The discovery of RCAR/PP2Cs as ABA receptors has made a paradigm shift in our understanding of the molecular basis of ABA action and has paved the way to comprehend the main signalling events leading to ABA-responsive gene regulation and ion channel control. PP2C coreceptors interact with SNF1-related protein kinases OST1/SnRK2.6/SnRK2E, SnRK2.2/SnRK2D and SnRK2.3/SnRK2I [57-59]. These protein kinases, which act as positive ABA key regulators, are structurally highly related and belong to the superfamily of sucrose-nonfermenting kinases (SNF) originally identified in yeast.

Guard cells provide an attractive single cell system to study ABA responses [60]. The ABA signalling pathway controlling ion channels in stomata appears to be surprisingly short (Fig. 2A). OST1 acts as positive regulator of stomatal closure [61]. It activates the anion channel SLAC1 [62,63] and inhibits the kation channel KAT1 [64] by phosphorylation. Both channels are reciprocally regulated by the ABA signalling pathway and by Ca^{2+} [18]. The Ca^{2+}-dependent regulation is probably provided by another SLAC1–stimulating protein kinase, a Ca^{2+}-dependent protein kinase

(unpublished results), and the related kinases CPK3 and CPK6 [65]. The ABA co-receptors ABI1 and the related PP2CA inhibit OST1-dependent SLAC1 activation via physical interaction [62,63]. ABA-and RCAR-mediated inactivation of the PP2C allows SLAC1 activation. It is tempting to speculate that control of ABA-responsive ion channels is imposed by a preformed signalling complex consisting of an ABA receptor and an associated protein kinase. Such a model is consistent with the findings of plasma membrane-associated ABI1 [30,66], stable OST1-PP2C complex formation [67], and holo-receptor formation in the absence of ABA in the cytosol [28,33,36]. OST1 also targets a plasma membrane-localized NADPH oxidase that generates H_2O_2 [68]. H_2O_2 increases mediate stomatal closure [69], probably by catalytic inactivation of ABI1 and ABI2, which are very sensitive to H_2O_2 and oxidation [70,71]. Marked rises in H_2O_2 are induced in guard cells by exposure to methyl jasmonate, bicarbonate and elicitors, such as chitosan known to regulate stomatal aperture [72,73]. It is conceivable that the different signal pathways target PP2Cs by the common secondary messenger H_2O_2.

Stomatal closure is initiated by the depolarisation of guard cells, which is triggered by anion release through SLAC1 [17,74]. Subsequently, the initial depolarisation activates outward-rectifying potassium channels. The loss of osmotically relevant ions subsequently leads to water and turgor loss causing stomatal closing.

ABA pathway controlling gene transcription

Key transcriptional regulators of ABA-dependent gene expression are ABFs/AREBs (ABA-responsive Element Binding Factor/Protein), basic region/leucine zipper (bZIP)-type transcriptional regulators with ABI5 as a prototype [75,76]. OST1 and the related SnRK2.2/SnRK2D and SnRK2.3/SnRK2I directly target ABF/AREBs in the nucleus (Fig. 2B), and ABF2/AREB1 is phosphorylated in vitro by this class of ABA-activated

protein kinases [77-80]. SnRK activation is promoted by ABA-mediated inactivation of the PP2Cs, which directly negatively regulate the protein kinases. Phosphorylation of ABI5 leads to its activation while sumoylation antagonises ABI5 action [81]. The principle mode of SnRK and bZIP interaction has been pioneered by Walker-Simmons in wheat [82]. ABF1 and ABF4/AREB2 are also phosphorylated by Ca^{2+}-dependent protein kinases CPK4 and 11 [83]. Other transcriptional regulators also contribute to ABA-specific transcription. ABI3, belonging to the B3 transcriptional regulators, binds to ABI5 and enhances its action. In addition, ABI4, an AP2-type transcription factor, and a number of additional transcription factors including MYC/MYB-type regulators act as positive ABA response regulators [84]. Finally, the homeodomain leucine zipper AtHB6 interacts with ABI1 and serves as a transcription factor to suppress ABA responses [85].

PP2Cs functioning as key regulators of ABA responses target a number of additional cellular components involved in abiotic stress responses. The interacting proteins comprise members of the SnRK3 class [86], the glutathione peroxidase as part of the redox homeostatic system [87], and fibrillin precursor, which is imported into plastids as a photosystem II protective and lipid-binding protein [29].

Perspectives

The principle pathways from ABA perception to ABA-dependent gene regulation and ion channel control are now elucidated. However, the intricacies and the orchestration of the numerous transcription factors involved remain to be fully characterised. Beside SnRK2s as key regulators of ABA responses, a prominent function of Ca^{2+}-regulated protein kinases, CPKs and CIPKs together with their regulatory Ca^{2+}-binding CBLs, is emerging in regulating ion channels and targeting

other ABA signalling components.The role and source of cytosolic Ca^{2+} increases in ABA responses is not fully understood. The generation and function of NAD-derived cADPR as a second messenger in the ABA signal cascade still remains a conundrum [88]. Regulation of ABA signaling implicates the control of physiologically active ABA. How ABA biosynthesis, transport, storage, and turnover are regulated by environmental cues such as cold and drought is a major challenge, we need to understand. Furthermore, the molecular mechanisms of crosstalk between ABA and other phytohormone signalling pathways needs to be elucidated in the future. Although many questions are still open, the current advances in ABA signalling in Arabidopsis pave the way to address the molecular events underlying stress responses in other plants, with the prospect to improve the abiotic stress performance of crop plants.

Acknowledgments

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Figure Legends

Fig. 1: The ABA-binding RCAR/PYR1/PYL proteins. A) Phylogenetic tree of ABA-binding proteins from Arabidopsis. The proteins can be grouped into three subfamilies I, II, and, III highlighted in yellow, blue, and red, respectively. The RCAR and PYR1/PYL numbering is given as well as the gene numbers. B) ABA binding by the heteromeric RCAR12/ ABI1 complex based on the crystal structure provided by [48]. The ABI1 protein is highlighted by a yellow backbone indicating the peptide linkages. A short and long arrow marks the RCAR-bound ABA molecule and Trp300 of ABI1, respectively. A white circle highlights a manganese ion bound the active site of the PP2C. The RCAR protein plugs the active site of the PP2C thereby inactivating ABI1. The secondary domains of α-helices and β-sheets are presented as pointed tubes and flat arrows, respectively.
Upper inset: Space filling presentation of the ABI1 surface in the vicinity of ABA bound to RCAR (RCAR residues are not shown). The Trp^{300} (indicated in yellow) is close to the ABA molecule (oxygen atoms are shown in red) and contacts it via a water molecule (not shown). Basic and acidic amino acid residues of ABI1 are marked by blue and red colour, respectively. Lower inset: Chemical structure of the physiologically

active *(S)*-ABA as well as of the ABA isomers *(R)*-ABA, in which the OH group faces opposite to *(S*)-ABA, and *(R,S)*-trans-ABA.

Fig. 2: ABA signalling to ion channels and to the nucleus.
The ABA receptor is formed by the heteromeric complex of a PP2C such as ABI1 and an ABA-binding RCAR member (both highlighted in pink). The receptor complex controls ABA signalling and is present in A) the cytosol and B) the nucleus. The phosphatase activity of the PP2C inhibits the action of the protein kinases (presented in green) OST1 and related SnRKs, and possibly of Ca^{2+}-dependent CPKs such as CPK23. In the presence of ABA, the phosphatase activity of the receptor is blocked. As a consequence, the protein kinases are released from inhibition and directly phosphorylate and regulate key targets of the ABA signal pathway. In guard cells, key targets are the ion channels SLAC1 and KAT1, which are activated and inhibited by OST1 action, respectively. In the nucleus, key targets are the basic leucine zipper transcription factor ABI5 and related ABFs. Phosphorylated ABFs bind as dimers to the ABA-responsive cis-element (ABRE) and provide in concert with other transcriptional regulators the ABA-responsive transcription (components are presented in steel-blue). ABI3 binds to ABI5 and enhances its action while ABI4 and related AP2-type transcription factors target a GC-rich coupling element (CE) for optimal regulation of ABA-dependent gene expression.

Tab.1: Reported ABA-binding proteins

Glossary

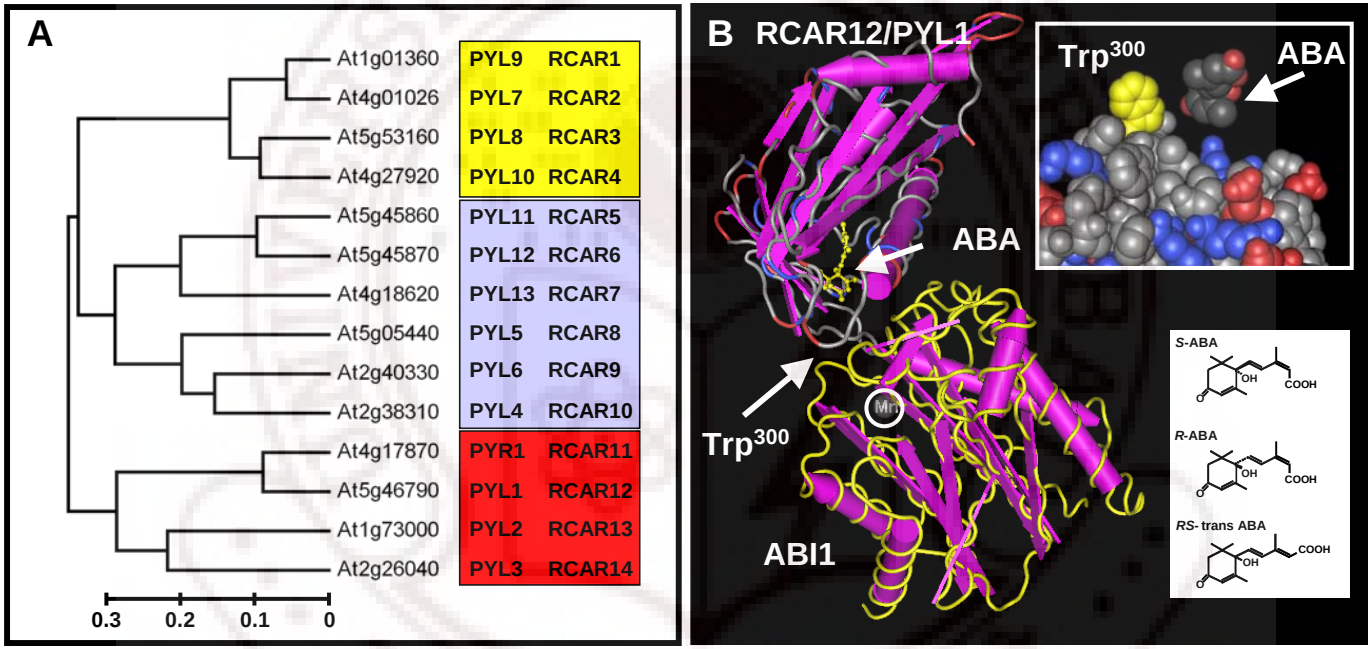
Table 1

Reported ABA-binding proteins	Localization	Study	Dissociation Constant -K_D (nM)	Reference	Comments
ABAR/ CHLH/ GUN5	Chloroplast & nucleus	Subunit of Mg-chelatase ^{3}H-ABA binding biochemistry & reverse genetics	32	Shen et al. 2006 ; Wu et al. 2009	Barley plants, with mutated/disabled CHLH gene normal in their response to ABA; selective binding to ABA; link to ABA signal pathway unknown.
GCR2	Plasma membrane	G protein-coupled receptor ^{3}H-ABA binding; homology modeling & reverse genetics	20	Liu et al. 2007	GCR2 likely a plant homologue of bacterial lanthionine synthetase; binding experiments are questioned.
GTG1 GTG2	Plasma membrane	G protein-coupled receptor -type G-proteins, ^{3}H-ABA binding; double knockout mutant hypersensitive to ABA	36 41	Pandey et al. 2009	ABA response in mutants only partially impaired. Mammalian homologue identified as an ion channel of the endoplasmatic reticulum
RCAR/PYL/ PYR	Nucleus & cytosol	Related to lipid-binding START proteins, Binding studies by isothermal calorimetry and NMR: Triple and quadruple mutants ABA-insensitive	64 for RCAR1/ABI2 38 for RCAR8/HAB1	Park et al. 2009 Ma et al. 2009 Santiago et al. 2009	Proteins inhibit negative key regulators of the ABA pathway, the PP2Cs ABI1, ABI2, HAB1, in the presence of ABA; Selective ABA interaction at molecular level confirmed by the use of nonactive ABA-stereomeres, reconstituted protein system, binding kinetics and mutagenesis

Glossary

ABF	ABRE binding factor
ABI	ABA insensitive, PP2C
AP	Apetala
ABRE	ABA response element
ARE	ABA-responsive element binding protein
Bet V 1	Birch pollen allergen
bZIP	Basic-leucine zipper transcription factor
cADPR	Cyclic ADP ribose
CIPK	CBL (calcineurin B-like proteins)-interacting protein kinase
CPK	Calcium-dependent protein kinase
DREB	Dehydration-responsive element binding
HAB	Hyper sensitive to ABA, PP2C
KAT	Inward-rectifying (transporting) K^+ channel
MYB	Myeloblastosis viral oncogene
MYC	Myelocytomatosis oncogene cellular
OST	Open stomata
PP2C	Protein phosphatase type 2C
PYL	Pyrabactin resistance like protein
PYR	Pyrabactin resistance protein
Pyrabactin	ABA agonist
RCAR	Regulatory component of ABA receptor
SLAC	Slow anion channel
SnRK	Sucrose non-fermenting related kinase

Fig. 1


A
At1g01360
At4g01026
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At4g27920
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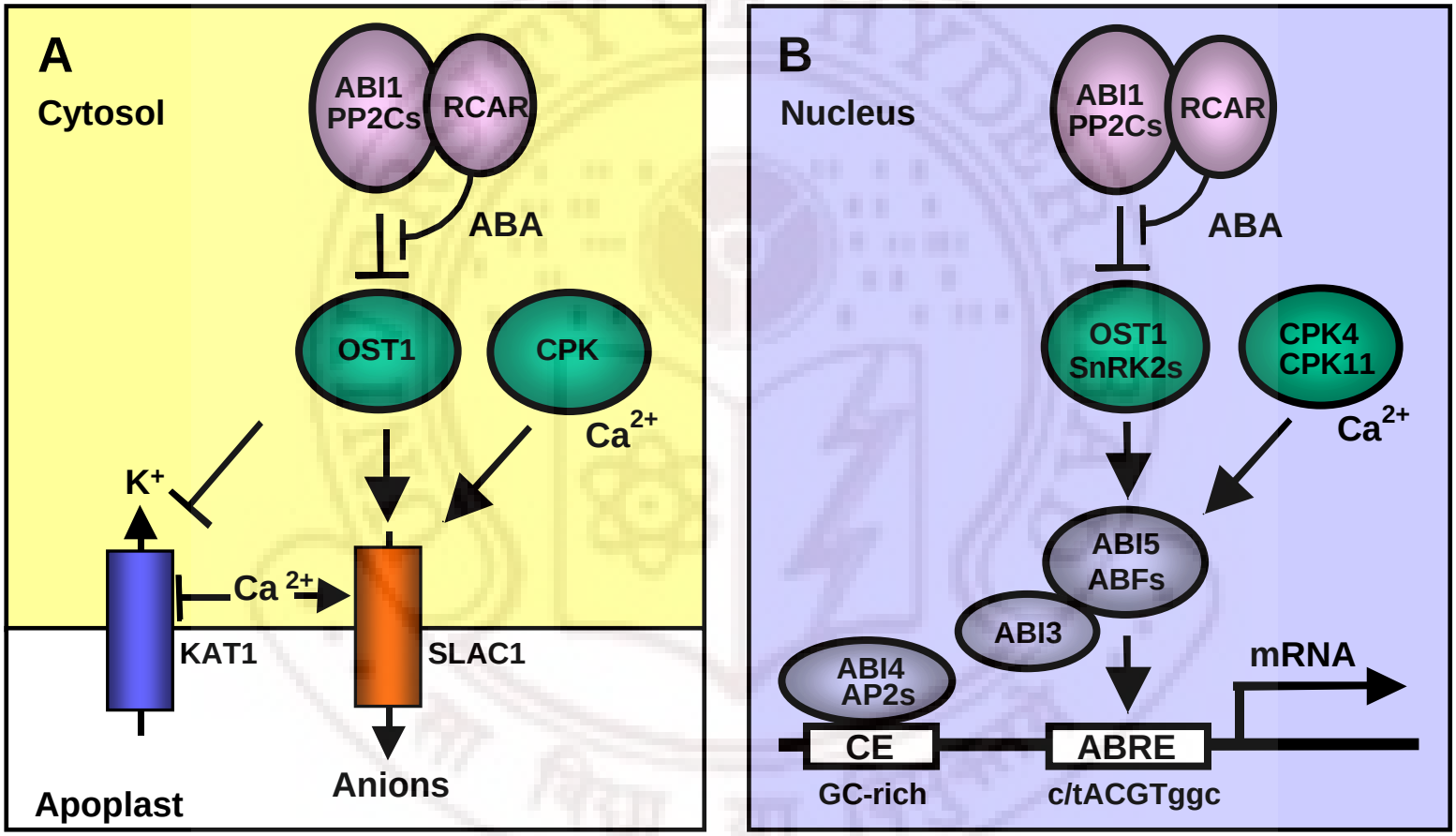
At5g45870
At4g18620
At5g05440
At2g40330
At2g38310
At4g17870
At5g46790
At1g73000
At2g26040
PYL9 RCAR1
PYL7 RCAR2
PYL8 RCAR3
PYL10 RCAR4
PYL11 RCAR5
PYL12 RCAR6
PYL13 RCAR7
PYL5 RCAR8
PYL6 RCAR9
PYL4 RCAR10
PYR1 RCAR11
PYL1 RCAR12
PYL2 RCAR13
PYL3 RCAR14
0.3
0.2
0.1
0
B RCAR12/PYL1
Trp300
ABA
ABA
Trp300
Mn
ABI1
S-ABA
R-ABA
RS- trans ABA
OH
COOH

Fig. 2


A
Cytosol
ABI1
PP2Cs
RCAR
ABA
OST1
CPK
Ca2+
K+
Ca 2+
KAT1
SLAC1
Anions
Apoplast
B
Nucleus
ABI1
PP2Cs
RCAR
ABA
OST1
SnRK2s
CPK4
CPK11
Ca2+
ABI5
ABFs
ABI3
ABI4
AP2s
mRNA
CE
ABRE
GC-rich
c/tACGTggc