Guard cell signal transduction: Role of secondary messengers during stomatal closure mediated by abscisic acid, pyrabactin and microbial elicitors

DOCTOR OF PHILOSOPHY

By

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Doctor of Philosophy

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Guard cell signal transduction: Role of secondary messengers during stomatal closure mediated by abscisic acid, pyrabactin and microbial elicitors"** submitted by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad is an original and independent research work and this work has not been submitted previously in part or in full for the award of any degree or diploma in this or any other University or Institution.

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CERTIFICATE

This is to certify that **Mr. Mallikarjuna Rao Puli** has carried out the research work embodied in the present thesis entitled **"Guard cell signal transduction: Role of secondary messengers during stomatal closure mediated by abscisic acid, pyrabactin and microbial elicitors"** for the degree of Doctor of Philosophy under my guidance and supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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Mallikarjuna Rao Puli

ABBREVIATIONS

A9C	:	Anthracene-9-carboxylic acid
ABA	:	Abscisic acid
ABI1	:	Abscisic acid insensitive1
ABI2	:	Abscisic acid insensitive 2
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 acetoxy methyl ester
BCECF-AM	:	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescien), acetoxy methyl ester
CM-H_2DCFDA	:	(5-(and-6)-chloromethyl-2',7' dichlorodihydro fluorescien diacetate
cPTIO	:	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF-2DA	:	4, 5-diaminofluorescein diacetate
DIDS	:	4, 4'-diisothiocyanatostilbene 2,2'-disulfonic acid
DL-*threo* DHS	:	DL-*threo* dihydrosphingosine
DMS		N', N'-dimethyl sphingosine
DPI	:	Diphenyleneiodonium chloride
H_2DCF-DA	:	2',7'-dichlorofluorescien diacetate
LCBP	:	Long chain base-phosphate
L-NAME	:	NG-nitro-L-arginine-methyl ester
LY294002	:	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
NO	:	Nitric oxide
NOS	:	Nitric oxide synthase
NR	:	Nitrate reductase

PA	:	Phosphatidic acid
PAMP	:	Pathogen associated molecular patterns
Phyto-S1P	:	Phytosphingosine-1-phosphate
PI3K	:	Phosphoinositide-3-kinase
PLDα1	:	Phospholipase Dα1
PP2C	:	Protein phosphatase 2C
PTI	:	Pattern triggered immunity
PYR1	:	Pyrabactin resistant1
PYL	:	Pyrabactin resistant1 like
RBOHD/F	:	Respiratory burst oxidise homologue D/F
RCAR	:	Regulatory component of abscisic acid receptor
ROS	:	Reactive oxygen species
S1P	:	Sphingosine-1-phosphate
SLAC1	:	S-type of anion channel1
SLAC1-OE	:	S-type of anion channel1 over-expressed
SLAH3	:	SLAC1 homologue
SPHK1	:	Sphingosine kinase 1
SPHK2	:	Sphingosine kinase 2

INDEX

Chapter1

INTRODUCTION AND REVIEW OF LITERATURE

Plants face severe challenges from their environments, which include abiotic and biotic factors. The abiotic factors are those related to plant hormones, drought, salinity, temperature, ozone and heavy metals, while biotic factors are exemplified by pathogens. The perception and integration of these signals to modulate the metabolism/physiology are essential for the plant survival. During these adaptations, one of the key factors is to conserve water loss, by regulating transpiration. Stomatal pores, flanked by a pair of guard cells, are the principal gateways for gaseous exchange and transpirational water loss. Stomata also facilitate the entry of pathogens. A pair of guard cells regulates the stomatal aperture by responding to interior and external signals. Opening and closing of stomatal pores is mediated by turgor-based volume changes in guard cells. During stomatal opening guard cells accumulate potassium ions, anions, malate and sucrose (Talbott and Zeiger, 1996) leading to osmotic water uptake, guard cell swelling and stomatal opening. Stomatal closure requires an efflux of potassium and anions, sucrose removal and the conversion of malate to osmotically inactive starch (Schroeder et al., 2001; Vavasseur and Raghavendra, 2005). Besides their importance in regulation of chief physiological functions, guard cells have been to be valuable system for dissecting signaling events in plant cells.

Among the plant hormones, abscisic acid (ABA) has a diverse role of not only regulating plant processes, but also to co-ordinate abiotic and biotic signals. Exposure to ABA leads to reduction of guard cell turgor and closure of stomata thereby facilitating

the conservation of water during periods of drought (Sirichandra et al., 2009; Kim et al., 2010).

ABA signaling in guard cells

Being a principal regulator of guard cell function, the signal transduction of ABA was extensively studied in plant cells. As many as 40 signaling molecules participate in ABA induced stomatal closure as well as other responses. Among these are protein phosphatases, protein kinases, phospholipases and lipid kinases. Small molecules like reactive oxygen species (ROS), nitric oxide (NO) and Ca^{2+} are important messengers in guard cells during ABA controlled stomatal responses. Changes in membrane potential and ion homeostasis play critical role in ABA signaling. Recently, ABA binding proteins that are required for ABA action in guard cells are identified based on genetical, molecular and biochemical approaches (Cutler et al., 2010; Raghavendra et al., 2010). Recent reviews provide as excellent summary of our current knowledge on signaling components in guard cells (Roelfsema and Hedrich, 2005; Neill et al., 2008; Wasilewska et al., 2008; Sirichandra et al., 2009; Kim et al., 2010; Cutler et al., 2010; Raghavendra et al., 2010).

Protein phosphatases

The most important group of protein phosphatases playing a critical role in ABA signaling are: PP2C and PP2A belonging to Ser/Thr phosphatases. At least six *Arabidopsis* PP2Cs belonging to clade A, act as negative regulators of ABA signaling. Among these six, three PP2Cs: ABI1, ABI2 and HAB1, form structural and functional complexes with ABA-receptors, to facilitate binding of ABA. In the absence of ABA,

PP2Cs inhibit the activity of positive regulators such as, sucrose non fermenting 1 related kinase 2s (SnRK2s) for regulating constitutive ABA responses. Upon binding to ABA-receptors, ABA prevents phosphatase activity of PP2Cs and as a result promotes SnRK (Cutler et al., 2010; Melcher et al., 2010; Raghavendra et al., 2010). Besides their role in direct ABA perception, PP2Cs may play critical role in integration of several signals during ABA induced stomatal closure. Lipid metabolite, phosphatidic acid, derived from PLD, directly binds to and inhibits ABI1 to promote ABA induced stomatal closure (Zhang et al., 2004). A triple loss of function mutant that lacks ABI1, ABI2 and HAB1 showed partial constitutive response to endogenous ABA, while extremely hypersensitive to external ABA (Lee et al., 2009).

Protein Kinases

ABA activates both calcium independent and calcium dependent protein kinases in guard cells as well as other cell types. Among these SnRK2s, SnRK3s/ calcineurin B like protein (CBL)-interacting protein kinases (CIPKs) and Ca^{2+} dependent protein kinases (CDPKs/CPKs) are the most important for ABA action, being positive regulators of ABA signaling. ABA activated protein kinase (AAPK) from *Vicia faba* is guard cell specific SnRK2 and regulates the ABA induced stomatal closure by the activation of slow anion channels (Li et al., 2000). Arabidopsis relative of AAPK is named ‘open stomata 1’ (OST1/SnRK2.6, a form of SnRK2), actively regulates the ABA signaling in guard cells. A triple loss of function mutant *snrk2.2/snrk2.3/snrk2.6* impaired in almost all ABA responses (Fujii et al., 2009). Active pools of SnRK2s regulate ABA signaling by phosphorylating ABA activated transcription factors, which

remain dephosphorylated in absence of ABA (Cutler et al., 2010; Raghavendra et al., 2010).

Four forms of CDPKs/CPKs: CPK3, CPK4, CPK6 and CPK11 were characterized to participate in calcium dependent ABA signaling of guard cells (Cutler et al., 2010; Kim et al., 2010). A *cpk3/cpk6* double mutant was impaired in ABA induced stomatal closure but not with seed germination or early seedling growth (Mori et al., 2006). CDPKs/CPKs are calcium dependent and promote Ca^{2+} activation of anion channels and phosphorylation of transcription factors (Cutler et al., 2010; Kim et al., 2010). CBLs are sensor relay proteins modulate the function of CIPKs/SnRK3s upon binding to Ca^{2+} (Cutler et al., 2010; Kim et al., 2010). Two CBLs: CBL1 and CBL9 act as negative regulators during ABA signaling. Two forms of CIPKs, CIPK3 and CIPK15, also are negative regulators of ABA in other signaling pathways (Cutler et al., 2010; Kim et al., 2010).

Lipids and related molecules

The components of ABA signaling in guard cells include a few bioactive lipids as positive regulators. Inositol triphosphate (IP3), phosphatidic acid (PA), sphingosine-1-phosphate (S1P)/ phytosphingosine-1-phophate (Phyto-S1P) and phosphoinositide-3-phosphate (PI3P) are all known to involve in ABA signaling. ABA activates enzymes such as phospholipase C, phospholipase D, sphingosine kinases (SphKs) and phosphoinositide-3-kinase (PI3K) to produce these messengers in guard cells. PLDα1 and PLDδ, have been shown to participate in ABA signaling and stomatal closure (Uraji et al., 2012). During stomatal closure, ABA activates PLDα1 to produce PA, which bound to and inactivates PP2C ABI1. Recently, two SphKs: AtSphK1 and AtSphK2

were characterized in *Arabidopsis*. SphKs and their products S1P/Phyto-S1P were considered as important messengers in ABA signaling especially during stomatal closure (Guo et al., 2012; Uraji et al., 2012). Plant PI3Ks phosphorylate D3 position of phosphatidylinositol to produce phosphoinositide-3-phosphate. Plants lacking AtPI3K (AtVpsp34P) were lethal, but pharmacological and biochemical studies revealed the participation of PI3K and its product PI3P in ABA signaling, during stomatal closure.

ROS, NO and Ca^{2+}

Among small molecules that act as secondary messengers in guard cell signaling are ROS, NO and Ca^{2+}. The levels of ROS, NO and cytosolic Ca^{2+} were actively altered in guard cells within few minutes after ABA exposure. ABA stimulates ROS production in guard cells by the activation of membrane bound NADPH oxidases called respiratory burst oxidase homologues (RBOHs). The *Arabidopsis* genome encodes several *rboh* genes (*rbohA to J*) of which *rbohD* and *rbohF* are guard cell expressed and are essential for ABA induced stomatal closure (Kwak et al., 2003; Torres et al., 2005).

Nitric oxide, a well-known intracellular molecule, is a key component of mechanism of ABA action. ABA triggers marked increase in NO of guard cells, well before stomatal closure and removal of NO with scavengers, prevents ABA response. Two enzymes: a L-NAME sensitive, nitric oxide synthase (NOS) and tungstate sensitive nitrate reductases (NRs), were reported to mediate ABA-induced NO rise in guard cells. In *Arabidopsis*, Guo et al. (2003) detected the enzyme activity of NOS based on *in vitro* assays and named the enzyme as AtNOS1. The Arabidopsis mutants lacking AtNOS1 showed reduced NO production and stomatal closure with ABA. However, the NOS nature of AtNOS1 was questioned and the protein was renamed as AtNOA1. Two NRs

encoded by *Arabidopsis* genome, NIA1 and NIA2, were required for ABA induced stomatal closure and NO production in guard cells (Desikan et al., 2002). NO appears to act downstream of H_2O_2 in ABA signaling (Bright et al., 2006).

Exposure to ABA elevates cytosolic free calcium in guard cells and induces oscillations known as calcium signatures (Allen et al., 2000; Grabov and Blatt, 1998; Marten et al., 2007). ABA can induce stomatal closure in both Ca^{2+} dependent and independent pathways. In guard cells, Ca^{2+} elevation activates S-type of anion channels by reversible phosphorylation, mediated by Ca^{2+} -dependent protein kinases CPK3 and CPK6 (Mori et al., 2006; Sirichandra et al., 2009). The rise in Ca^{2+} by ABA can inhibit the plasma membrane H^+-ATPases and K^+-inward rectifying channels in guard cells (Sirichandra et al., 2009).

Cytoplasmic pH

The participation of pH as a signal in ABA induced stomatal closure is strongly indicated by two independent observations. Exposure to ABA rises the intracellular pH of guard cells up to 0.1 to 0.3 units (Irving et al., 1992; Blatt and Armstrong, 1993). Lowering the intracellular pH by a weak acid, butyrate, prevents the K^+ efflux and activates K^+ influx by the differential regulation of K^+ inward and outward rectifying channels (Blatt, 1992; Blatt, 2000; Blatt and Armstrong, 1993). Cytoplasmic pH increase was an early signal in guard cells after exposure to ABA as well as MJ and preceded ROS and NO production in guard cells of *Arabidopsis thaliana* and *Pisum sativum* (Suhita et al., 2004; Gonugunta et al., 2008). Butyrate prevented the stomatal closure and pH increase by ABA in both *Arabidopsis thaliana* and *Pisum sativum* guard

cells. Changes in intracellular pH might be independent of Ca^{2+} increase in guard cells (Sirichandra et al., 2009).

Plasma membrane depolarization and ion flux

Stomatal movements are the result of guard cell turgor which depends on guard cell solute contents. During stomatal opening, guard cells perceive signals and activate plasma membrane H^+-ATPases to cause hyperpolarization. Membrane hyperpolarization and proton motive force, generated by H^+ active pumping outwards, directs the exchange of solutes across the plasma membrane. Upon plasma membrane hyperpolarization, guard cells start accumulating K^+ ions from apoplast through voltage-dependent K^+ inward rectifying channels. During stomatal closure, ABA causes depolarization of plasma membrane by inhibiting plasma membrane H^+-ATPases and promotes solute efflux, leading to decrease guard cell turgor (Gaxiola et al., 2007; Roelfsema et al., 2012). Membrane depolarization by ABA causes the K^+ efflux by activation of outward rectifying K^+ channels.

There are two types of anion channels present in guard cells, denoted as rapid (R)-type and slow (S)-type, which differ in the velocity of their voltage dependent activation and de-activation. ABA activates both S-type and R-type anion channels in *Vicia faba* guard cells (Roelfsema et al., 2004, 2012). R-type anion channels or quick anion channels represented by AtALMT12/QUAC1 (for quick anion channel 1) in *Arabidopsis* and mutants lacking AtALMT12/QUAC1 were impaired in ABA-activation of rapid anion currents and stomatal closure (Meyer et al., 2010; Roelfsema et al., 2012). S-type anion channels also called slow anion channels are encoded by 5 homologues of SLAC1 in *Arabidopsis*. *Arabidopsis* single or double mutants lacking SLAC1 and/or

SLAH3 were impaired in stomatal closure by ABA (Negi et al., 2008; Vahisalu et al., 2008).

Gene regulation

Exposure to ABA markedly modulates the transcription patterns in guard cells as well as other cell types, as almost 5-10% of total transcripts are regulated by ABA. Many ABA upregulated genes contain ABA response elements (ABREs) in their promoters which interact with ABA response element binding factors (AREBs/ABFs/DPBFs) and phosphorylated by ABA-activated SnRK2 kinases (Cutler et al., 2010; Kim et al., 2010; Sirichandra et al., 2009). Another family of ABA-responsive guard cell expressed components is MYB transcription factors, particularly MYB60 and MYB61, which participate in light induced stomatal opening. MYB44, another guard cell expressed MYB transcription factor participate in ABA induced stomatal closure. *Arabidopsis* plants over-expressing MYB44 were hypersensitive to ABA in stomatal closure (Cutler et al., 2010; Kim et al., 2010; Sirichandra et al., 2009). In addition to the positive regulation by above transcription activators, ethylene transcription factor 7 (AtERF7) and nuclear protein X 1 (NPX1) act as negative regulators of ABA signaling in guard cells. Mutants lacking AtERF7 or NPX 1 showed reduced sensitivity to ABA during stomatal movements (Kim et al., 2010; Sirichandra et al., 2009). Transcriptome analysis of *Arabidopsis* guard cells revealed the presence of a unique *cis*-acting motif ‘GTCGG’ in guard cells and among 1173ABA-regulated genes identified, about 300 are unique to this cell type (Wang et al., 2011). Details on ABA regulated genes and transcription regulation in guard cells are explained in recent

publications (Leonhardt et al., 2004; Sirichandra et al., 2009; Kim et al., 2010; Wang et al., 2011).

ABA receptors and ABA analogues

Since 2006 several groups of scientists worked on ABA binding proteins and proposed a number of putative ABA receptors, but many of these studies were debated in subsequent investigations (Cutler et al., 2010; Habbard et al., 2010; Raghavendra et al., 2010). Among such putative/suggested ABA receptors is an ABA binding protein, ABAR (for ABA receptor) isolated from broad bean. The *Arabidopsis* relative of ABAR encodes the H-sub unit of Mg-chelatase (CHLH), a key component of chlorophyll biosynthesis and chloroplast to nucleus signaling. ABAR/CHLH is stereospecific, binds to (+) ABA and regulates the ABA responses such as seed germination, post-germination changes and stomatal movement (Shen et al., 2006). However, ABA binding nature of CHLH was questioned based on technical grounds based on the inability of XanF (a barley homologue of CHLH) to bind ABA (Müller and Hansson, 2009). Further studies with ABA-affinity chromatography confirmed the ABA binding nature of *Arabidopsis* CHLH and genetic dissection revealed its involvement in ABA responses such as seed germination and gene expression but not stomatal movements (Wu et al., 2009).

A G-protein coupled receptor, called GCR2 was assumed to bind ABA, but its GPCR nature and ABA binding ability were under debate. Two unconventional GPCRs: GPCR-type G proteins, GTG1 and GTG2 from *Arabidopsis* genome appeared to be ABA receptors based on their topological similarity to GPCRs. GTGs redundantly function in ABA responses such as seed germination, root growth, stomatal responses

and gene- expression (Panday et al., 2009). GTGs were stereo specific, recombinant GTGs binds to (+) ABA in presence of phosphatidyl choline and localized to plasma membrane (Panday et al., 2009). However, the binding experiments involved in these experiments were also questioned (Risk et al., 2009).

Finally, a major breakthrough in identification of ABA-receptors occurred, when four separate research groups isolated PYR/PYL/RCAR proteins belongs to START-domain superfamily or Bet v I-fold superfamily and characterized as cytoplasmic ABA receptors (Ma et al., 2009; Nishimura et al., 2010; Park et al., 2009; Santiago et al., 2009). Based on a newly discovered ABA analogue, pyrabactin, Park et al. (2009) identified a 14 member family of *pyrabactin resistant 1/ pyrabactin resistant 1-like* (PYR/PYL) proteins in *Arabidopsis thaliana*. Ma et al. (2009) independently discovered the same proteins and named as *regulatory component of abscisic acid receptors* (RCARs). PYR/PYL/RCAR dimers posseses a central hydrophobic region, and upon ABA binding forms a trimeric complex with PP2Cs and inhibits PP2C activity (Cutler et al., 2010; Raghavendra et al., 2010).

Microbial elicitors: perception and signaling in guard cells

Pathogens utilize natural openings of plant leaf surface, such as stomata or hydathodes as gateways for successful entry in to the plant interiors (Melotto et al., 2006, 2008). Hence, the tight regulation of stomatal opening and promotion of closure is essential for plant defense. Plants evolved two levels of defense for preventing the entry of pathogens in to plant tissues. Recognition of pathogen specific effector molecules (elicitors) called pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs) by specific transmembrane pattern recognition receptors

confers first level of defense by inducing pattern triggered innate-immunity. Successful pathogens can suppress PTI to cause disease in host plants but not in other plants. Some of the pathogens suppress the innate-immunity by secreting effector proteins inside the plant cells. Plants can recognize some of these effectors through intra-cellular immune receptors to trigger second level of defense i.e. effector triggered immunity (Jones and Dangl, 2006; Dodds and Rathjen, 2010).

The plant model *Arabidopsis thaliana* actively recognizes several bacterial elicitors like flagellin (or its derived peptide Flg22), lipopolysaccharides (LPS) and closes stomata to defend the successful entry of respective pathogens (Melotto et al., 2006). A fungal elicitor, chitosan, triggers defence responses by inducing stomatal closure in guard cells of *Pisum sativum* and *Hordeum vulgare* (Srivastava et al., 2009; Koers et al., 2011). Elicitors/PAMPs are expected to be recognized by receptors located on the plasma membrane of plant cells. Such receptors are identified in case of flagellin, elf18/26, or chitin (Nicaise et al., 2009; Park et al., 2012). Receptors for several other elicitors, including LPS, are not yet known.

Elicitor signaling in guard cells

Guard cells also seem to perceive the elicitor signals through the membrane bound receptors such as FLS2. For example, flg22 failed to induce stomatal closure in *Arabidopsis fls2* mutants (Melotto et al., 2006). There is some parallelism reported in elicitor signaling and hormonal signaling of guard cells. Stomatal closure by flg22 and LPS was impaired in ABA biosynthesis mutant *aba3-1* and ABA signaling mutant *ost1-2*. Salicylic acid deficient mutants (*nahG* or *eds16*) did not respond to *Pseudomonas syringae pv. tomato* strain DC3000 (Melotto et al., 2006, 2008).

Bacterial PAMPs (flg22 and LPS) induced NO production in guard cells, while NOS inhibitor, Nω-nitro-L-arginine (L-NNA), prevented stomatal closure by flg22 or LPS in wild type *Arabidopsis thaliana* (Melotto et al., 2006). Fungal elicitor chitosan triggered increase of ROS and NO in guard cells of *Pisum sativum* epidermis in as early as 10 minutes after treatment (Srivastava et al., 2009). Flg22 prevented light induced stomatal opening by inhibiting inward K^+ currents. Flg22 inhibition of stomatal opening and K^+ influx was impaired in *Arabidopsis* mutants *fls2* and *gpa1* (deficient in G-protein α-subunit GPA1) (Zhang et al., 2008). Further, pathogen virulence factors such as coronatine and fusicoccin counter stomatal closure caused by environmental factors as well as PAMPs to cause successful infection (Melotto et al., 2008; Zeng et al., 2010).

Gaps in our present knowledge

Based on the similarities with ABA in inhibition seed germination and triggering gene expression, pyrabactin (an ABA analogue) is considered as a potential anti-transpirant and useful in agriculture (Park et al., 2009). Pyrabactin was able to bind to PYR/PYL/RCAR proteins in *in vitro* assays (Park et al., 2009; Melcher et al., 2010; Cutler et al., 2010). However, there are no direct experiments on the ability of pyrabactin to trigger stomatal closure and if it affects any of the guard cell signal transduction events.

Our knowledge on lipid messengers and related kinases is also limited. For example, SPHKs and S1P/Phyto-S1P are known to involve in ABA induced stomatal closure (Ng et al., 2001; Coursol et al., 2003, 2005; Guo and Wang, 2012). Such S1P/Phyto-S1P induced stomatal closure required the participation of Ca^{2+}, ion channels, GPA1 and PLD (Coursol et al., 2003, 2005; Guo et al., 2012). However, their

interaction with other signaling elements like ROS, NO and cytoplasmic pH was not known. Similarly, the interaction of S1P/Phyto-S1P with PP2Cs or SnRKs is yet to be studied. There are no direct reports on the interaction of SPHKs with other protein kinases such as CIPKs and CDPKs.

Despite the vast literature on microbial elicitor induced defence responses in other plant tissues, there is only limited data on guard cells. ROS production and S-type anion channel activation are two important steps during ABA induced stomatal closure (Kwak et al., 2003; Roelfsema et al., 2012). Besides guard cells, the system of cell cultures have been quite useful for studies on signal transduction, particularly elicitors and ABA. Microbial elicitors can induce apoplastic ROS production in plant cell suspension cultures (Kadota et al., 2004). However there are no experiments on the importance of S-type anion channels in elicitor signaling in guard cells and stomatal closure.

In this report we studied some of these aspects, where a knowledge gap exists and compared with ABA. We focused our experiments to examine the ability of pyrabactin to induce stomatal closure and modulate guard cell signaling components; interaction of S1P with other signaling elements such as ROS, NO or pH; importance and interaction of ROS and S-type of anion channels in elicitor induced changes in guard cells as well as cell suspension cultures. The specific objectives and approaches in this work are described in the next chapter.

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Chapter 2

OBJECTIVES AND APPROACHES

Stomatal guard cells can respond to different stimuli, including plant hormones whether in an intact leaf or in isolated epidermis. Thus guard cells offer a versatile system for the dissection of signal transduction events in plants. Plants like *Pisum sativum*, *Vicia faba* and *Arabidopsis thaliana* are used as model plants for studying guard cell function, in view of their ease in isolation of epidermis.

Besides guard cells, plant suspension cell cultures are useful for the signal transduction studies. Homogenous cultures of tobacco bright yellow (BY2) cells and *Arabidopsis* T87 cells are classical examples of the plant suspension cultures derived from tobacco callus cells and *Arabidopsis* mesophyll cells, respectively, these cell cultures are extensively utilized in physiological studies.

Objectives

The following objectives were set for the present work.

1. To characterize the effects of pyrabactin, an ABA analogue, on stomatal closure: Changes in signaling components and possible site of action.
2. To study the role of S1P/Phyto-S1P and SphK in ABA induced stomatal closure and importance of NO and ROS; to assess the importance of PLD and PI3K in stomatal closure by ABA and their interaction with NO and ROS relative to S1P/Phyto-S1P.
3. To study the importance of cytoplasmic pH in stomatal closure caused by S1P/Phyto-S1P in relation to PLD and PI3K.

4. To study the pattern and role of ROS production induced by ABA or microbial elicitors: S-type of anion channels in Arabidopsis and responses of two different cell cultures (Tobacco BY2 and Arabidopsis T87).

Approaches

For studying the above objectives, pharmacological, fluorimetric, luminescence and genetic tools were used based on their requirement and availability. The effects of pyrabactin or S1P were compared with those of ABA. The role and importance of ROS, NO or cytoplasmic pH in stomatal closure caused by various signals were studied by using respective modulators and suitable fluorescent probes. ROS modulators - DPI (NADPH oxidase inhibitor) and catalase (ROS scavenger); NO modulators - cPTIO (NO scavenger), L-NAME (nitric oxide synthase inhibitor) and tungstate (nitrate reductase inhibitor) as well as pH modulator - butyrate (a weak acid that neutralize increasing pH), were used to assess the importance of these respective messengers in guard cell signaling. The real time monitoring of ROS, NO or pH were studied by confocal microscope and fluorescent probes: H_2DCFDA/CM-H_2DCFDA (for ROS), DAF-2DA (for NO) and BCECF-AM (for cytoplasmic pH) respectively. The results related to ROS production, were validated by NADPH oxidase deficient *Arabidopsis thaliana* double mutant *rbohD/F*.

The role and importance of SPHKs were studied by using SPHK inhibitors DL-*threo* DHS and DMS while LCBPs action was analyzed using S1P and Phyto-S1P. The relative position of PLD and PI3K were studied using PLD inhibitor (1-butanol), PLD product (PA) and PI3K inhibitors (wortmannin and LY294002). We used *Arabidopsis*

thaliana mutants *abi1* and *abi2* for studying relative position of ABI1 and ABI2, respectively, in ABA signaling.

The importance and interaction of cytoplasmic pH with S1P were studied by monitoring pH by the fluorescent dye BCECF-AM. The pH of guard cells was modulated by using butyrate, a weak acid. The effect of S1P was assessed in presence of SPHK inhibitors, DL-*threo* DHS and DMS.

The importance of anion channels in elicitor signaling was monitored using anion channel inhibitors A9C and DIDS, and the results further validated using *Arabidopsis thaliana* single mutants (*slac1, slah3*), double mutant (*slac1slah3*) and overexpression line (*slac1-OE*). Two different types of suspension cell cultures i.e. tobacco BY2 cells and *Arabidopsis* T87 cells, were used for studying their responses to ABA or microbial elicitors. The apoplastic ROS production was monitored by luminol and the differential sensitivity of different cell types for different elicitors was assessed. The role of anion channels in apoplastic ROS production was further studied in different suspension cell cultures using anion channel inhibitors and luminol.

The results and discussion were described in four chapters, corresponding to four objectives, listed above.

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Chapter 3

MATERIALS AND METHODS

Plant materials and growth conditions

Pea plants

Plants of pea (*Pisum sativum* L., cv. Arkel) (Fig. 3.1A) were raised from seeds, procured from either Pocha Seeds (Pune) or India seeds (Delhi). The seeds were soaked in water for overnight and then surface sterilized with sodium hypochlorite solution (0.2% v/v). Then, seeds were covered in moist cotton black cloth or wet filter papers for 3 d and allowed to germinate. The germinated seeds with protruded hypocotyls were sown in the plastic trays containing 1:1 mixture of soil and farmyard manure and watered daily. The plants were grown in a green house under natural photoperiods of approximately 12 h and an average temperature of 30 ^{0}C day / 20 ^{0}C night. The second pair of fully unfolded leaves was picked at about 9 am from 9-15 day old plants and was used for experimentations.

Arabidopsis plants

Seeds of *Arabidopsis thaliana* wild type (Fig. 3.1 B) and mutants were surface sterilized with 5% sodium hypochlorite and 0.05% Tween 20 for 10 min and washed off for 5-6 times. Then after, seeds were spread on to the petridishes containing 0.6% agar and ½ MS medium (detailed composition in Table 3.1). Seeds were vernalized in a cold-chamber for 2 days to remove dormancy and grown in a controlled chamber (light 120 µmol m^{-2} s^{-1}, temperature 25^{0}C, long photoperiods of 16 h light and 8 h dark). Two-

Figure 3.1: Plants used in present work. A, *Pisum sativum* L., cv. Arkel (10 d old) B, *Arabidopsis thaliana* (4-week old).

Table 3.1: Composition of ½ MS medium for growing *Arabidopsis thaliana* plants

Macronutrients	mg/L	Micronutrients	mg/L
KNO_3	1900	$ZnSO_4.7H_2O$	2
NH_4NO_3	1650	$CuSO_4.5H_2O$	0.025
$CaCl_2.2H_2O$	440	$CoCl_2.6H_20$	0.025
$MgSO_4$	370	$FeSO_4.7H20$	27.8
KH_2PO_4	170	Na_2-EDTA.$2H_2O$	37.26
		$MnSO_4.H_2O$	10
		H_3BO_3	3
		KI	0.75
		$Na_2MoO_4.2H_2O$	0.25

week-old seedlings were transferred to plastic cups containing 1:1:1 mixture of pearlite, soil rite and vermiculite and growth continued under same photoperiods.

Arabidopsis T87 cells

Arabidopsis thaliana (ecotype Columbia) suspension cultured cells T87 (Fig. 3.2 A) were grown in 330 ml of Jouannéau and Peaud-Lenoël (JPL) medium (composition mentioned in Table 3.2) by gentle agitation (120 rpm) under continuous illumination of 60 µmols^{-1}m^{-2} at 22^{0}C (Takahashi et al., 2004). An aliquot of cell suspension (0.5 ml) was transferred to 100 ml fresh medium every week.

Tobacco BY2 cells

Tobacco BY2 (Nicotiana tabacum L. cv. Bright Yellow 2) (Fig. 3.2 B) cell suspensions were maintained by weekly dilution (1/100) with fresh modified Linsmaier and Skoog (LS) medium (composition mentioned in Table 3.3) and maintained at 28^{0} C with aeration in the dark under continuous shaking at 100 rpm.

Bioassays of stomatal closure in abaxial epidermis of *Pisum sativum* and *Arabidopsis thaliana*

The abaxial epidermis was peeled off from the leaves and cut in to pieces of ca. 0.4 cm^{2}. 25 epidermal strips were transferred to 3-cm diameter petridishes containing 3 ml of opening medium (10 mM MES-KOH, pH 7.0, 50 mM KCl) and allowed stomata to open under light (200-250 µmol m^{-2} s^{-1}) for 150 min. Then, epidermal strips were transferred to each of 24 well plates containing medium and required concentrations of test compounds (inhibitors or scavengers). Illumination was continued for next 120 min,

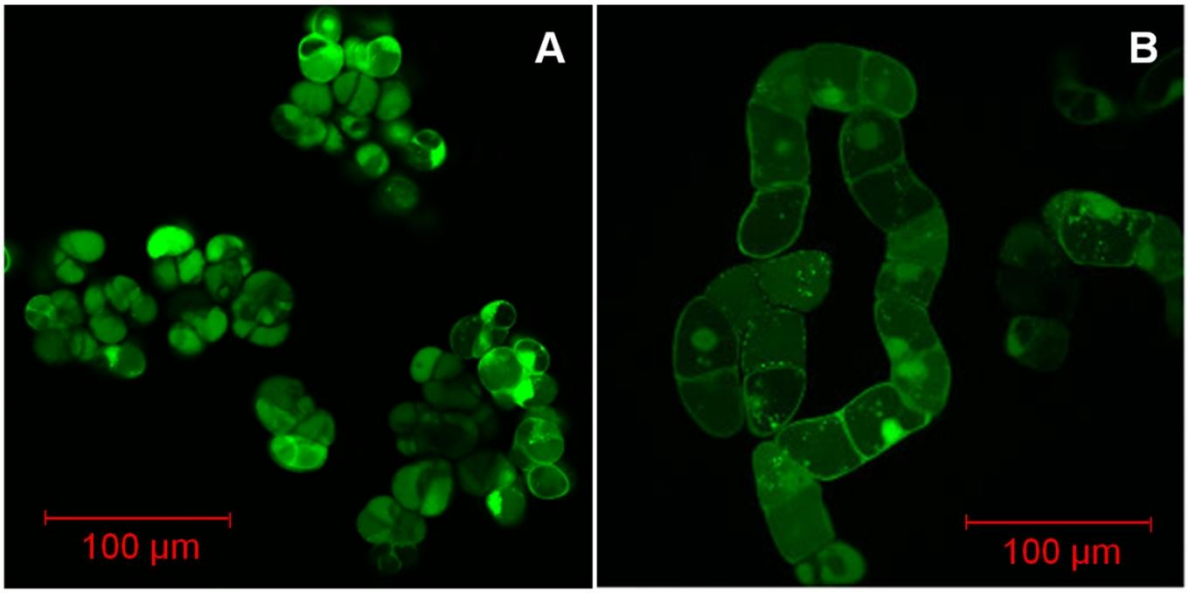

Figure 3.2: Cell suspension cultures loaded with fluorescent probe CM-H_2DCFDA. A, *Arabidopsis*T87 cells. B, Tobacco BY2 cells.

Table 3.2: Composition of modified JPL medium for growing *Arabidopsis* T87 cells

JPL mineral solution (1L):

JPL A	g/L	JPL B	g/L	JPL C	g/L
KNO_3	65.5	$MnSO_4.4H_2O$	22.3	$FeSO_4.7H20$	2.78
$CaCl_2.2H_2O$	4.4	H_3BO_3	6.2	Na_2-EDTA	3.730
$MgSO_4$	3.7	KI	0.83		
KH_2PO_4	1.7	$ZnSO_4.7H_2O$	10.6		
		$CuSO_4.5H_2O$	0.025		
		$CoCl_2.6H_20$	0.025		
		$Na_2MoO_4.2H_2O$	0.25		

JPL mineral solution (1L) was prepared by adding 37.5 ml JPL A, 0.375 ml JPL B and 2.5 ml JPL C to milli Q water. Medium pH was adjusted to 5.7 with 0.2N KOH.

JPL organic solution (100 ml)		**JPL sucrose solution (100 ml)**	
Casein hydrolysate	100 mg	Sucrose	15 g
myo-Inositol	100 mg	200 mM KH_2PO4	195 µl
Glycine	2 mg	Glycine	305 µl
Nicotinic acid	0.5 mg	1 mM NAA	1 ml
pH adjusted to 5.7 with 0.2 N HCl			

1L of JPL medium was prepared by adding 800 ml of JPL mineral solution, 100 ml of JPL organic solution and 100 ml of JPL sucrose solution.

Table 3.3: Composition of modified LS medium for growing tobacco BY2 cells

Nutrients	mg/L	Nutrients	mg/L
KNO_3	1900	$ZnSO_4.7H_2O$	8.6
NH_4NO_3	1650	$CuSO_4.5H_2O$	0.025
$CaCl_2.2H_2O$	440	$CoCl_2.6H_20$	0.025
$MgSO_4$	180.69	$FeSO_4.7H20$	27.8
KH_2PO_4	370	Na_2-EDTA.$2H_2O$	37.26
$MnSO_4.H_2O$	16.9	*myo*-Inositol	100
H_3BO_3	6.2	Thiamine hydrochloride	1
KI	0.83	$Na_2MoO_4.2H_2O$	0.25

before measuring stomatal apertures. When used together, the test compounds were added 15 min prior to the addition of ABA or pyrabactin.

In case of *Arabidopsis thaliana* wild type and mutants, second whorl of leaves (from bottom) collected into 3 cm diameter petridishes containing opening medium (10 mM MES KOH pH 6.16 + 50 mM KCl) from 3-4 week old plants. These leaves were incubated under white light of 150-200 mol m^{-2} s^{-1}, for 150 min for inducing stomatal opening. The leaves were transferred in to the 24 well plates containing opening medium with or without text compounds and illumination continued for next 120 min before measuring stomatal apertures. Abaxial sides of treated leaves were pressed to cover glasses, with medical adhesive Telesis V (Premiere Products Inc., Pacaima, California, USA). Leaving the abaxial epidermis, stuck to the cover glass, other tissues were carefully scrapped off with the help of another cover glass. The stuck epidermis was washed with double distilled water and placed on a glass slide to take the pictures under a pre-calibrated research microscope.

The width of stomatal aperture was measured under a research microscope with the help of a precalibrated ocular micrometer. 10-15 apertures were monitored at random in each of three different epidermal strips, from each treatment. The experiments were repeated at least on three different days, making each measurement of stomatal aperture an average of a minimum of 90 stomata.

Levels of ROS, NO or cytoplasmic pH

Changes in ROS, NO or cytoplasmic pH levels in guard cells were monitored by using respective fluorescent probes, 2',7' dichlorofluorescien diacetate (H_2DCF-DA) or

(5-(and-6)-chloromethyl-2',7' dichlorodihydro fluorescien diacetate (CM-H_2DCFDA) for ROS; 4, 5-diaminofluorescein diacetate (DAF-2DA) for NO; and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescien), acetoxymethyl ester (BCECF-AM) for pH (Gonugunta *et al.,* 2008). Epidermal peels were mounted on a microscope slide with Telesis V. Stomata were allowed to open by incubating epidermal strips under 200-250 µmol m^{-2} s^{-1} white light for 150 min, in a medium of 10 mM MES-KOH, pH 7.0, 50 mM KCl. After 150 min, the epidermal tissues were loaded with 30 µM H_2DCF-DA or 25 µM CM-H_2DCFDA, 25 µM DAF-2DA or 5 µM BCECF-AM (30 min in dark), respectively at 25 ± 1 ^{0}C. The epidermis was rinsed with incubation buffer twice, to wash off excessive fluorophore. For studying time course changes in ROS/NO/pH levels, the epidermal tissues were treated with test compounds and the changes in fluorescence levels were measured at every 3 min intervals. For studying the effect of respective modulators on ROS/NO/pH changes by epidermal tissues were pre-loaded with modulators 10 min prior to the addition of effectors and measured the changes in fluorescence levels after 20 min.

The fluorescence of guard cells was observed under fluorescence microscope (Optiphot-2, Nikon, Japan) fitted with monochrome high-resolution digital cooled CD camera (Cool snap FX) or confocal laser scan microscope (TCSSP-2, AOBS 4 channel UV and visible; Leica, Heidelberg, Germany or Zeiss-LSM 710 NLO, Carl Zeiss, Jena, Germany) and saved as JPEG files. The captured images and the relative fluorescence emission of guard cells were analysed by using NIH Image for Windows (Scion Image/Image J) or Leica Image Analysis software. The images were opened in either one of the above, created a circle and measured the fluorescent intensity in each stomata.

The average of 90 stomata were measured and deducted from the average background fluorescence. The fluorescence intensity in the guard cells, without any effectors (at the beginning of the experiment), was taken as 100% (Suhita *et al.,* 2004; Gonugunta *et al.*, 2008).

Measurement of ROS (H_2O_2) in suspension cultured cells

Protocols for ROS studies in suspension cultured cells were modified from Kadota et al. (2004). Tobacco BY2 cells or Arabidopsis T87 cell cultures were washed thrice and resuspended in a buffer solution containing 5 mM HEPES (pH 7.0), 175 mM mannitol, 3 mM $CaCl_2$ and 0.5 mM K_2SO_4. After that, cell suspensions were equilibrated for 3 h on a gyratory shaker (100 rpm, 28^0 C). 250 µl of cell suspension was transferred to the culture tube (1.1 cm diameter) before adding 0.5 mM luminol and 5 mM potassium phosphate buffer (pH 7.0) and set in a luminometer (Lumicounter 2500, Microtech Nition, Chiba, Japan). The culture tube rotates 17 times per 3 s in the luminometer, both clockwise and counter-clockwise, respectively to agitate the cells. Cell suspension was agitated for 2 min before adding test compounds and changes in H_2O_2 indicative luminescence was measured at 30 s intervals up to 15 min. Each value represents an average of triplicates studied on three different days.

Solvents, chemicals and materials

S1P, DMS and DAF-2DA were purchased from Calbiochem. BCECF-AM and CM-H_2DCFDA was from Invitrogen. Phyto-S1P was from Avanti Polar Ltd. All other chemicals were from Sigma-Aldrich. S1P was dissolved in warm ethanol and slightly sonicated. ABA was in ethanol. Phyto-S1P and PA were dissolved in chloroform, dried

under nitrogen gas followed by liquid nitrogen and resuspended in opening medium by brief sonication just before usage. Pyrabactin, apyrabactin, fluorescent probes, wortmannin, LY294002 and DPI were dissolved in DMSO. All other compounds were dissolved in milli Q water. Wherever necessary the controls included requisite quantities of solvents. The data presented are the average values (± SEM) of results from three to four experiments conducted on different days.

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Chapter 4

PYRABACTIN, AN ABA AGONIST, INDUCED STOMATAL CLOSURE AND CHANGES IN SIGNALING COMPONENTS OF GUARD CELLS IN ABAXIAL EPIDERMIS OF *PISUM SATIVUM*

Introduction

Stomatal closure is an adaptation to conserve water loss during drought/water stress conditions. During the stress conditions, the synthesis and mobilization of abscisic acid (ABA) form key physiological events, facilitating stomatal closure by ABA (Seo and Koshiba, 2002, Christmann et al., 2006). In view of the powerful effects of ABA, the signaling components during ABA induced stomatal closure were examined extensively (Hetherington, 2001, Wasilewska et al., 2008, Acharya and Assmann 2009, Kim et al., 2010). Protein phosphatases such as ABI1, ABI2 and HAB1 are negative regulators during ABA induced stomatal closure, while protein kinases such as SnRK2s (including OST1) are positive regulators (Mustilli et al., 2002, Li et al., 2006, Kim et al., 2010, Hubbard et al., 2010). Other protein kinases such as CBLs and CIPKs also play crucial role in ABA induced stomatal closure. Besides the above proteinacious secondary messengers, participation of several small molecules like reactive oxygen species (ROS), nitric oxide (NO) and ions like Ca^{2+}, besides a rise in guard cell pH are all essential during ABA-mediated stomatal closure (Neill et al., 2002, Suhita et al., 2004, Gonugunta et al., 2008, 2009). In addition, ABA promotes the activity of anion channels (e.g. SLAC1, AtALMT12) and down regulates the activity of inward K^+ channels (KAT1and KAT2) in stomatal guard cells (Geiger et al., 2009, Lee et al., 2009, Sirichandra et al., 2009, Kim et al., 2010).

Despite the repeated attempts, the identity of ABA putative receptors was not established for a long time. In 2009, two independent groups identified and established that PYR/PYL/RCAR proteins, that belong to cyclase subfamily of START/Bet v I protein superfamily, acted as ABA receptors in *Arabidopsis* (Ma et al., 2009, Park et al., 2009). Soon after, the crystallization, molecular modelling and simulation of the structure of PYR/PYL/RCAR proteins, unravelled the novel mechanisms of their function (Nishimura et al., 2009, Yin et al., 2009, Melcher et al., 2010a). In the absence of ABA, PP2Cs keep the pool of SnRK2s dephosphorylated and limit the phosphorylation of transcription factors involved in ABA induced gene expression. When present, ABA binds to PYR/PYL and then to PP2C making a functional complex, and blocks the normal function of PP2C. As a result, the SnRK2s stay in phosphorylated state and activate the transcription factors and induce ABA-activated gene expression (Cutler et al., 2010, Raghavendra et al., 2010, Melcher et al., 2010b).

The identification of PYR/PYL proteins as ABA receptors was made possible with the discovery of pyrabactin (4-bromo-N- (pyridine-2-yl methyl) naphthalene-1-sulfonamide), a synthetic compound. Pyrabactin was found to suppress markedly seed germination and hypocotyl growth, besides promotion of gene expression, very similar to the pattern with ABA (Park et al., 2009). It became clear that pyrabactin was acting as agonist during ABA action. The expression of *pyr/pyl* mRNA was quite high in not only the seeds, but also guard cells. In addition, the Arabidopsis quadruple mutants lacking *pyr1pyl1pyl2pyl4* were impaired in ABA-induced stomatal closure and ABA-inhibition of stomatal opening (Nishimura et al., 2010). Besides it, pyrabactin was considered as a potential anti-transpirant/stress adaptor and had the potential in field application. All

these studies imply that pyrabactin must affect guard cell function and stomatal closure. However, there have been no direct detailed experiments on stomatal closure in response to pyrabactin. Here in this report we studied the response of *Pisum sativum* guard cells to pyrabactin during stomatal closure and compared the effects with ABA. We examined in detail the effect of ABA on the stomatal closure as well as changes in signaling components, including pH, ROS and NO. We have then examined the influence of pyrabactin on stomata in the absence/presence of ABA *vice versa*. Attempts were made to determine the apparent K_D for pyrabactin and ABA.

Results

Pyrabactin induced stomatal closure and changes in ROS, NO and cytoplasmic pH levels in guard cells

ABA or its analog pyrabactin caused stomatal closure in a concentration dependent manner. The concentrations of ABA or pyrabactin, required for maximal stomatal closure, were quite similar (Fig. 4.1A, B). In contrast, apyrabactin, an inactive analog of ABA, did not have any significant effect on stomata (Fig. 4.1C). The presence of fusicoccin (FC, a fungal toxin) prevented the stomatal closure caused by either ABA or pyrabactin (Fig. 4.2).

Pyrabactin increased ROS, NO and cytoplasmic pH levels in guard cells within few minutes after treatment, when compared to their respective controls (Fig. 4.3). Apyrabactin, an inactive analogue of ABA, did not cause noticeable changes in ROS/NO/pH of guard cells (Fig. 4.3d, h and l). The initial rise of H_2DCF-DA fluorescence (indicating ROS levels) was seen at 6 min after treatment and fluorescence

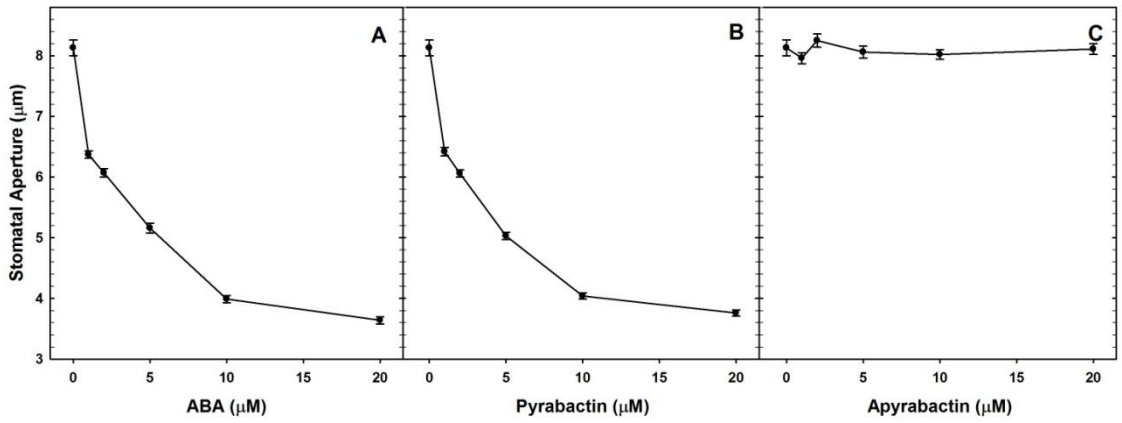

Fig. 4.1: Effect of ABA, pyrabactin or apyrabactin concentrations on stomata in abaxial epidermis of *Pisum sativum*. ABA or pyrabactin caused marked stomatal closure in a similar pattern, while apyrabactin did not have any significant effect. The data are averages of three independent experiments ± SE.

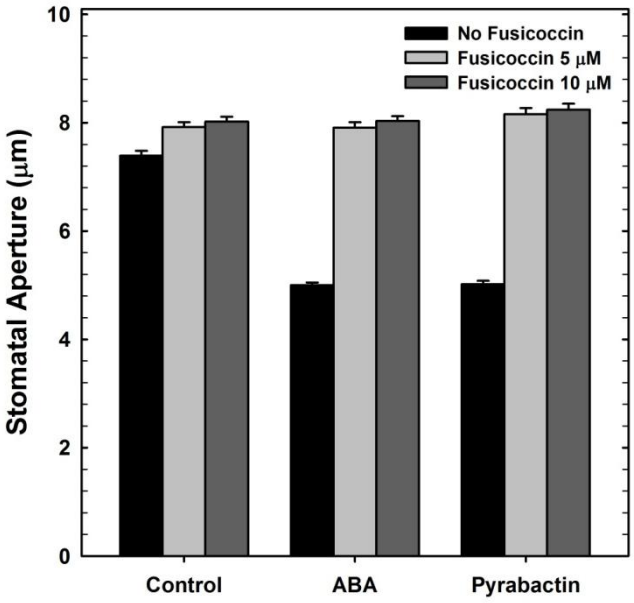

Fig. 4.2: Effect of fungal toxin, fusicoccin (FC), on stomatal closure caused by ABA or pyrabactin. Fusicoccin completely relieved stomatal closure by ABA or pyrabactin. The data are means of three experiments ± SE.

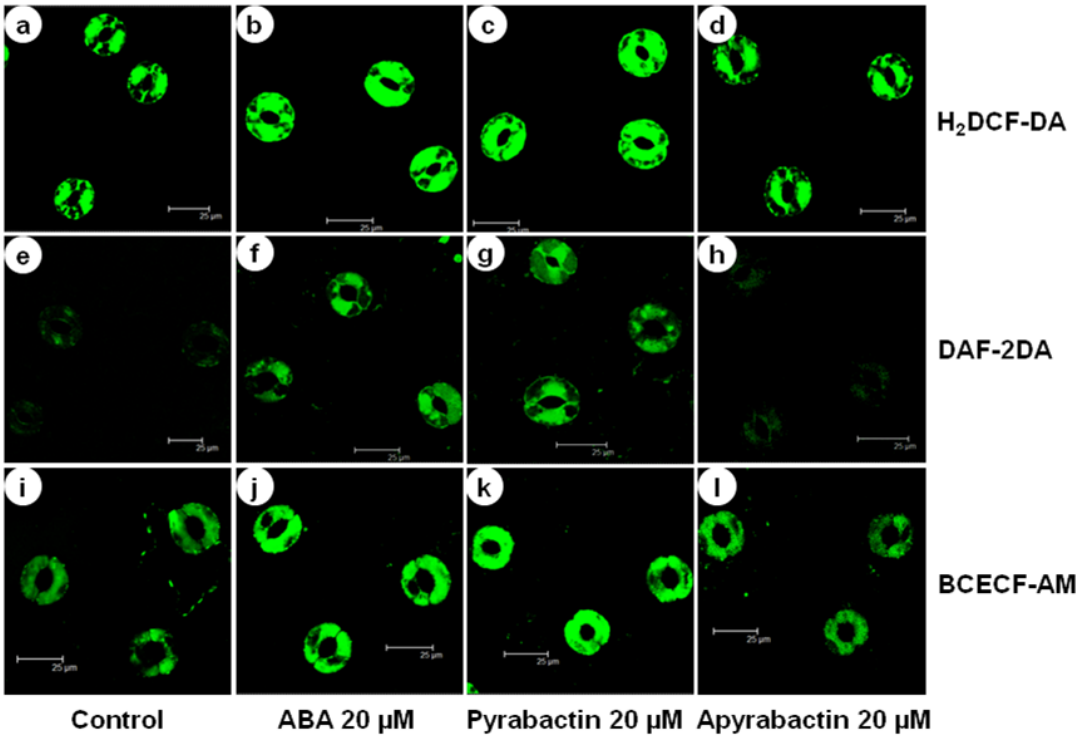

Fig. 4.3: Panels were confocal images showing the changes in ROS, NO and cytoplasmic pH changes in *Pisum sativum* guard cells in presence or absence of ABA, pyrabactin or apyrabactin. a-d, Changes in ROS levels as indicated by H_2DCF-DA fluorescence. e-h, Changes in NO levels as indicated by DAF2-DA fluorescence. i-l, Changes in cytoplasmic pH levels as indicated by BCECF-AM fluorescence. ABA or pyrabactin increased the levels of ROS, NO and cytoplasmic pH, compared to respective controls.

peaked in between 18-24 min (Fig. 4.4A). Similarly, NO specific DAF-2DA fluorescence (indicating NO) showed initial rise at 9 min after treatment and fluorescence peaked after 18 min of treatment (Fig. 4.4B). The BCECF-AM fluorescence (reflecting the pH) initially increased within 3 min and peaked after 24 min (Fig. 4.4C). Similar pattern of changes were observed with ABA.

Modulators of ROS/NO/pH can relieve pyrabactin induced stomatal closure and dampen the rise in ROS, NO or pH levels of guard cells

ROS modulators: DPI (NADPH oxidase inhibitor) or catalase (H_2O_2 scavenging enzyme), partially relieved stomatal closure by ABA or pyrabactin. Similarly, stomatal closure by ABA or pyrabactin was compromised in presence of either cPTIO (NO scavenger), or L-NAME (nitric oxide synthase inhibitor) or tungstate (nitrate reductase inhibitor). Butyrate (a weak acid), relieved stomatal closure induced by ABA or pyrabactin (Fig. 4.5).

Catalase completely relieved the increase in H_2DCF-DA fluorescence by pyrabactin or ABA, while DPI had a partial effect, conforming the increase in fluorescence due to ROS (Fig. 4.6). DAF-2DA fluorescence increase by ABA or pyrabactin was abolished by cPTIO (NO scavenger). Similarly, L-NAME or tungstate (inhibitors of NO synthase or nitrate reductase) restricted the DAF-2DA fluorescence increase by ABA or pyrabactin. Butyrate restricted the rise in BCECF-AM fluorescence by ABA or pyrabactin.

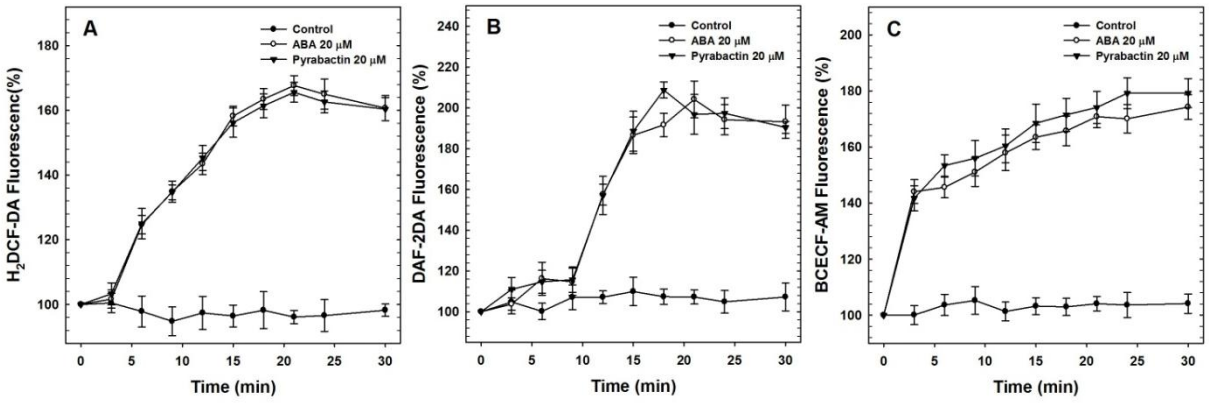

Fig. 4.4: Changes with time in fluorescence of guard cells, loaded with fluorescent probes specific for ROS, NO or pH. The fluorescence was monitored at different times after exposure to pyrabactin or ABA, using an inverted fluorescence microscope. The details are described in materials & methods. ABA or pyrabactin increased with time in the fluorescence intensities of H_2DCF-DA, DAF2-DA, and BCECF-AM reflecting the rise in ROS, NO and pH of guard cells. The effects of pyrabactin and ABA were quite similar. The data are representative of the average mean fluorescence of three independent experiments represent a minimum of 90 individual stomata ± SE.

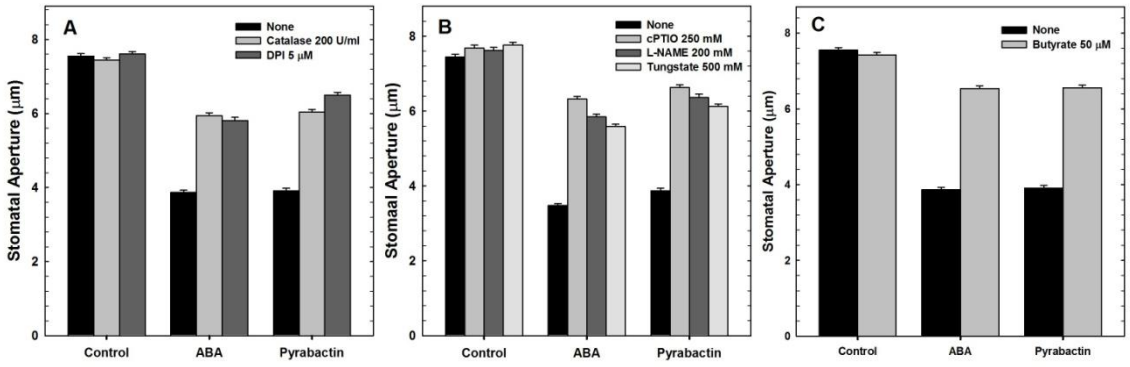

Fig. 4.5: The effect of ROS, NO or pH modulators on stomatal closure caused by ABA or pyrabactin. The decrease in stomatal aperture by ABA or pyrabactin was relieved by ROS modulators, catalase or DPI (A), NO modulators cPTIO and L-NAME or tungstate (B) and pH modulator, butyrate (C). The data are representative of the means of three independent experiments represent a minimum of 90 individual stomata ± SE.

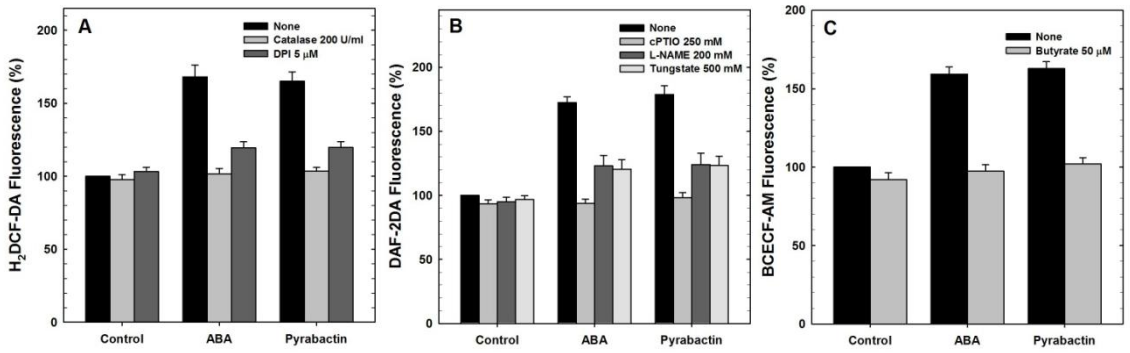

Fig. 4.6: Effect of ROS, NO or pH modulators on H2DCF-DA, DAF2-DA or BCECF-AM fluorescence levels respectively. Changes in fluorescence levels were monitored by using an inverted fluorescence microscope. A, ROS modulators catalase or DPI, prevented ABA or pyrabactin induced increase of H_2DCF-DA fluorescence. B, NO modulators, cPTIO and L-NAME or tungstate, prevented ABA or pyrabactin induced increase of DAF2-DA fluorescence. C, Butyrate, a pH modulator, restricted the ABA or pyrabactin induced increase in BCECF-AM fluorescence. The data are representative of the average mean fluorescence of three independent experiments represent a minimum of 90 individual stomata ± SE.

Pyrabactin competes with ABA during stomatal closure

Experiments employing varied concentrations of pyrabactin (0-100 µM) in the absence or presence of a 5 µM ABA and *vice-versa* (varied concentrations of ABA in the absence or presence of 5 µM pyrabactin) revealed that the effects of ABA and pyrabactin were additive (Fig. 4.7). Kinetic analyses of these data indicated that the apparent IC_{50} of pyrabactin or ABA did not change much in presence of ABA or pyrabactin (Fig. 4.7A, B). In contrast, the double reciprocal plots employing low concentrations of varied concentrations of ABA or pyrabactin (0.5-5 µM) in the absence or presence of 5 µM pyrabactin or ABA demonstrated that the K_D of ABA increased by almost 4-fold in presence of pyrabactin and the K_D of pyrabactin increased by nearly 3-fold in presence of ABA (Fig. 4.7C, D).

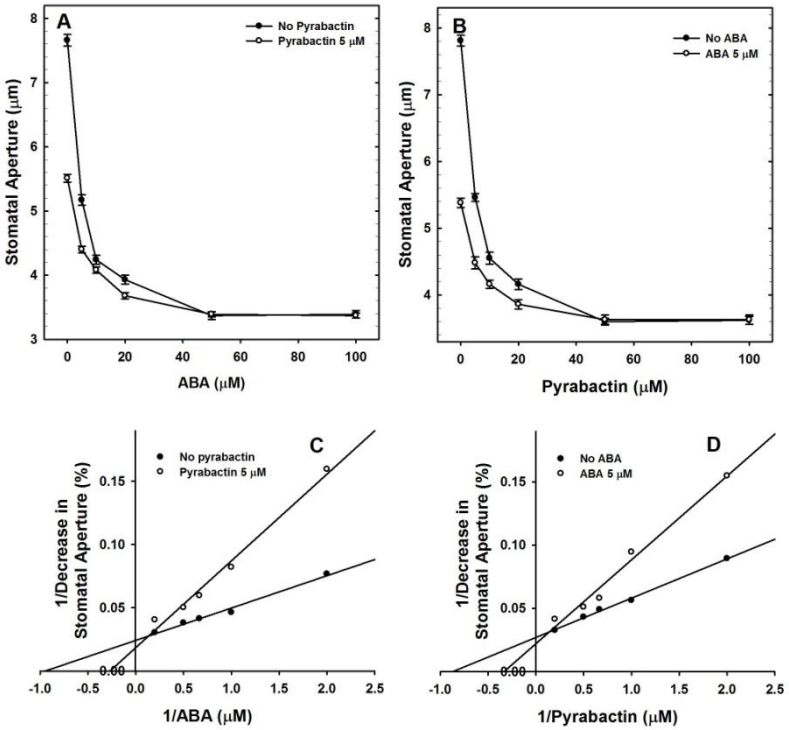

Fig. 4.7: Additive effect of ABA and pyrabactin during stomatal closure. A, The apparent IC_{50} of ABA changed from 4.26 µM to 4.88 µM in presence of 5 µM pyrabactin, when the change in stomatal closure was plotted as a function of concentration of ABA. B, The apparent IC_{50} of pyrabactin changed from 4.47 µM to 5.05 µM in presence of 5 µM ABA, when the change in stomatal closure was plotted as a function of concentration of pyrabactin. C, The double reciprocal plot showing the increase of the apparent K_D of ABA, almost 4-fold, from 1.08 µM to 3.82 µM in presence of 5 µM pyrabactin. D, The double reciprocal plot demonstrated that the apparent K_D of pyrabactin increased by almost 3-fold from 1.17 µM to 3.05 µM in presence of 5 µM ABA. The marked increase in K_D of ABA/pyrabactin in presence of pyrabactin/ABA suggested their competition at or near their binding site during stomatal closure. The data are the average means of three independent experiments.

Discussion

Pyrabactin, a synthetic ABA analog, is considered as a potential tool in future agriculture. Initially identified as a seed germination inhibitor, pyrabactin led the way for identification, purification and characterization of ABA receptors. Most of the earlier experiments with pyrabactin were done on either germinating seeds or *in vitro* reconstituted systems. Our results present an unequivocal and direct demonstration that pyrabactin is as powerful as ABA in promoting stomatal closure in abaxial epidermis.

Pyrabactin is as powerful as ABA in inducing stomatal closure

Pyrabactin caused a marked reduction in stomatal aperture. The effective concentrations as well as the effect of pyrabactin on stomatal closure were quite similar to ABA (Fig. 1). In contrast, apyrabactin did not induce closure of stomata suggesting that only pyrabactin can mimic ABA during stomatal closure. Park et al. (2009) have reported that pyrabactin caused inhibition of seed germination like ABA, and facilitated the binding of PP2C with PYR1 during seed germination, while apyrabactin did not. These observations demonstrate that pyrabactin is an agonist of ABA, while apyrabactin is not an agonist. Several effects of ABA, such as inhibition of seed germination or promotion of stomatal closure are reversed by FC (She et al., 2010, Zeng et al., 2010). The stomatal closure caused by pyrabactin was also reversed completely by FC (Fig. 2), reconfirming our observations that pyrabactin is a strong mimic of ABA, in its effect on stomata.

Signaling components during pyrabactin-induced stomatal closure

The signaling components involved during ABA induced stomatal closure have been extensively studied. After the initial recognition of ABA signal, through ABA-PYR/PYL/RCAR-PP2C complex, the ABA-responsive kinases are activated. Subsequently, the guard cell pH becomes alkaline, the membrane bound NADPH oxidase becomes active, the ROS levels are elevated, followed by rise in NO levels (Suhita et al., 2004, Bright et al., 2006, Gonugunta et al., 2008, Kim et al., 2010). On exposure to pyrabactin too, there were marked increases in pH, ROS and NO levels (Fig. 3). Again, apyrabactin, an inactive analogue, did not cause any significant changes in the signaling components of guard cells (Fig. 3). We therefore conclude that pyrabactin is an active analogue of ABA, in relation to its influence on stomatal function and signal transduction in guard cells. The present study illustrated that pyrabactin could successfully induce stomatal closure and generate small intracellular components ROS, NO during stomatal closure besides increasing cytoplasmic pH. However the exact mechanism of the induction of these signaling events is not yet known. It is quite possible that pyrabactin induces ROS, NO production in guard cells by the mediation of PYR/PYL/RCARs, PP2Cs and OST1/SnRK2.6.

The kinetics of rise in pH/ROS/NO as indicated by respective fluorophores (Fig. 4) suggested that there was marked similarity in the sequence of changes due to pyrabactin or ABA. The ability of DPI and L-NAME to dampen the pyrabactin-induced rise in ROS/NO indicates that NADPH oxidase and putative NOS play an important role during pyrabactin effects. That the action of either pyrabactin or ABA, required the alkalinization of guard cells was evident by the ability of butyrate to prevent the rise in

pH as well as closure (Fig. 5 and 6). The effect of pyrabactin on stomatal closure and its dependence on rise in pH/ROS/NO of guard cells strikes a strong similarity with the action of ABA as well as methyl jasmonate (Suhita *et al.,* 2004; Gonugunta *et al.,* 2008, 2009).

ABA and pyrabactin compete during stomatal closure

Further experiments on stomatal closure in response to varying concentrations of pyrabactin, in presence of fixed concentration of ABA and *vice versa* revealed interesting information on the apparent IC_{50} and K_D values of pyrabactin in relation to ABA (Fig. 7). The method we followed is similar to that used for examining the ethylene effects on bud and flower drop of *Begonia* in presence of gaseous ethylene binding inhibitor, silver thiosulfate (Serek et al., 1994). The apparent IC_{50} for pyrabactin did not change much in presence of ABA or *vice versa* (Fig. 4.7A, B). In contrast, the K_D of pyrabactin or ABA (about 4-5 mM) was elevated in presence of ABA or pyrabactin (Fig. 4.7C, D). These results suggested that pyrabactin was competing with ABA during the induction of stomatal closure, either at the active site or very close to the active site on ABA receptors. These values of IC_{50} or K_D values for pyrabactin or ABA (4-5 µM) appear high compared to IC_{50} values reported for ABA (60 to 125 µM) during interaction with PP2C *in vivo* in using a reconstituted system (Park et al., 2009, Ma et al., 2009). However, it has already been noticed that the IC_{50} values of ABA to interact *in vivo* with PP2C (60 nM) can vary with that for suppressing root growth (3 µM), as observed by Ma et al. (2009). Similarly IC_{50} values of 2 to 4 µM were reported for pyrabactin during the inhibition of seed germination and hypocotyl elongation (Park et al., 2009).

We acknowledge the limitations of our experiments. For example, we know only the amount of pyrabactin or ABA in the external medium. The actual concentrations of pyrabactin or ABA, within the cells (at the ABA receptor level) would be much less. Further, the rate of movement of pyrabactin or ABA across the guard cell could also vary. These factors can explain the differences in the observed K_D for pyrabactin/ABA in our experiments (done *in vivo*) and the values obtained during reconstitution attempts (*in vitro*) by Park et al. (2009) and Hao et al. (2010). In *Arabidopsis* seed germination assays, pyrabactin concentration (100 µM) required to get an effect similar to ABA (10 µM) was almost 10 times higher (Park et al., 2009; Melcher et al., 2010b). However, our major point, that pyrabactin and ABA are competiting with each other, seems to be certain.

Concluding remarks

The ability of pyrabactin, an analogue of ABA, to induce stomatal closure in *Pisum sativum* leaf abaxial epidermis was as powerful as ABA. This observation opens up an exciting possibility of using pyrabactin as an anti-transpirant. However, it is necessary to explore the possibility of synthesizing pyrabactin and/or analogues at an affordable price for suitable application in agriculture. Since, the pattern of signaling components in response to pyrabactin and reversal of pyrabactin effects by modulators was quite similar to that of ABA, we suggest that pyrabactin and similar synthetic compounds could be quite useful in studying the signal transduction mechanisms in guard cells as well as other plant tissues.

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Chapter 5

SPHINGOSINE-1-PHOSPHATE OR PHYTOSPHINGOSINE-1-PHOSPHATE INDUCED STOMATAL CLOSURE IN *PISUM SATIVUM* DEPENDS ON RISE IN NO, BUT NOT ON ROS OF GUARD CELLS

Introduction

Sphingoid long chain bases, such as sphingosine or phytosphingosine are constituents of membrane lipids in plasma membrane and tonoplast of plant cells. Sphingosine kinases (SPHKs) mediate the phosphorylation of sphingosine or phytosphingosine (Worrall et al., 2008). The phosphorylated derivatives, namely S1P or Phyto-S1P, are biologically active and regulate several physiological processes (Chalfant and Speigel, 2005; Spiegel and Milstien, 2002; Guo and Wang, 2012). Active SPHKs capable of phosphorylating sphingosine and phytosphingosine were reported in *Arabidopsis* (Coursol et al., 2005; Worrall et al., 2008; Guo et al., 2011).

The effects of S1P and SPHKs on guard cells are of great interest, as the reports on the effects of S1P on stomata are ambiguous. When *Commelina communis* plants were exposed to water deficit conditions, there was an increase in S1P biosynthesis (Ng et al., 2001). In *Arabidopsis*, S1P and Phyto-S1P promoted stomatal closure (Coursol et al., 2003; 2005). The ABA-induced stomatal closure in *Commelina* and *Arabidopsis* was impaired in presence of SPHK inhibitor, DL-*threo* dihydrosphingosine (DL-*threo* DHS) (Ng et al., 2001; Coursol et al., 2003). In contrast, the existence of S1P in *Arabidopsis* was questioned (Lynch et al., 2009; Michaelson et al., 2009). However, Michaelson et al. (2009) suggested that, despite the lack of S1P, the stomatal regulation by other LCBPs would be possible. Recently, the importance of S1P has again come into focus,

with the observations on *Arabidopsis* mutants deficient in SPHK or Long Chain Base Phosphate-phosphatase. *Arabidopsis* knockout mutants lacking SPHK were insensitive to ABA (Worrall et al., 2008; Guo et al., 2012), while those deficient in LCBP-phosphatase were more sensitive to ABA than the wild type (Nakagawa et al., 2011).

Besides the ambiguity on the role of S1P and SPHK, studies on changes in guard cell signaling components such as ROS and NO after exposure to S1P or Phyto-S1P, have been limited. The available reports suggested that S1P modulated stomatal responses involved Ca^{2+} (Ng et al., 2001), G-protein α subunit (Coursol et al., 2003, 2005), phospholipase Dα1 (PLD α1) (Guo et al., 2012; Guo and Wang, 2012), phosphatidic acid (PA) (Guo et al., 2011) and phosphoinositide-3-kinase (PI3K) (Jung et al., 2002; Park et al., 2003).

In plants, sphingolipid composition varies among the species (Islam et al., 2009; Lynch et al., 2009; Minamioka and Imai, 2009). Leaves of *Pisum sativum* have measurable levels of several LCBs, including S1P (Minamioka and Imai, 2009). We therefore chose *Pisum sativum* to examine in detail the effects of S1P and Phyto-S1P on stomatal closure and their interaction with other signaling elements. The changes as well as real time kinetics of NO and ROS in guard cells of pea were studied in presence of S1P/Phyto-S1P. We extended our studies to assess modulation of S1P-induced stomatal closure by PLD, PA and PI3K. Our experiments indicated that the S1P or Phyto-S1P induced stomatal closure was not completely dependent on ROS, unlike the case with ABA. The effect of S1P/Phyto-S1P on stomatal closure and guard cell signaling components was therefore checked in *Arabidopsis* mutants, with impaired ROS production in guard cells such as those deficient in ABI1 or RBOHD/F (Murata et al.,

2001; Kwak et al., 2003). Another mutant, deficient in ABI2, but capable of ROS production was included for comparison.

Results

S1P or Phyto-S1P caused stomatal closure and marked rise in NO but not of ROS in guard cells

The presence of S1P as well as Phyto-S1P induced stomatal closure in abaxial epidermis of *Pisum sativum*, in a concentration dependent manner, quite similar to ABA (Fig. 5.1). Exposure to S1P or Phyto-S1P caused a rise in ROS, as well as NO of guard cells (Fig. 5.2). The kinetics of rise in ROS/NO in response to S1P, Phyto-S1P or ABA were quite interesting. ABA increased gradually the levels of NO in guard cells by 2.5 folds of original level upto 21 min and thereafter declined. S1P or Phyto-S1P caused a rapid rise in NO of guard cells within 6 min, stable till 21 min (Fig. 5.3A). Similarly, ABA raised the ROS-levels by almost 2.5 fold (with a lag of 6 min), and the levels declined after 21 min. However, S1P or Phyto-S1P caused only a marginal increase of <1.5 fold within 3 to 9 min in ROS, which was stable thereafter. Such rise in ROS levels was far less than that by ABA (Fig. 5.3B).

SPHK inhibitors relieved stomatal closure and prevented the ABA-induced rise in NO but not of ROS

Since active SPHKs are known to operate in leaves, to produce S1P or Phyto-S1P during drought or ABA, the effects of SPHK inhibitors on ABA induced stomatal closure were assessed. Two of such SPHK inhibitors, DL-*threo* DHS and DMS,

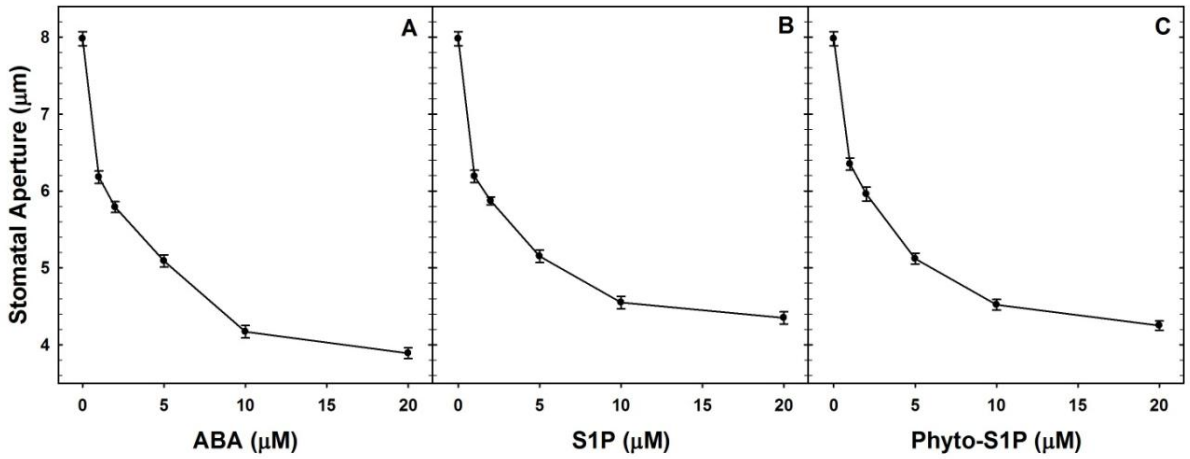

Figure 5.1: Stomatal closure in abaxial epidermis of *Pisum sativum* by S1P or Phyto-S1P in a concentration-dependent manner. The patterns of closure by S1P or Phyto-S1P is similar to the effect of ABA.

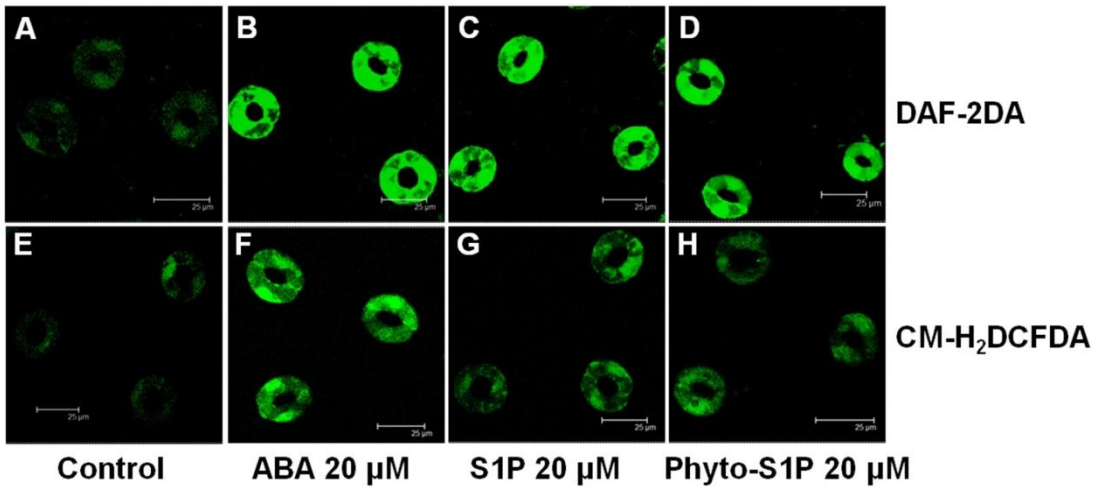

Figure 5.2: Confocal images of NO and ROS in guard cells of *Pisum sativum* on exposure to ABA, S1P or Phyto-S1P. A-D, Change in NO levels, as indicated by DAF2-DA fluorescence. E-H, ROS levels, as indicated by CM-H_2DCFDA fluorescence. ABA elevated the levels of NO and ROS, compared to the control. Similarly, S1P or Phyto-S1P increased NO, as effective as ABA. However, the rise in ROS by S1P or Phyto-S1P was less than that by ABA.

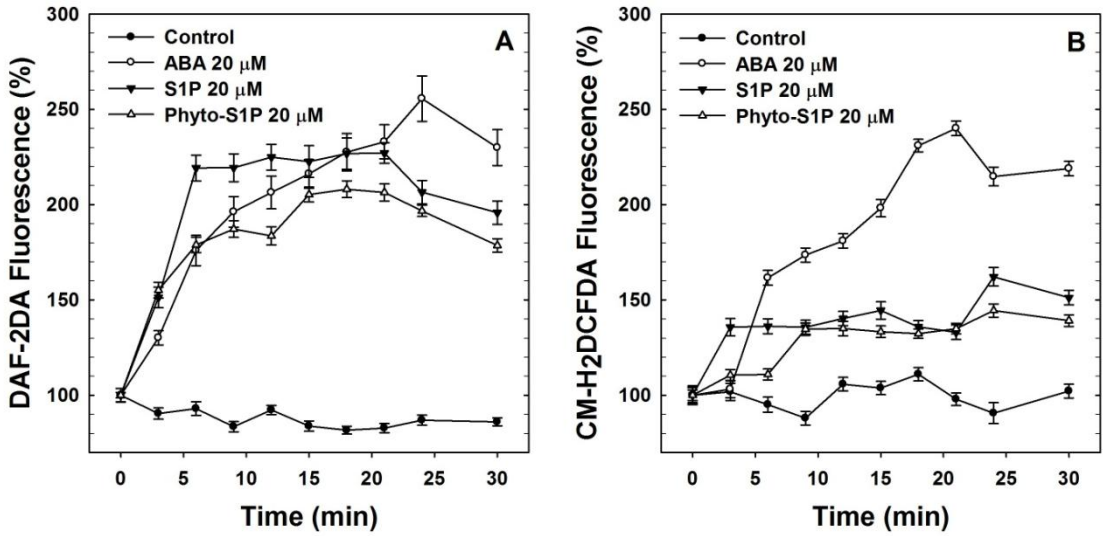

Figure 5.3: Time-dependent changes in levels of NO or ROS in *Pisum sativum* guard cells, when exposed to (20 μM) ABA, S1P or Phyto-S1P. The levels of NO (A) or ROS (B) were monitored with fluorescent probes of DAF-2DA or CM-H_2DCFDA, respectively. Again, S1P or Phyto-S1P were as effective as ABA in elevating NO but not ROS.

prevented stomatal closure by ABA (Fig. 5.4). DL-*threo* DHS or DMS considerably prevented the ABA-induced rise in NO (Fig. 5.5A) by ABA. However, DL-*threo* DHS or DMS did not affect the increase of ROS by ABA (Fig. 5.5B).

Modulators of NO, but not of ROS, attenuated S1P/Phyto-S1P induced stomatal closure and rise in NO/ROS

ABA, S1P or Phyto-S1P induced stomatal closure was relieved and the rise in NO was prevented in presence of carboxy-PTIO, a NO scavenger. Similarly, tungstate (nitrate reductase inhibitor) or L-NAME (nitric oxide synthase inhibitor) prevented stomatal closure and NO production by ABA as well as S1P/Phyto-S1P. However, tungstate had only limited effect on stomatal closure and NO production by S1P or Phyto-S1P (Fig. 5.6A, 5.7A). Two ROS modulators: DPI (NADPH oxidase inhibitor) and catalase (H_2O_2 scavenger), relieved stomatal closure and prevented the rise in ROS, by ABA. Either DPI or catalase could not affect the stomatal closure by S1P or Phyto-S1P (Fig. 5.6B). However, catalase attenuated the limited rise in ROS caused by S1P or Phyto-S1P (Fig. 5.7B).

Role of PLD and PI3K during stomatal closure by S1P/Phyto-S1P or ABA and of SPHK during stomatal closure by PA

Presence of PLD inhibitor, 1-butanol prevented stomatal closure, increase of NO by S1P/Phyto-S1P as well as ABA (Fig. 5.8). In contrast, 2-butanol (that does not inhibit PLD) did not prevent stomatal closure or the increase of NO by ABA or S1P/Phyto-S1P (Fig. 5.8). PA (a product of PLD) induced stomatal closure (Fig. 5.9A).

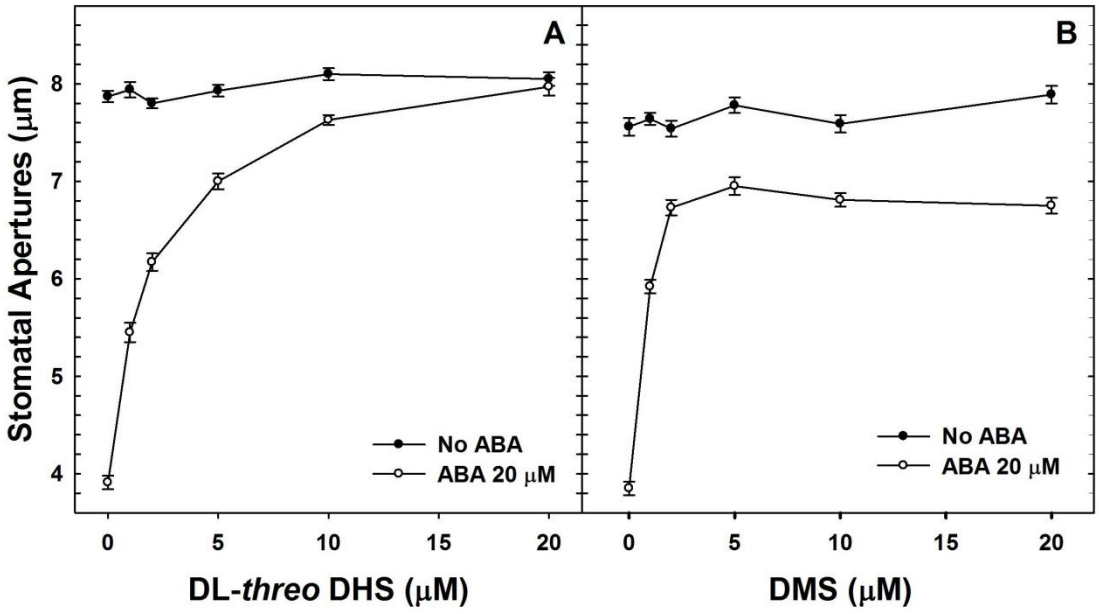

Figure 5.4: Reversal by SPHK inhibitors of stomatal closure by ABA. DL-*threo* DHS could completely prevent the stomatal closure by 20 μM ABA (A), while DMS could partially (yet significant) reverse the ABA effect (B).

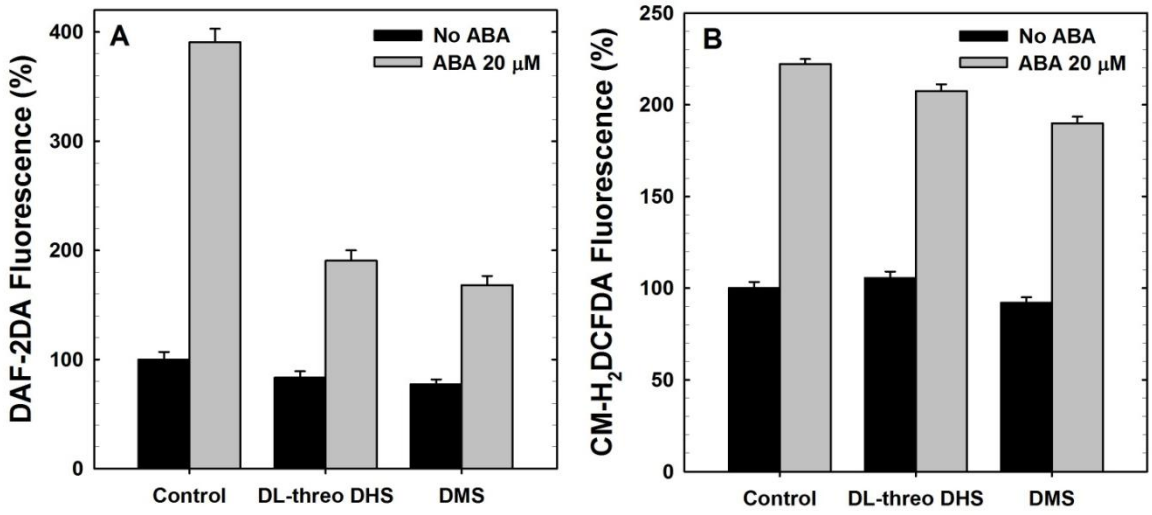

Figure 5.5: The effect of SPHK inhibitors on the elevation of NO/ROS by ABA. A, DL-*threo* DHS (10 µM) and DMS (5 µM) prevented the rise in NO induced by 20 µM ABA. B, In contrast, DL-*threo* DHS and DMS did not affect the rise in ROS caused by ABA.

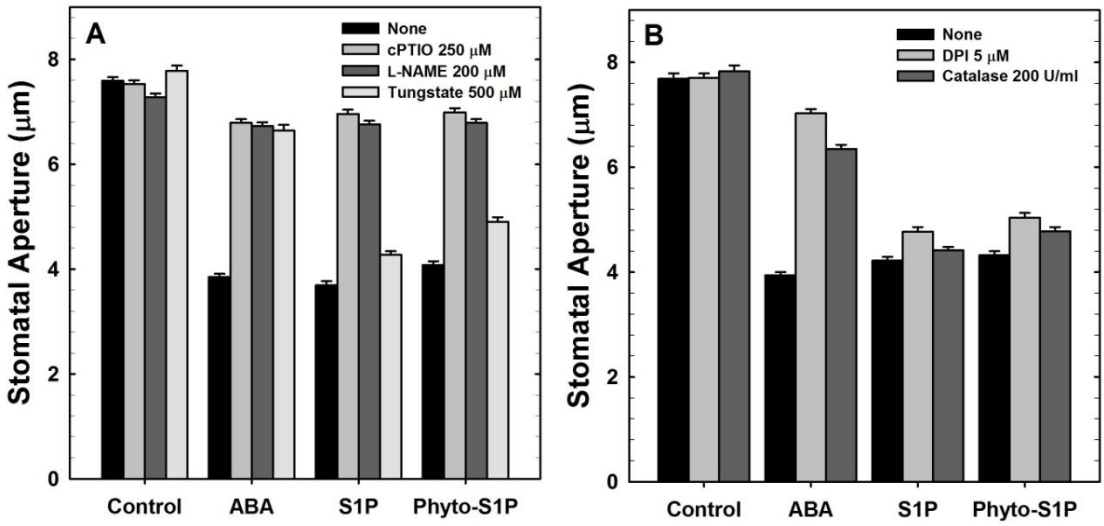

Figure 5.6: Effect of NO/ROS modulators on stomatal closure by ABA, S1P or Phyto-S1P. A, cPTIO (NO scavenger) or L-NAME (NOS inhibitor) relieved stomatal closure by ABA, S1P or Phyto-S1P. However, tungstate (NR inhibitor) relieved stomatal closure only by ABA, but not by S1P or Phyto-S1P. B, Catalase or DPI (ROS modulators) relieved stomatal closure by ABA, but had negligible effect on stomatal closure by S1P or Phyto-S1P.

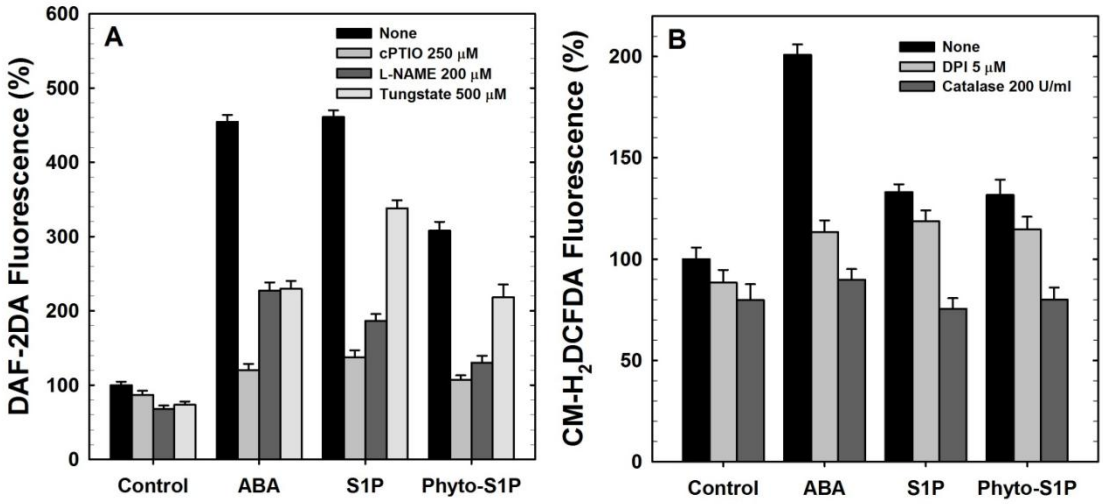

Figure 5.7: Effect of NO/ROS modulators on the rise in NO/ROS induced by ABA, S1P or Phyto-S1P. The levels of NO or ROS were monitored by DAF-2DA and CM-H_2DCFDA fluorescence, respectively. A, cPTIO (NO scavenger) relieved the rise in NO by ABA, S1P or Phyto-S1P. However, L-NAME (NOS inhibitor) or tungstate (NR inhibitor) could prevent only partially such rise in NO. In particular, the effect of tungstate was quite weak in case of S1P induced NO production. B, Catalase or DPI (ROS modulators) relieved the rise in ROS induced by ABA, S1P or Phyto-S1P.

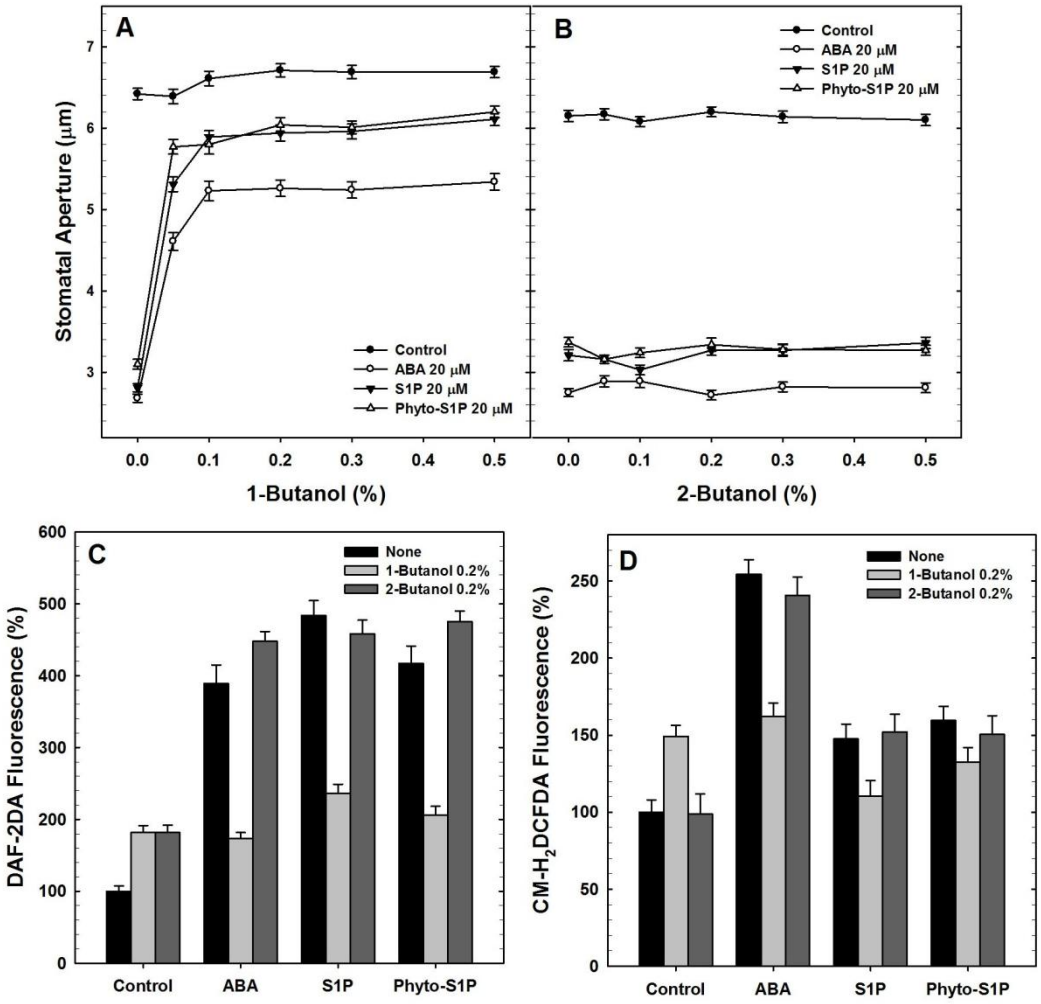

Figure 5.8: Effect of PLD modulators on stomatal closure in abaxial epidermis of pea and increase of NO/ROS in guard cells by ABA, S1P, Phyto-S1P or PA. A, Stomatal closure by ABA/S1P/Phyto-S1P was prevented by 1-butanol (PLD inhibitor). B, 2-butanol could not impair stomatal closure by ABA/S1P/Phyto-S1P. C, Increase of NO by ABA/S1P/Phyto-S1P was prevented by 1-butanol but not by 2-butanol. (D) Elevation of ROS in guard cells by ABA/S1P/Phyto-S1P was prevented by 1-butanol but not by 2-butanol.

Such stomatal closure by PA was not prevented by SPHK inhibitors, DL-threo DHS and DMS 5.9B). However, the PA induced stomatal closure was prevented by wortmannin or LY294002, inhibitors of PI3K (Fig. 5.9C). PI3K inhibitors, wortmannin and LY294002 prevented stomatal closure by ABA as well as S1P/Phyto-S1P (Fig. 5.10 A, B). Maximum reversal of stomatal closure occurred at 0.5 µM wortmannin and 100 µM LY294002. Both wortmannin and LY294002 completely prevented the increase in ROS by ABA/S1P/Phyto-S1P (Fig. 5.10D), while partially preventing the increase of NO (Fig. 5.10C).

Differential responses of *Arabidopsis* Mutants: ABI1 and ABI2 or RBOHD/F in terms of stomatal closure and rise in NO/ROS of guard cells

Experiments were performed with three mutants (lacking ABI1 and ABI2 or RBOHD/F). ABA/S1P/Phyto-S1P caused marked stomatal closure in wild type (La er) *Arabidopsis thaliana*. ABA induced stomatal closure was impaired in both *abi1* closure was impaired in *abi1* and *abi2* mutants. S1P and Phyto-S1P induced normal stomatal closure in *wild type* and *abi2*, but partially impaired closure in *abi1*. B, ABA, S1P and Phyto-S1P induced normal stomatal closure in wild type (Col-0). ABA induced stomatal closure was completely impaired in *rbohD/F*. Stomatal closure by S1P/Phyto-S1P was marginally impaired. and *abi2* mutants. In contrast, S1P/Phyto-S1P induced stomatal closure was partially relieved in *abi1* but not in *abi2* (Fig. 5.11 A). Similarly, ABA/S1P/Phyto-S1P induced ROS and NO production also was impaired in *abi1* but not in *abi2* (Figs. 5.12 to 5.14).

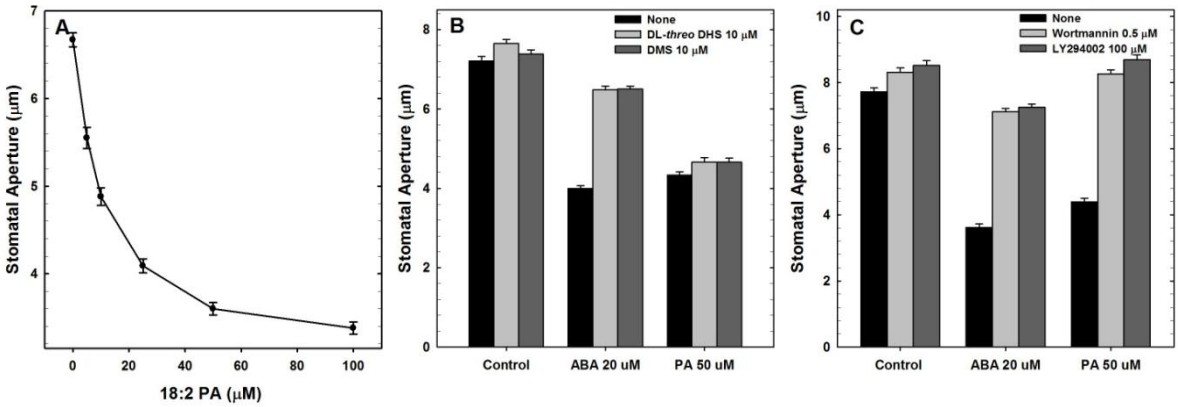

Figure 5.9: PA induced stomatal closure and effect of SPHK/PI3K inhibitors on it. A, PA caused stomatal closure in concentration dependent manner. B, SPHK inhibitors DL-*threo* DHS or DMS have no considerable effect on stomatal closure by PA. C, Two PI3K inhibitors wortmannin or LY294002 prevented PA induced stomatal closure as in case of ABA.

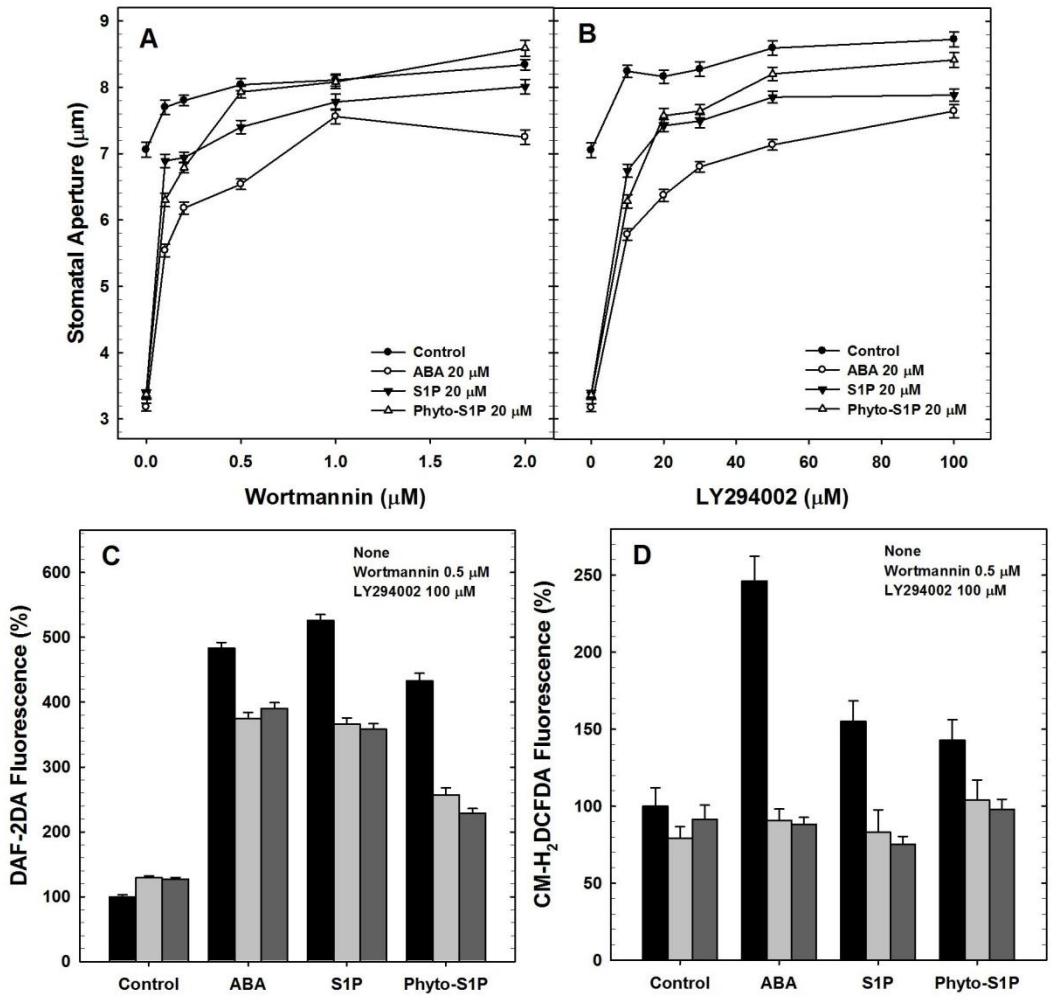

Figure 5.10: Effect of PI3K modulators on stomatal closure and increase of NO/ROS by ABA/S1P/Phyto-S1P. The levels of NO or ROS were monitored by DAF-2DA and CM-H_2DCFDA fluorescence, respectively. Stomatal closure by ABA/S1P/Phyto-S1P was prevented by (A) wortmannin or (B) LY294002 in concentration dependent manner. C, Wortmannin or LY294002 partially prevented increase of NO by ABA/S1P/Phyto-S1P. D, ROS production by ABA/S1P/Phyto-S1P was completely prevented by wortmannin or LY294002.

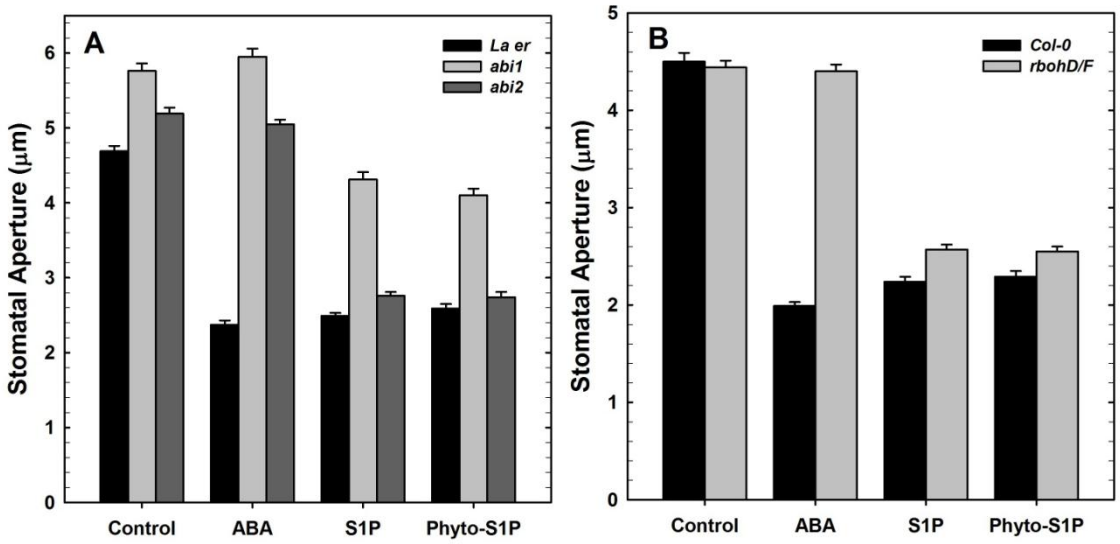

Figure 5.11: Stomatal closure by ABA, S1P/Phyto-S1P in *Arabidopsis* PP2C mutants *abi1* or *abi2* and ROS deficient double mutant *rbohD/F*. A, ABA induced normal stomatal closure in wild type (La er) and ABA induced stomatal

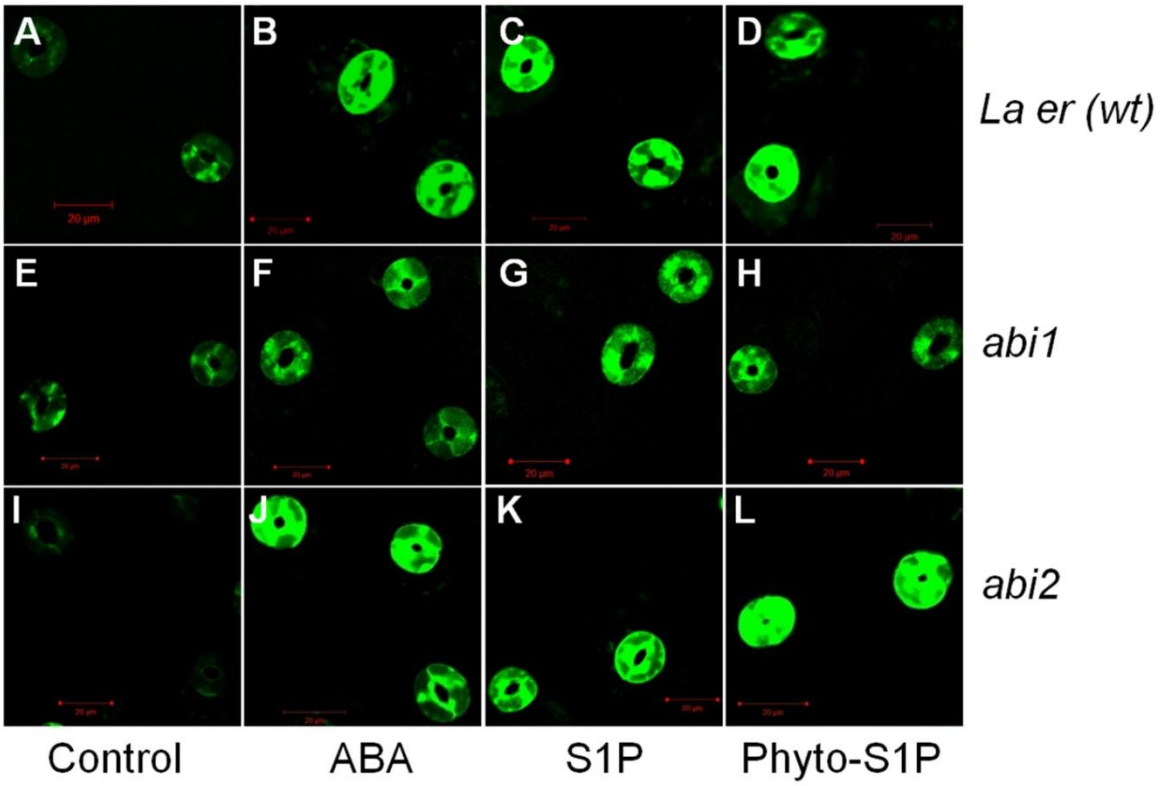

Figure 5.12: Confocal images of NO in guard cells of *Arabidopsis thaliana* wild type and PP2C mutants *abi1* and *abi2* in response to (20 µM) ABA, S1P or Phyto-S1P. A-D, *wild type* (La er); E-H, *abi1*; I-L, *abi2*. ABA, S1P or Phyto-S1P induced NO increase in *wild type* and *abi2* mutants but partially impaired NO increase in *abi1*.

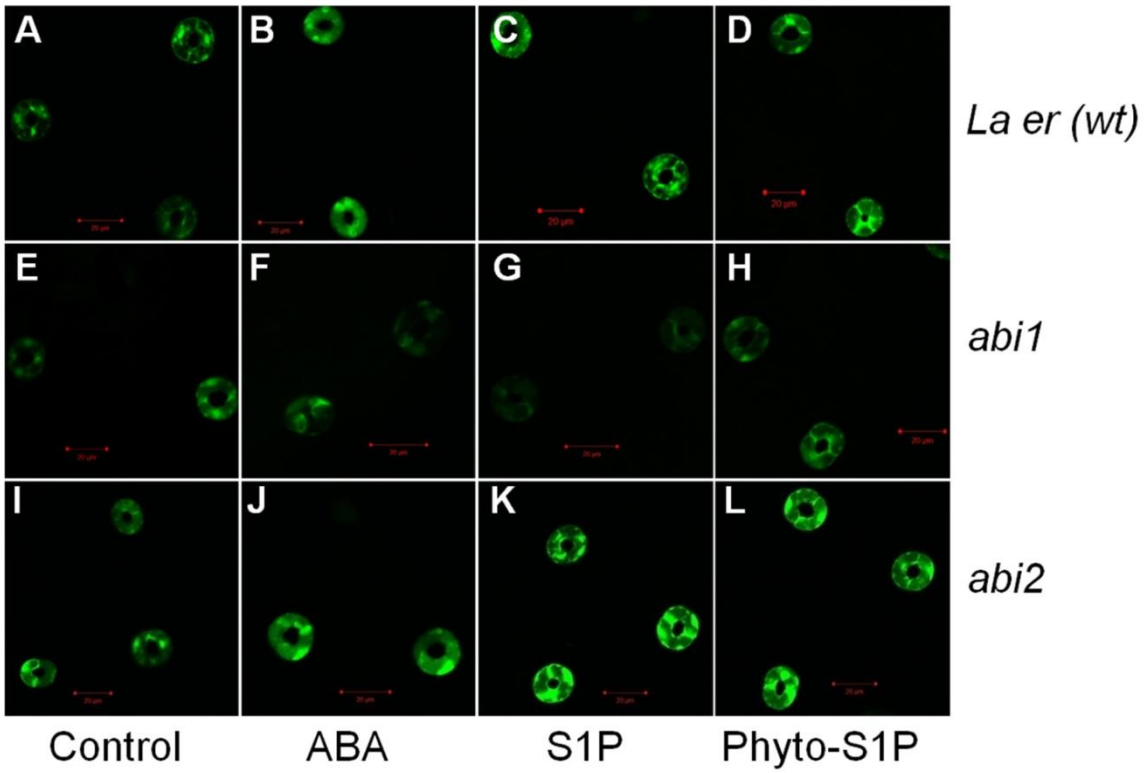

Figure 5.13: Confocal images of ROS in guard cells of *Arabidopsis thaliana* wild type and PP2C mutants *abi1* and *abi2* in response to (20 µM) ABA, S1P or Phyto-S1P. A-D, *wild type* (La er); E-H, *abi1*; I-L, *abi2.* ABA induced rapid ROS production in wild type and abi2. In contrast, S1P or Phyto-S1P induced marginal ROS production in both *wild type* and *abi2* compare to ABA similar to *Pisum sativum* guard cells. ABA, S1P or Phyto-S1P failed to increase ROS production in *abi1*.

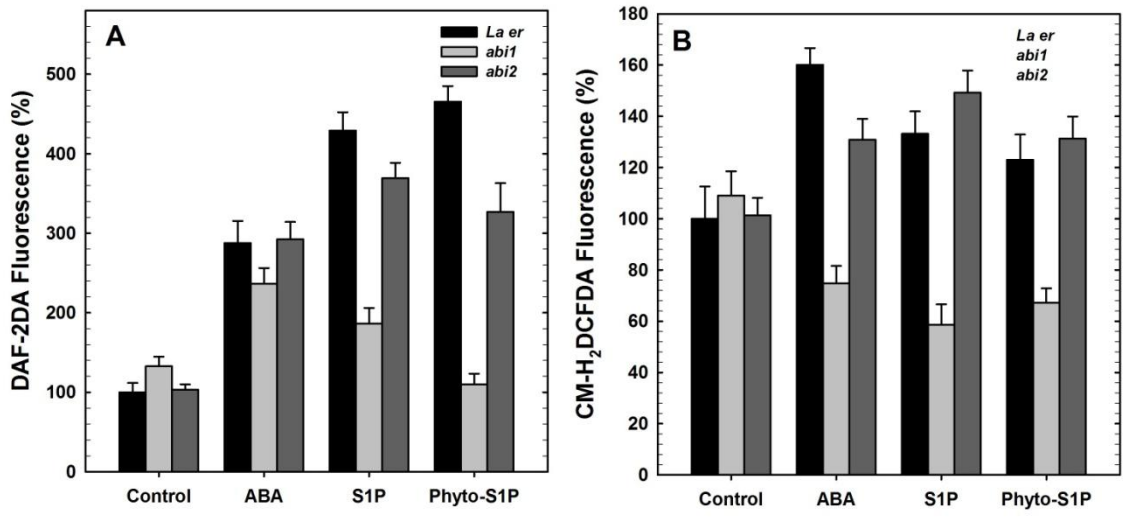

Figure 5.14: Quantitative changes in NO/ROS of guard cells, by ABA, S1P/Phyto-S1P in *Arabidopsis* PP2C mutants *abi1* or *abi2*. The levels of NO or ROS were monitored by DAF-2DA and CM-H_2DCFDA fluorescence, respectively. A, ABA, S1P and Phyto-S1P induced NO production in wild type as in case of *Pisum sativum*. NO production by ABA/S1P/Phyto-S1P was partially impaired in *abi1* but not in *abi2*. B, ABA, S1P and Phyto-S1P induced ROS production was similar to *Pisum sativum* in wild type. ROS production by ABA/S1P/Phyto-S1P was impaired in *abi1* but not in *abi2*.

ABA/S1P/Phyto-S1P caused stomatal closure and rise in NO and ROS of guard cells in wild type (Col-0) *Arabidopsis thaliana*. Stomatal closure and ROS production by ABA as well as S1P/Phyto-S1P were impaired in *rbohD/F* (Fig. 5.11B). The increase of NO by S1P/Phyto-S1P was only partially affected in *rbohD/F* (Figs.5.15 to 5.17).

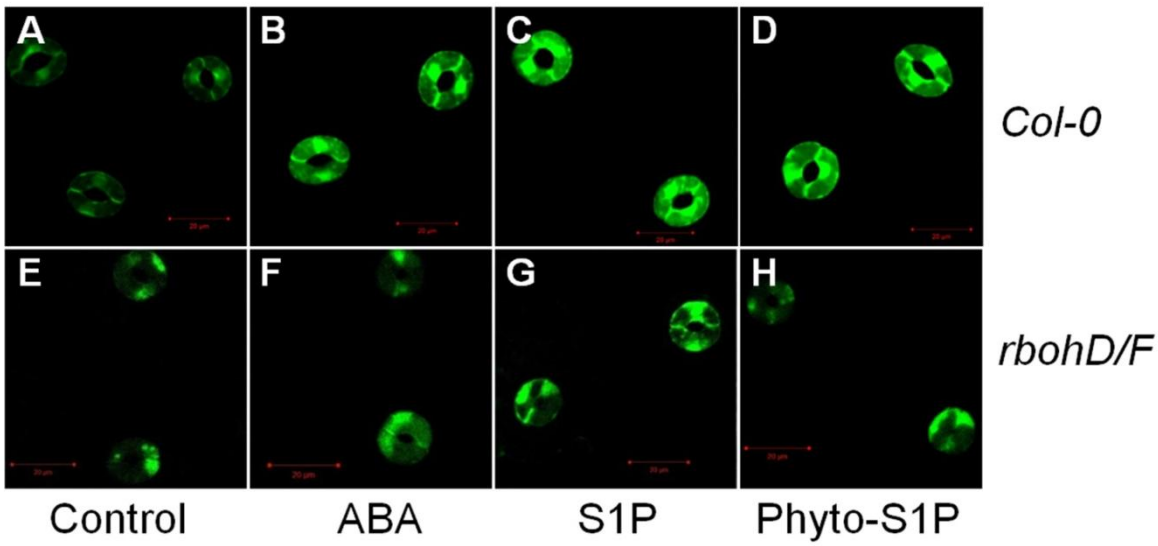

Figure 5.15: Confocal images of NO in guard cells of *Arabidopsis thaliana* wild type and *rbohD/F* mutants in response to (20 µM) ABA, S1P or Phyto-S1P. A-D, *wild type* (*Col-0*); E-H, *rbohD/F*. ABA, S1P or Phyto-S1P induced NO increase in *wild type* but partially impaired NO increase in *rbohD/F*.

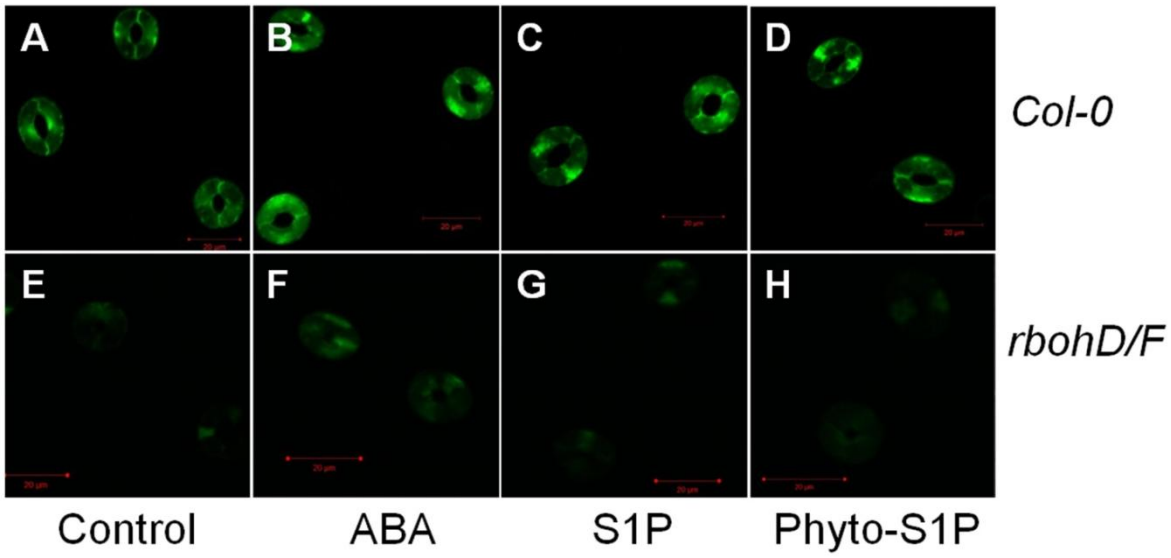

Figure 5.16: Confocal images of ROS in guard cells of *Arabidopsis thaliana* wild type and *rbohD/F* mutants in response to (20 µM) ABA, S1P or Phyto-S1P. A-D, *wild type* (*Col-0*); E-H, *rbohD/F*. In *wild type,* ABA induced rapid ROS production, while S1P or Phyto-S1P induced marginal ROS production and ABA, S1P or Phyto-S1P failed to increase ROS production in *rbohD/F*.

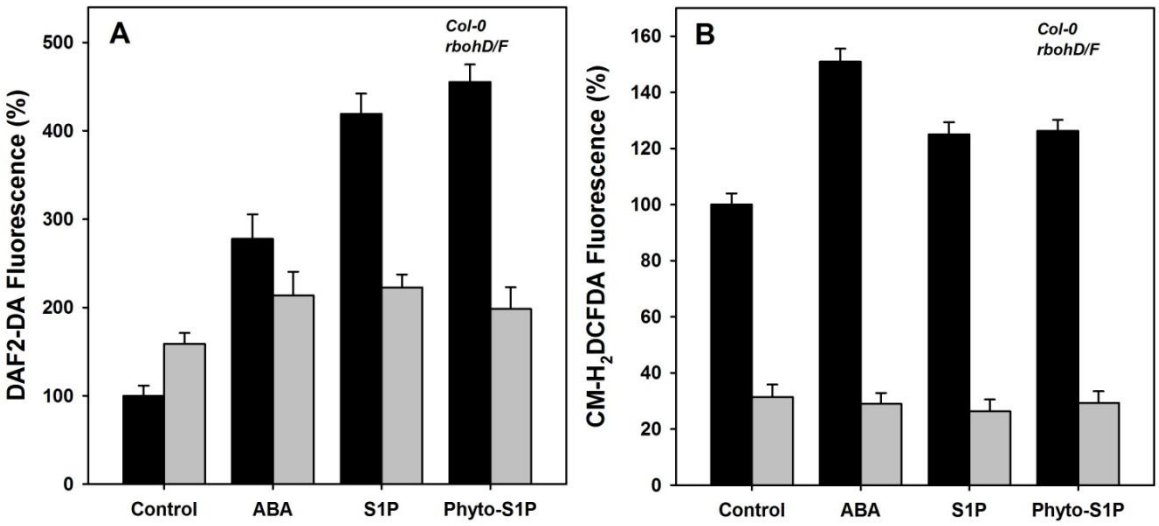

Figure 5.17: Stomatal closure and changes in NO/ROS by ABA, S1P/Phyto-S1P in *Arabidopsis* ROS deficient double mutant *rbohD/F*. The levels of NO or ROS were monitored by DAF-2DA and CM-H_2DCFDA fluorescence, respectively. A, ABA, S1P or Phyto-S1P induced normal NO increase in wild type but decreased NO increase in *rbohD/F*. B, ABA and S1P/Phyto-S1P increased ROS production was normal in wild type but completely impaired in *rbohD/F*.

Discussion

The present article confirms that the marked stomatal closure in abaxial epidermis of pea by S1P and Phyto-S1P was as strong as with ABA. Our results suggest that such S1P/Phyto-S1P induced stomatal closure depends on the marked rise in NO, but not that of ROS. The relative independence of S1P/Phyto-S1P-induced stomatal closure on ROS was validated by studies on *Arabidopsis* mutants, with limited capacity of ROS production in their guard cells, such as *abi1* and *rbohD/F*.

S1P and Phyto-S1P promote stomatal closure, while elevating NO of guard cells

The marked promotion of stomatal closure in abaxial epidermis of pea by S1P/Phyto-S1P as efficient as ABA (Fig. 5.1) and the prevention by SPHK inhibitors (DL-*threo* DHS and DMS) the stomatal closure (Fig. 5.4), together suggest that S1P and Phyto-S1P play an important role in regulating ABA-induced stomatal closure. Obviously, ABA promotes the activity of SPHK, facilitates the production of S1P/Phyto-S1P and leads to stomatal closure (Guo et al., 2012). Our results clear the ambiguity and endorse the view that S1P and Phyto-S1P are important signaling components during ABA-induced stomatal closure (Ng et al., 2001; Coursol et al., 2003, 2005; Worrall et al., 2008). The prevention of ABA-induced stomatal closure by DL-threo DHS/DMS (Fig. 5.4) complements the report of Worrall et al. (2008) that SPHK can modulate ABA responses in guard cells.

Kinetics of ROS production in guard cells and its role during stomatal closure by S1P/Phyto-S1P different from that in case of ABA

Increase of ROS as well as NO in guard cells is an early event during stomatal closure by signals such as ABA, methyl jasmonate, and chitosan (Kwak et al., 2003, Suhita et al., 2004, Srivastava et al., 2009). In *Pisum sativum,* ABA induced a rapid increase of ROS during stomatal closure, while S1P or Phyto-S1P caused only a limited production of ROS (Fig. 5.3B). In contrast, the extent of NO production by S1P or Phyto-S1P was quite similar to that by ABA (Fig. 5.3A). The importance of NO along with relatively subdued role of ROS, was confirmed by experiments employing NO/ROS modulators. ABA induced stomatal closure was impaired in presence of DPI (NADPH oxidase inhibitor) or catalase (ROS scavenger), while catalase or DPI had no significant effect on stomatal closure by S1P or Phyto-S1P (Fig. 5.6B).

The difference in the role of ROS during the effects of ABA and S1P/Phyto-S1P was also noticed in relation to kinetics of changes in ROS/NO in guard cells. Unlike in case of ABA, the increase in ROS was quite limited, while the rise in NO was quite pronounced in case of S1P or Phyto-S1P (Fig. 5.3). These results reaffirm that the S1P or Phyto-S1P induced stomatal closure differs from that of ABA, in the dominant role of NO but not of ROS, in guard cells.

Interaction with other signaling components: PLD, PA and PI3K

Like in case of ABA, S1P/Phyto-S1P interacts with PLD, PA and PI3K during stomatal closure. The prevention by 1-butanol (a PLD inhibitor) of S1P/Phyto-S1P-induced stomatal closure and increase of NO/ROS (Fig. 5.8) implying the participation

of PLD downstream of S1P, is in conformity with earlier reports (Guo et al., 2011; 2012). *Arabidopsis* mutants, lacking PLDα1, did not exhibit increase in ROS or NO by ABA (Uraji et al., 2012). Although, PA triggered stomatal closure in *Pisum sativum* (Fig. 5.9A), the inability of SPHK inhibitors to prevent stomatal closure by PA (Fig. 5.9B), in comparison with the effect of wortmannin or LY294002 (Fig. 5.9C) demonstrating the participation of PI3K downstream of S1P/Phyto-S1P during stomatal closure by ABA. The participation of PI3K in ABA-induced ROS-generation and stomatal closure was observed by Park et al. (2003).

The importance of PI3K is further reflected in our observations, where in Wortmannin or LY294002 (two PI3K inhibitors) prevented stomatal closure as well as ROS production by ABA/S1P/Phyto-S1P (Figs. 5.10A-D). All these results suggest that the signaling pathway deviates from PLDα1 towards PA and then to RBOHD/F, leading to H_2O_2 production. We therefore propose that ABA as well as S1P/Phyto-S1P interact with PLD/PA/PI3K, with divergence of signaling events downstream of PLDα1.

Stomatal closure by S1P/Phyto-S1P is not impaired in mutants deficient in ROS production

The relative independence of S1P/Phyto-S1P stomatal closure on ROS production, in contrast to that of ABA, is quite interesting but not surprising. Such ROS-independent stomatal closure is known in mutants of *Arabidopsis*. For example, stomatal closure as well as ROS production is impaired in *abi1* (Murata et al., 2001). Similarly, *gpa1* mutants are deficient in ROS production but close their stomata in response to ABA or H_2O_2 (Mishra et al., 2006; Zhang et al., 2011). The *rbohD/F* mutants, lacking NADPH oxidases, are classic examples, incapable of ROS production

and stomatal closure in response to ABA (Kwak et al., 2003). All the three mutants we examined (*abi1, abi2, rbohD/F*), closed their stomata (to varying extent) in response to S1P/Phyto-S1P while showing up a detectable rise in NO of guard cells (Figs. 11-17). However, there were no significant increases in ROS levels of their guard cells, when exposed to S1P/Phyto-S1P or even ABA in two mutants: *abi1* and *rbohD/F*. Our results with *Arabidopsis* mutants reaffirm that the stomatal closure induced by S1P/Phyto-S1P is associated with NO production but not always with ROS. Functional differences between ABI1 and ABI2 are realized. ABA induced ROS production was impaired in *abi1* but not *abi2* (Murata et al., 2001). ABA and H_2O_2 activation of S-type anion channels was inhibited by ABI2 but not ABI1 (Hua et al., 2012).

Concluding Remarks

The stomatal closure caused by ABA is well known to be associated with increases in ROS/NO/Ca^{2+} of guard cells. However, S1P-induced stomatal closure occurs with marked rise in NO but not always of ROS. Obviously, the production of NO is not completely dependent on RBOHD/F or H_2O_2. The ROS-independent signal transduction appears to be mediated by the divergence of pathway either above or below RBOHD/F or both. In case of ABA, closure was dependent on both ABI1 & ABI2 besides RBOHD/F. In contrast, S1P effect appears to be partly dependent on ABI1 but quite independent of either ABI2 or RBOHD/F. Based on the differential responses of mutants (*abi1, rbohD/F* and *abi2*) to S1P, a scheme can be proposed. The signaling cascade initiated by ABA, leading to stomatal closure, involves ABI1, RBOHD/F, H_2O_2, ABI2, and NO. In contrast, the effect of S1P is partially dependent on ROS production mediated by PLDα1, PA, PI3K, ABI1 and RBOHD/F. The events leading to S1P/Phyto-

S1P induced NO-production appear to involve PLDα1, independent of RBOHD/F & ABI2. Alternatively NO production can be stimulated by H_2O_2 produced due to the activation of PLDα1, PA and finally RBOHD/F. Stomatal closure by NO, H_2O_2 and Ca^{2+} depends on slow type anion channels (SLACs) (Vahisalu et al., 2008). The proposed scheme also explains the possibility of stomatal closure in response to ABA, independent of SPHK and S1P, besides illustrating the distinction of ABI1 & ABI2. We suggest that S1P and Phyto-S1P could be important tools to distinguish the role of NO and ROS during stomatal closure.

A scheme incorporating the points and proposing the possible scheme of events during stomatal closure by ABA or S1P is presented in Fig. 5.18

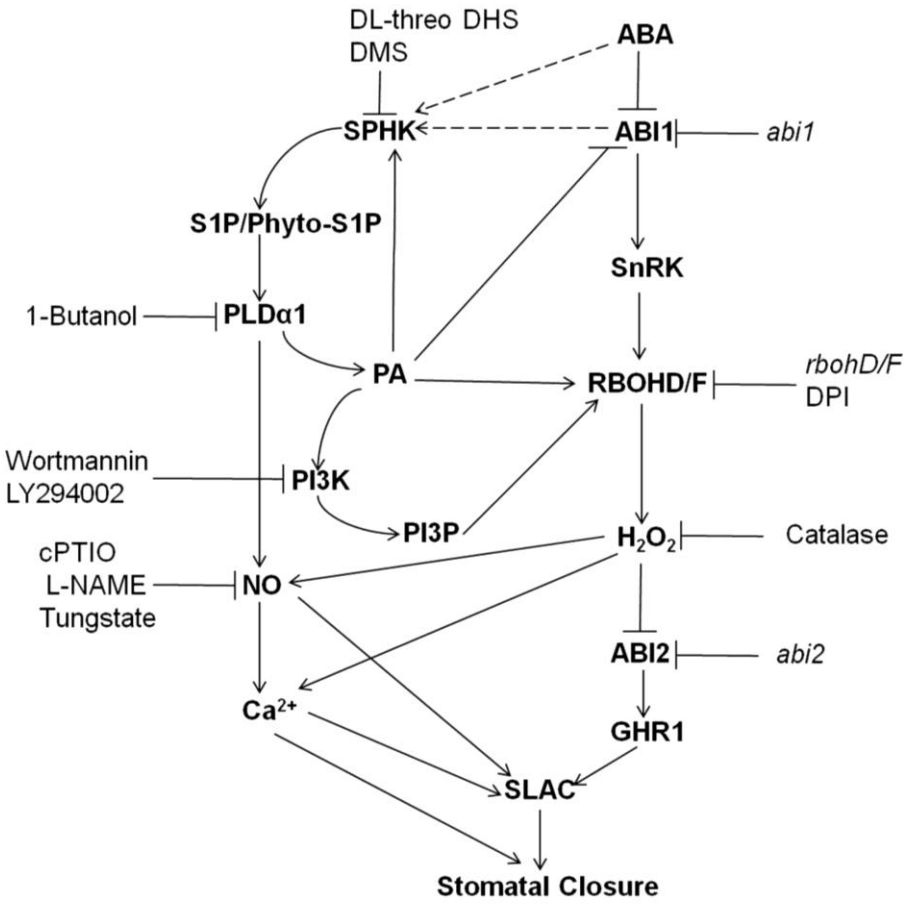

Figure 5.18: Scheme proposed to explain the signaling cascade in guard cells. The signaling cascade initiated by ABA, leading to stomatal closure, involves ABI1, RBOHD/F, H_2O_2, ABI2, and NO. In contrast, the effect of S1P is partially dependent on ROS production mediated by PLDα1, PA, PI3K, ABI1 and RBOHD/F. The events leading to S1P/Phyto-S1P induced NO-production appear to involve PLDα1, independent of RBOHD/F & ABI2. Alternatively NO production can be stimulated by H_2O_2 produced due to the activation of PLDα1, PA and finally RBOHD/F. Both ROS and NO converge at S-type anion channels (SLAC) to induce stomatal closure. The proposed scheme also explains the possibility of stomatal closure in response to ABA, independent of SPHK and S1P, besides illustrating the distinction of ABI1 & ABI2.

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Chapter 6

CYTOPLASMIC pH RISE AS AN EARLY SIGNAL DURING SPHINGOSINE-1-PHOSPHATE/PHYTOSPHINGOSINE-1-PHOSPHATE INDUCED STOMATAL CLOSURE

Introduction

Bioactive lipids such as sphingosine-1-phosphate (S1P)/phytosphingosine-1-phophate (Phyto-S1P), phosphatidic acid (PA) and phosphatidylinositol-3-phosphate (PI3P) are important regulators and act as ABA signaling molecules in guard cells. ABA activates enzymes such as sphingosine kinases (SPHKs), phospholipase Ds (PLDs), phosphatidylinositol-3-kinase (PI3K) to produce the above lipid messengers. In addition, ABA enhances the levels of ROS, NO and cytoplasmic pH in guard cells. In our previous chapter, we reported that, S1P or Phyto-S1P induced stomatal closure by marked elevation of NO but not of ROS. We further reported that PLD/PA, PI3K and ABI1 acts downstream of SPHK/S1P/Phyto-S1P during ABA induced stomatal closure.

The participation of pH as a signal in ABA induced stomatal closure is supported by two observations: ABA exposure raises the intracellular pH of guard cells by 0.1 to 0.3 units (Irving et al., 1992). Decreasing of intracellular pH by a weak acid (e.g. butyrate) prevents the K^+ efflux and activate K^+ influx by the regulation of K^+ inward/outward rectifying channels (Blatt, 2000). Cytoplasmic pH increase was an early signal in guard cells after exposure to ABA and precedes ROS and NO in guard cells of *Arabidopsis thaliana* and *Pisum sativum* (Suhita et al., 2004; Gonugunta et al., 2008). Butyrate prevented the stomatal closure and pH increase by ABA in both *Arabidopsis thaliana* and *Pisum sativum* guard cells. Changes in intracellular pH might be acts

independent of Ca^{2+} increase in guard cells (Sirichandra et al., 2009). However, there have been no reports on cytoplasmic pH change in guard cells during S1P or Phyto-S1P induced stomatal closure. The present chapter explains the importance of SPHK and S1P/Phyto-S1P in ABA induced cytoplasmic pH raise and effect on guard cell pH. We further examined the interaction of S1P/Phyto-S1P induced cytoplasmic pH rise with PLD/PA, PI3K and Ca^{2+}.

Results

S1P/Phyto-S1P caused a marked rise in cytoplasmic pH during induction of stomatal closure

Presence of S1P/Phyto-S1P induced stomatal closure in *Pisum sativum* epidermis and the decrease in width of stomatal apertures was observed as early as 30 min, as in case of ABA (Fig. 6.1). The maximum stomatal closure was observed after 120 min (Fig. 6.1). Further, S1P/Phyto-S1P raised the levels of cytoplasmic pH of guard cells, again similar to the effect of ABA (Fig. 6.2). However, the kinetics of pH rise by ABA, S1P or Phyto-S1P, in guard cells, were quite different. ABA-triggered pH rise was gradual upto 1.7 fold within 18 min and then decreased. S1P raised the levels of pH upto 2.3 fold within 9 min. Phyto-S1P triggered the pH rise upto 1.8 fold within 3 min, and was stable until 21 min (Fig. 6.3).

Butyrate, a weak acid, prevented S1P/Phyto-S1P induced stomatal closure. The ABA induced stomatal closure was partially prevented by butyrate (Fig. 6.4A). Further, presence of butyrate prevented the increase of cytoplasmic pH by S1P/Phyto-S1P as in

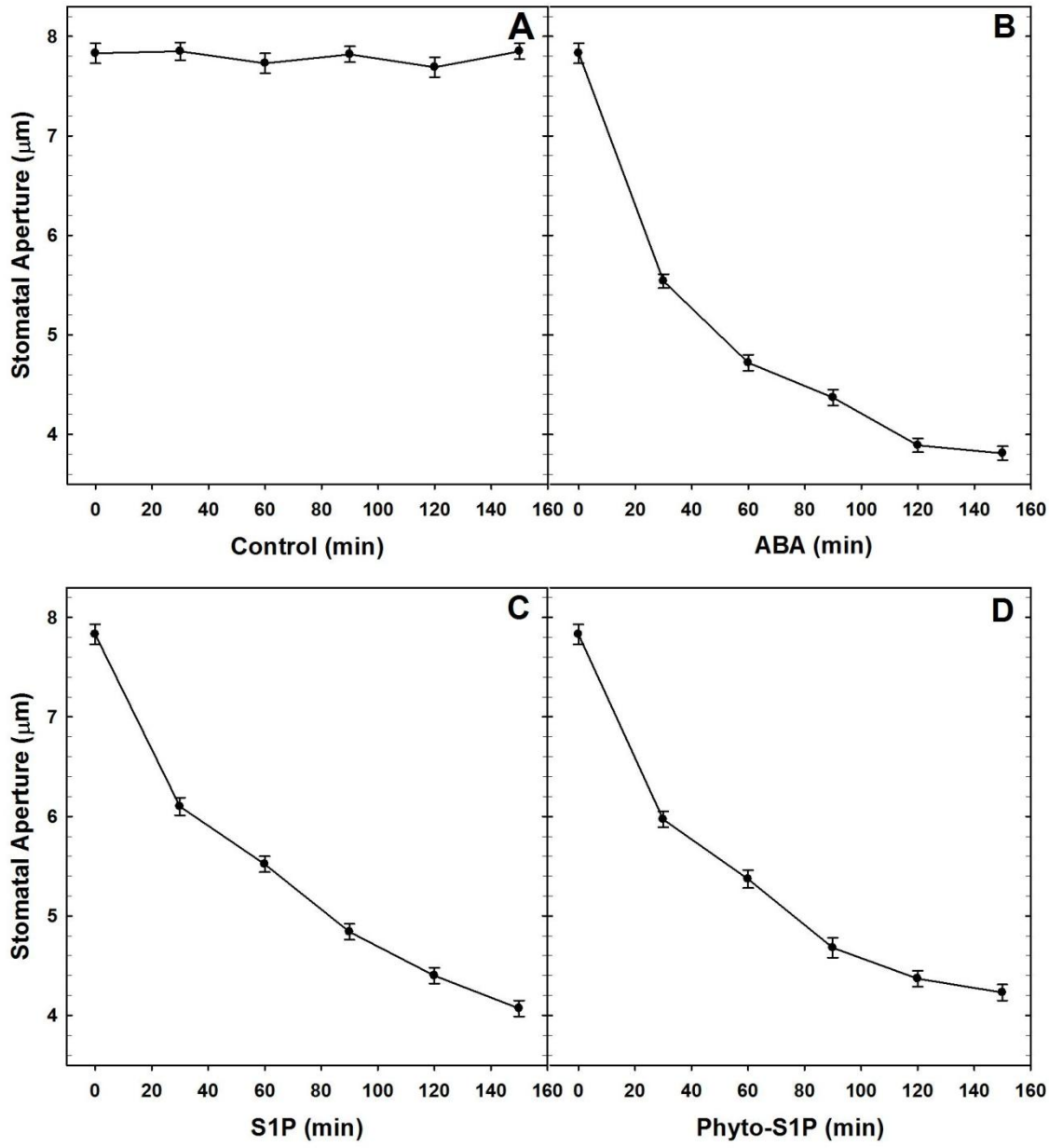

Fig. 6.1: Stomatal closure in *Pisum sativum* epidermis caused by ABA or S1P/Phyto-S1P. A, Control; B, ABA: C, S1P; D, Phyto-S1P (all at 20 μM). ABA, S1P or Phyto-S1P induced stomatal closure with time. The maximum stomatal closure occurs after 120 min. However, there was no significant change observed in control set.

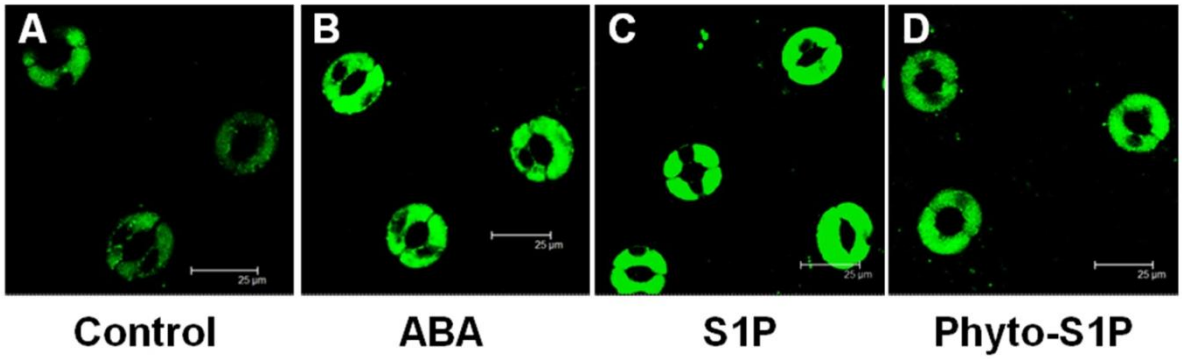

Fig. 6.2: Panels showing representative images of pH indicative BCECF-AM fluorescence after 20 min treatment with different modulators. ABA, S1P or Phyto-S1P (at 20 µM) increased cytoplasmic pH levels in guard cells, compared to control.

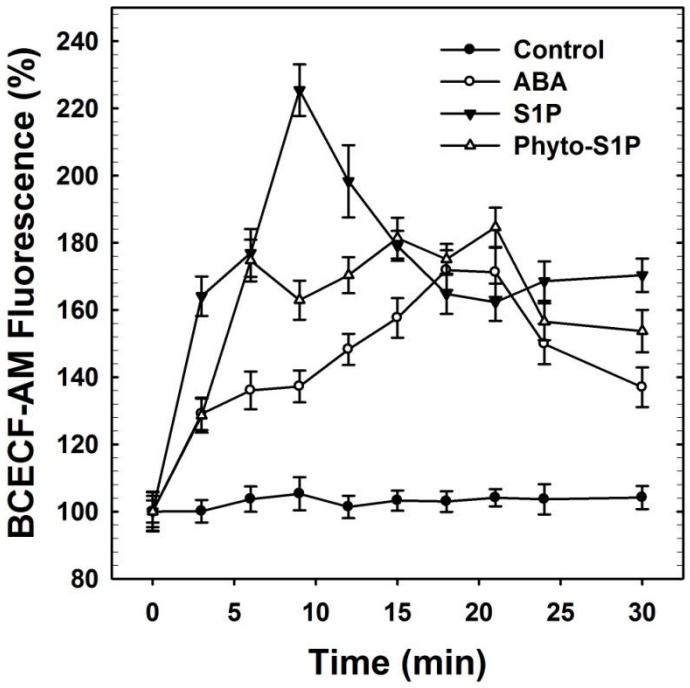

Fig. 6.3: Patterns of cytoplasmic pH change in guard cells by ABA, S1P or Phyto-S1P (at 20 µM). ABA increased pH of guard cells until 18 min and then decreased. The increase of guard cell pH by S1P is rapid and the peak reached within 9 min. Phyto-S1P caused rise in pH of guard cells by 3 min and the rise was stable until 21 min. S1P and Phyto-S1P triggered stronger pH rise than ABA.

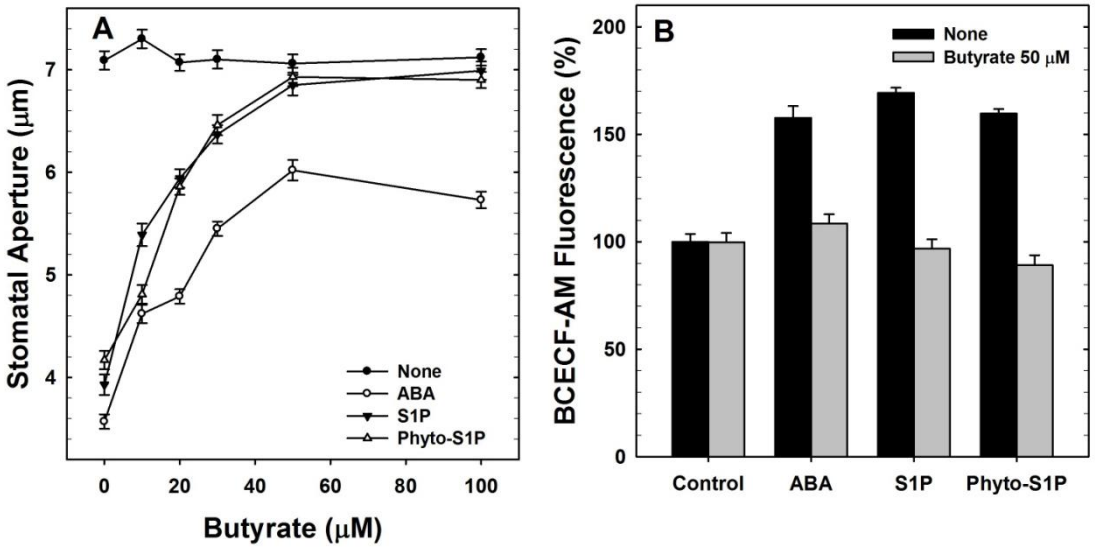

Fig. 6.4: Importance of cytoplasmic pH during stomatal closure by ABA, S1P or Phyto-S1P. A, Butyrate, a weak acid prevented stomatal closure by ABA, S1P or Phyto-S1P. B, Butyrate prevented the increase of pH by S1P or Phyto-S1P besides ABA.

case of ABA (Fig. 6.4B). SPHK inhibitors DL-threo DHS and DMS prevented both stomatal closure and increase of pH by ABA (Fig. 6.5).

Stomatal closure and pH rise by S1P/Phyto-S1P in presence of PLD inhibitor and PA

PLD inhibitor 1-butanol (but not 2-butanol) prevented stomatal closure and increase of guard cell pH by ABA, S1P/Phyto-S1P (Fig. 6.6). Presence of PA, (a product of PLD) caused stomatal closure in time dependent manner (Fig. 6.7A). Further, in kinetic studies, PA gradually triggered the cytoplasmic pH rise in guard cells upto 1.4 fold within 25 min (Fig. 6.7B). PA induced stomatal closure was prevented by butyrate (Fig. 6.7C).

Role of PI3K and Ca^{2+} in stomatal closure and pH rise by ABA/S1P/Phyto-S1P

Wortmannin and LY294002, two inhibitors of PI3K, prevented stomatal closure as well as cytoplasmic pH rise by S1P or Phyto-S1P, as in case of ABA (Fig. 6.8), suggesting the participation of PI3K upstream of cytoplasmic pH raise and downstream of SPHK/S1P/Phyto-S1P. In addition, calcium chelators BAPTA (for extracellular Ca^{2+}) and BAPTA-AM (for intracellular Ca^{2+}) prevented stomatal closure by ABA, S1P as well as by Phyto-S1P (Fig. 6.9A). However, BAPTA or BAPTA-AM failed to prevent the cytoplasmic pH rise by S1P or Phyto-S1P (Fig. 6.9B).

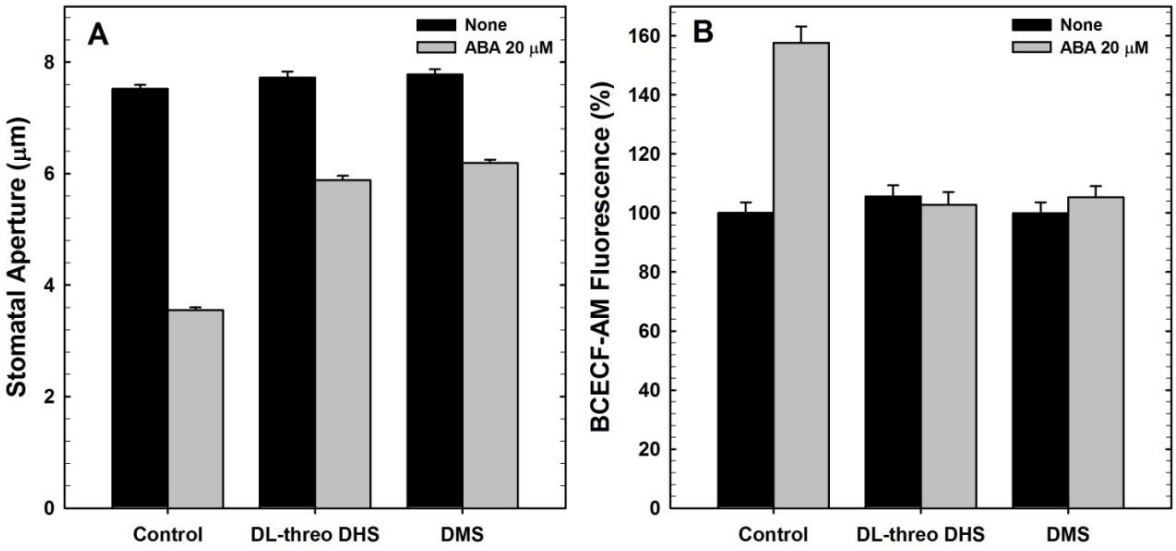

Fig. 6.5: Effect of SPHK inhibitors on stomatal closure and pH increase caused by ABA. DL-threo DHS (10 µM) or DMS (10 µM) partially prevented both stomatal closure (A), and rise in pH (B), by ABA.

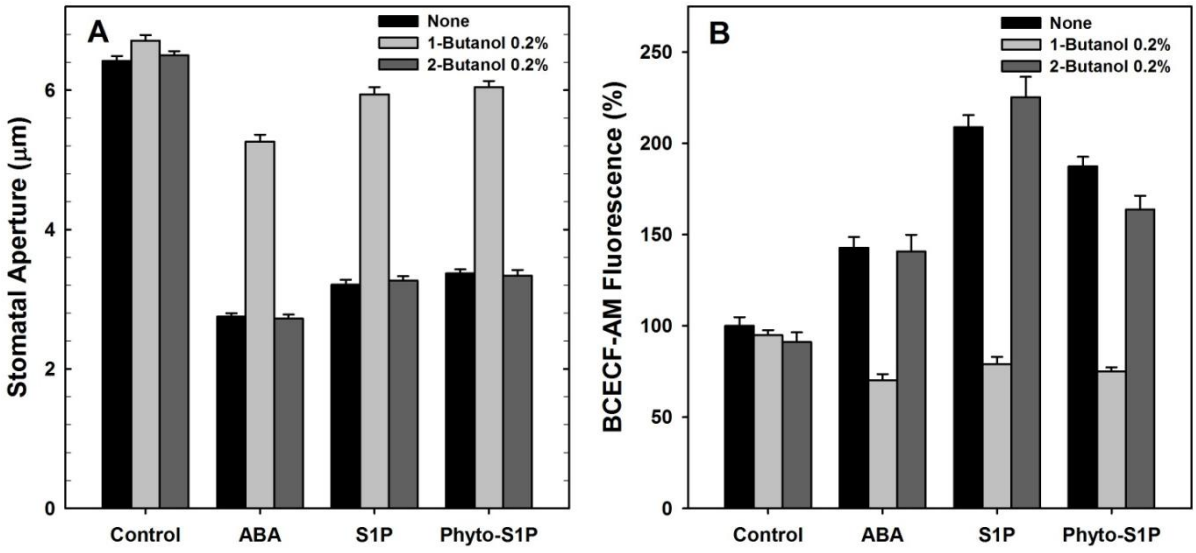

Fig. 6.6: Role of PLD in stomatal closure and increase of pH by ABA, S1P or Phyto-S1P. A, Only 1-butanol (PLD inhibitor), but not 2-butanol prevented stomatal closure by S1P or Phyto-S1P as in case of ABA. B, Similarly, 1-butanol but not 2-butanol prevented cytoplasmic pH increase by S1P or Phyto-S1P.

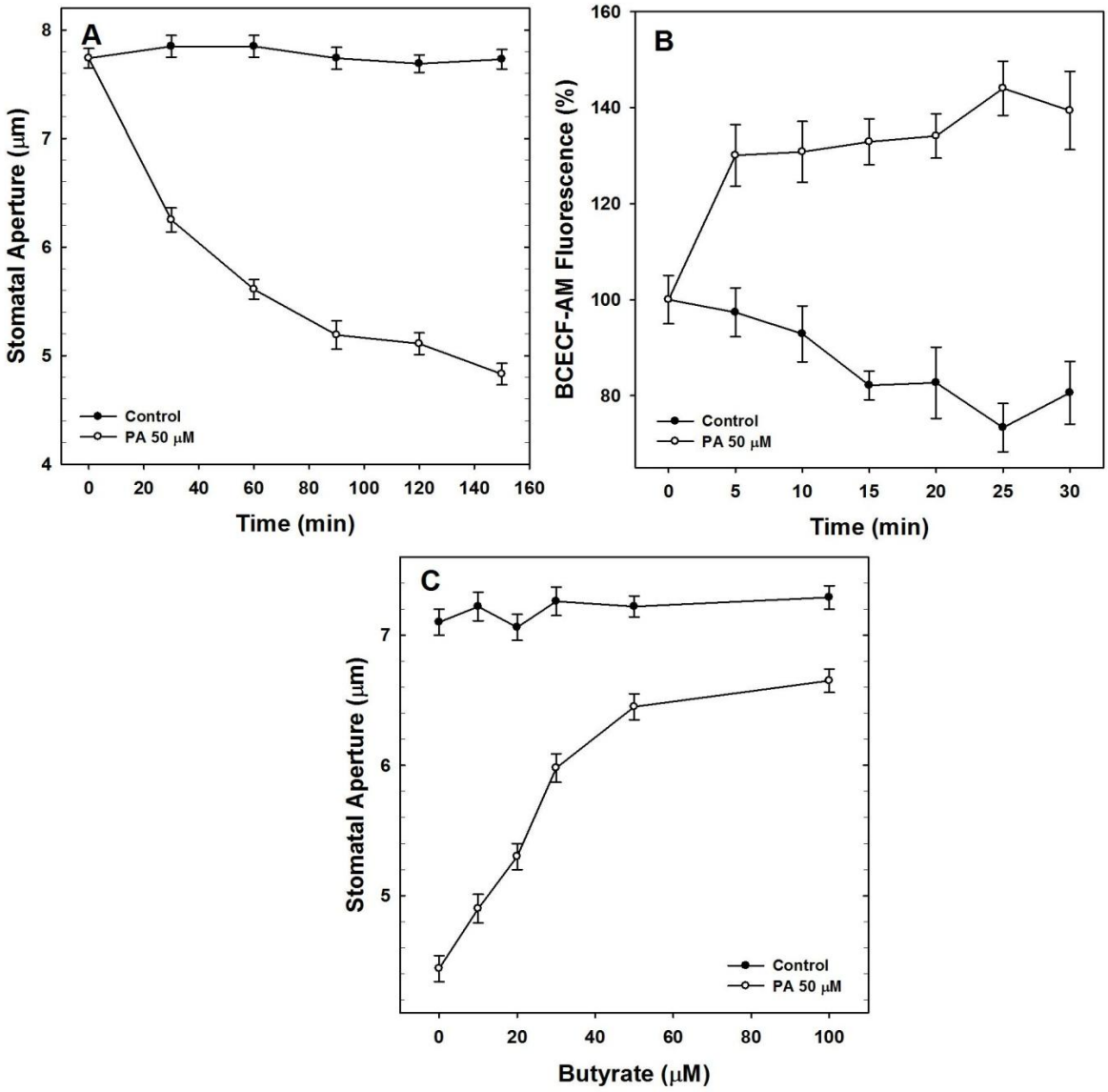

Fig. 6.7: Effect of PA on stomatal closure and guard cell pH. A, PA induced stomatal closure with time. B, PA triggered increase of pH with time. The increase of pH was started within 5 min, peaked at 25 min and thereafter declined. C, Butyrate prevented PA induced stomatal closure in concentration dependent manner.

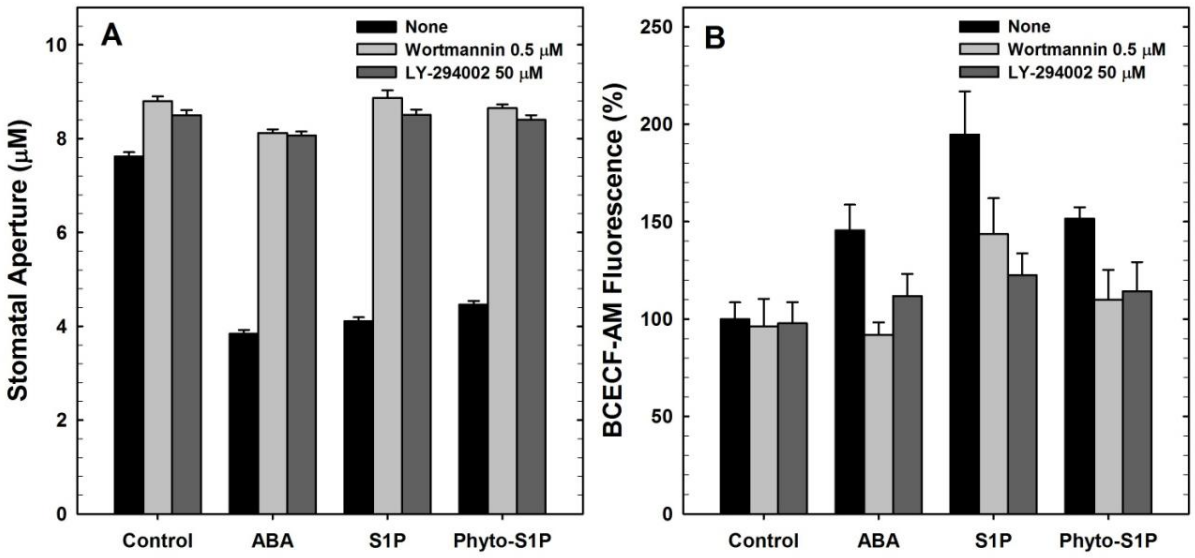

Fig. 6.8: Effect of PI3K inhibitors on stomatal closure and increase of pH. A, PI3K inhibitors wortmannin or LY294002 prevented stomatal closure by ABA, S1P or Phyto-S1P. B, Cytoplasmic pH increase by ABA, S1P or Phyto-S1P was prevented by wortmannin or LY294002.

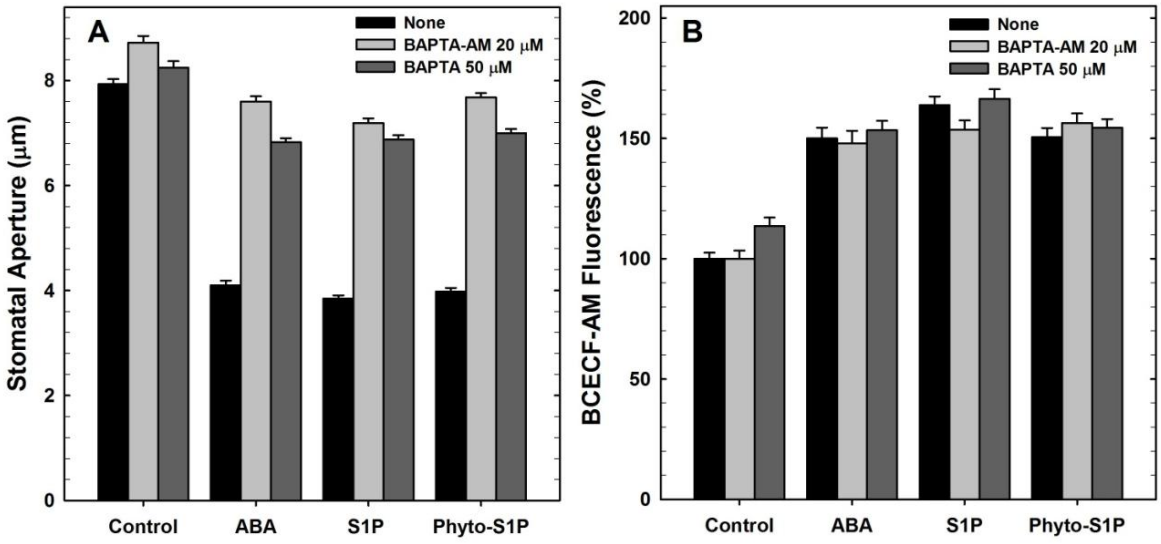

Fig. 6.9: Effect of calcium chelators on stomatal closure and cytoplasmic pH raise by ABA, S1P or Phyto-S1P. A, Both membrane permeable BAPTA-AM and impermeable BAPTA prevented stomatal closure by ABA, S1P or Phyto-S1P. B, BAPTA-AM and BAPTA failed to prevent increase of pH by S1P or Phyto-S1P as well as ABA.

Discussion

Sphingosine kinases and their products S1P/Phyto-S1P, play critical role in stomatal closure caused by ABA (Guo et al., 2012; Guo and Wang 2012). It is known that, ABA while inducing stomatal closure, triggers a marked increase of cytoplasmic pH in guard cells (Irving et al., 1992). Additional reports appeared to suggest an association of pH rise in guard cells as an early event during stomatal closure (Suhita et al., 2004; Gonugunta et al., 2008). Our experiments revealed the participation of cytoplasmic pH downstream of S1P/Phyto-S1P in guard cells of *Pisum sativum* prior to stomatal closure.

S1P/Phyto-S1P induced stomatal closure requires the elevation of cytoplasmic pH in guard cells

Cytoplasmic pH rises within few minutes in guard cells of *Pisum sativum* and *Arabidopsis thaliana,* on exposure to ABA or methyl jasmonate (Suhita et al., 2004; Gonugunta et al., 2008). In the present work too, S1P/Phyto-S1P triggered the rise in pH as early as by 3 min (Fig. 6.2; 6.3). Further, S1P/Phyto-S1P induced stomatal closure and increase of cytoplasmic pH by S1P/Phyto-S1P was prevented by weak acid, butyrate (Fig. 6.4). These points suggest strongly the participation of SPHK/S1P upstream of cytoplasmic pH raise during stomatal closure by ABA. The prevention of ABA induced stomatal closure by SPHK inhibitors, DL-threo DHS and DMS mimicked the effects of *sphk* mutants of *Arabidopsis thaliana* (Worrall et al., 2008; Guo et al., 2012). Hence the validation of above concept using SPHK inhibitors further confirmed the participation of SPHK/S1P upstream of cytoplasmic pH rise during ABA induced

stomatal closure as both DL-threo DHS and DMS prevented ABA induced cytoplasmic pH rise (Fig. 6.5).

Interactions of S1P/Phyto-S1P-induced cytoplasmic pH rise with PLD, and PI3K

PLD and Ca^{2+} two important messengers in guard cells during ABA induced stomatal closure, act downstream of S1P/Phyto-S1P (Ng et al., 2001; Guo et al., 2012). In Arabidopsis, PA triggered SPHK activation, while Phyto-S1P triggered the PA formation by PLD (Guo and Wang 2012). Inhibition of ABA/S1P/Phyto-S1P induced stomatal closure by PLD inhibitor 1-butanol and Ca^{2+} chelators BAPTA and BAPTA-AM (Fig. 6.6A, 6.9A) confirms the participation of PLD and Ca^{2+} downstream of S1P/Phyto-S1P during ABA induced stomatal closure in *Pisum sativum* epidermis. The prevention of ABA induced cytoplasmic pH rise by 1-butanol is consistent with the report of Uraji et al. (2012) with *Arabidopsis thaliana*, where ABA failed to induce cytoplasmic pH raise in a double mutant, *pldα1pldδ*. Further 1-butanol prevented the increase of cytoplasmic pH by S1P/Phyto-S1P (Fig. 6.5B). In addition, PA triggered stomatal closure and cytoplasmic pH rise in guard cells of *Pisum sativum* (Fig. 6.7). Hence, we can conclude that PLD/PA acts upstream of cytoplasmic pH rise, while being downstream of SPHK/ S1P/Phyto-S1P.

The dependence of ABA as well as S1P/Phyto-S1P induced pH rise on Ca^{2+} was studied using Ca^{2+} chelators. Both BAPTA (chelator of extracellular Ca^{2+}) and BAPTA-AM (chelator of intracellular Ca^{2+}) failed to prevent the cytoplasmic pH rise by not only ABA but also S1P/Phyto-S1P (Fig. 6.9B). Both BAPTA and BAPTA-AM prevented stomatal closure by S1P/Phyto-S1P besides ABA. Thus Ca^{2+} was required for the induction of stomatal closure but not increase of cytoplasmic pH by S1P/Phyto-S1P.

Our results are in contrast with the results of Islam et al. 2010, who found the co-ordinated function of Ca^{2+} and cytoplasmic pH during stomatal closure by ABA or MJ in guard cells of *Arabidopsis thaliana*. Islam et al. (2010) found that, addition of 2.5 mM $CaCl_2$ induced about 18% increase in BCECF-AM fluorescence and butyrate prevented ABA activation of Ca^{2+} currents. However they didn't observed the effect of Ca^{2+} removal on ABA induced pH rise.

The prevention of stomatal closure by PI3K inhibitors is known in *Arabidopsis thaliana* and *Vicia faba* guard cells (Jung et al., 2002; Park et al., 2003). These inhibitors (Wortmannin and LY294002) further prevented stomatal closure and increase of pH by ABA as well as S1P/Phyto-S1P (Fig. 6.8). Hence, PI3K might be required for ABA induced cytoplasmic pH rise downstream of SPHK/S1P/Phyto-S1P. We conclude that cytoplasmic pH is an important component during stomatal closure by S1P/Phyto-S1P and acts downstream of SPHK, PLD, and PI3K during ABA induced stomatal closure. Although it appears that Ca^{2+} is not required for ABA/S1P/Phyto-S1P induced cytoplasmic pH rise in guard cells, further work is needed to confirm the suggestion.

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Chapter 7

ROLE OF S-TYPE ANION CHANNELS IN ELICITOR INDUCED ROS PRODUCTION IN GUARD CELLS AND TWO DIFFERENT TYPES OF CELL SUSPENSION CULTURES

Introduction

Plants face a continuous exposure to several pathogenic and nonpathogenic microbes and have evolved two levels of defense for preventing the entry of pathogens into plant tissues. Recognition of pathogen specific effector molecules (elicitors) called pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs) by specific receptors confers the first level of defense. The interaction of PAMPs with such receptors leads to signaling events within plant cells to induce pattern triggered immunity (PTI). Successful pathogens in turn try to suppress PTI to cause disease in host plants. Some of the pathogens suppress the PTI by secreting effector proteins inside the plant cells. Plants can recognize some of these effectors through intra-cellular immune receptors to trigger second level of defense i.e. effector triggered immunity (Jones and Dangl, 2006; Nicaise et al., 2009; Coll et al., 2011; Dodds and Rathjen, 2012).

Several pathogenic utilize natural openings of plant leaf surface, such as stomata or hydathodes, as gateways for their entry into the plant interiors (Melotto et al., 2006 & 2008). Hence, the promotion of stomatal closure in response to PAMPs can offer first line of defense in plants. The plant model *Arabidopsis thaliana* actively recognizes several bacterial PAMPs like flagellin (or its derived peptide Flg22), lipopolysaccharides (LPS) and close stomata to restrict the entry of pathogens (Melotto et al., 2006).

Chitosan, a fungal elicitor, triggers defence responses by inducing stomatal closure in guard cells of *Pisum sativum* and *Hordeum vulgare* (Srivastava et al., 2009; Koers et al., 2011). Studying guard cell function in response to such microbial elicitors provides a valuable system for studying the molecular mechanisms of PTI.

The PAMPs trigger an array of events in guard cells to cause stomatal closure, as a part of PTI. Induction of ROS and NO, elevation of Ca^{2+} signatures and extrusion of K^+ and Cl^- by membrane depolarization are common and early events in PAMP triggered immunity. Chitosan triggered ROS and NO in *Pisum sativum* guard cells as early as 10 minutes during induction of stomatal closure (Srivastava et al., 2009). Flg22 and LPS trigger the activation of NO and guard cell specific OST1 kinase in *Arabidopsis thaliana* (Melotto et al., 2006). Flg22 inhibited stomatal opening by prevention of inward K^+ currents by the mediation of heterotrimeric G-proteins. Barley mildew (*Blumeria graminis*) and its elicitor chitosan nanoparticles triggered the activation of S-type anion channel currents in *Hordeum vulgare* guard cells (Koers et al., 2011).

Guard cells of *Arabidopsis thaliana* express two kinds of anion channels: slow type (S-type) and rapid type (R-type) encoded by two gene families (Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010). S-type of anion channels encoded by slow anion channel (SLAC) gene family play critical role in stress responses, ABA signaling and plant immunity (Roelfsema et al., 2012). Slow anion channel associated 1 (SLAC1) was the founding member of S-type of anion channels in plants and homologues of SLAC1 are represented as SLAHs (Negi et al., 2008; Vahisalu et al., 2008). Two homologues SLAC1 and SLAH3 are highly expressed in guard cells. *Arabidopsis*

mutants lacking SLAC1 was insensitive to stomatal closure by ABA, CO_2, ozone, dark-light transitions, H_2O_2 and nitric oxide (Negi et al., 2008; Vahisalu et al., 2008).

The importance of S-type of anion channels in plant immune response is of great interest and not yet fully studied. In this report, we studied the importance of S-type of anion channels SLAC1 and SLAH3 in regulation of stomatal closure by microbial elicitors cryptogein (98 amino acid peptide elicitor from *Phytophthora cryptogea*) or Flg22 (22 amino acid peptide elicitor from Eubacteria) and compared with ABA. We then extended our studies on the impact of SLAC1/SLAH3 double mutation and SLAC1 over-expression on ROS production by cryptogein, Flg22 or ABA in guard cells. We studied the ROS production by cryptogein, Flg22 or ABA in *Arabidopsis* T87 and tobacco BY2 cultured cells. In addition we checked the anion channel inhibitors effect on ROS production by cryptogein, Flg22 or ABA in *Arabidopsis* T87 cultured cells.

Results

Cryptogein or Flg22 induced stomatal closure in *Arabidopsis thaliana* leaves and its prevention by anion channel inhibitors

Cryptogein or Flg22 caused marked stomatal closure in leaf abaxial epidermis of *Arabidopsis thaliana* (Fig. 7.1A). The magnitude of stomatal closure by Flg22 was as effective as ABA, while the effect by cryptogein was less than that of by both Flg22 and ABA (Fig. 7.1). Anthracene-9-carboxylic acid (A9C) and 4,4’-diisothiocyanatostilbene 2,2’-disulfonic acid (DIDS), two known anion channel inhibitors, prevented stomatal closure by cryptogein or Flg22 besides ABA in concentration dependent manner (Fig. 7.1B & C). A9C was more effective than that of DIDS in prevention of stomatal closure

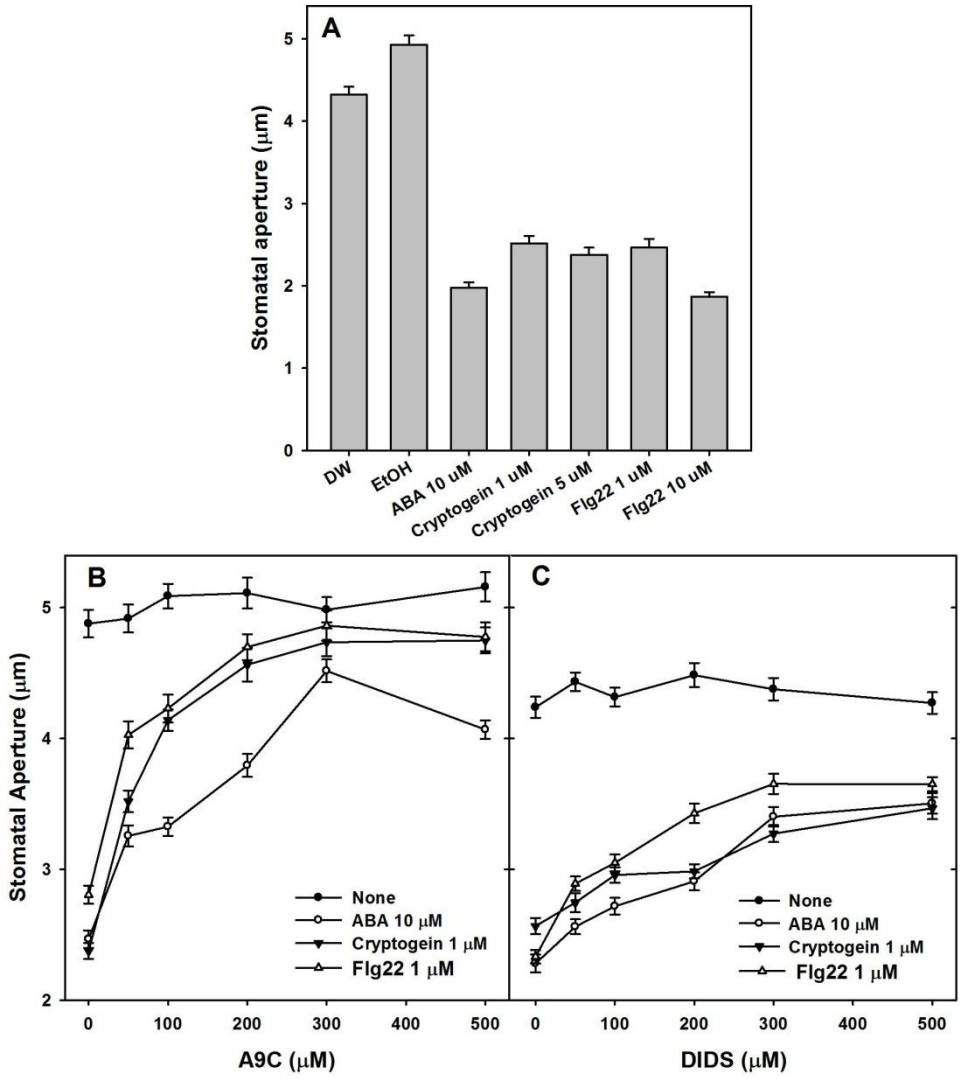

Fig 7.1: ABA, cryptogein and Flg22 induced stomatal closure in *Arabidopsis thaliana* and their inhibition by anion channel inhibitors. ABA as well as microbial elicitors cryptogein and Flg22 promoted stomatal closure compare to control sets added with distilled water (DW) or ethanol (EtOH) (A). Anthracene-9-carboxylic acid (A9C) (B) and 4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid (DIDS) (C) prevented stomatal closure by ABA, cryptogein and Flg22. A9C was more effective in such prevention than DIDS.

by cryptogein, Flg22 or ABA.

Arabidopsis mutants lacking SLAC1 were impaired in elicitor induced stomatal closure while overexpression line was hypersensitive

Stomatal closure by cryptogein or Flg22 was impaired in *Arabidopsis thaliana* mutant *slac1* (deficient in guard cell expressed S-type of anion channel SLAC1), as in case of ABA. Mutation in SLAH3, (a homologue of SLAC1 and another guard cell expressed S-type anion channel) partially impaired the stomatal closure by these microbial elicitors. Double mutant *slac1slah3* was more insensitive to cryptogein or Flg22 than either one of *slac1* or *slah3* (Fig. 7.2).

Arabidopsis line overexpressing SLAC1 (*slac1-OE*) was hypersensitive to cryptogein or Flg22 (Fig. 7.2). Comparative concentration dependent studies of wild type and *slac1-OE* revealed that SLAC1 overexpression caused hypersensitivity towards cryptogein, Flg22 and ABA in induction of stomatal closure. At very low concentration the percentage of stomatal closure by these compounds in *slac1-OE* is almost 2-2.5 fold higher than that of wild type (Fig. 7.3).

Anion channel inhibition or mutation in SLAC1/SLAH3 genes did not prevent ROS production by cryptogein or Flg22 in guard cells

Studies on ROS production in guard cells revealed that cryptogein or Flg22 induced rapid increase of ROS indicative CM-H_2DCFDA fluorescence compared to control set (Fig. 7.4). However, the anion channel inhibitors, A9C or DIDS did not affect ROS production by cryptogein, Flg22 or ABA in guard cells (Fig. 7.4). To confirm these

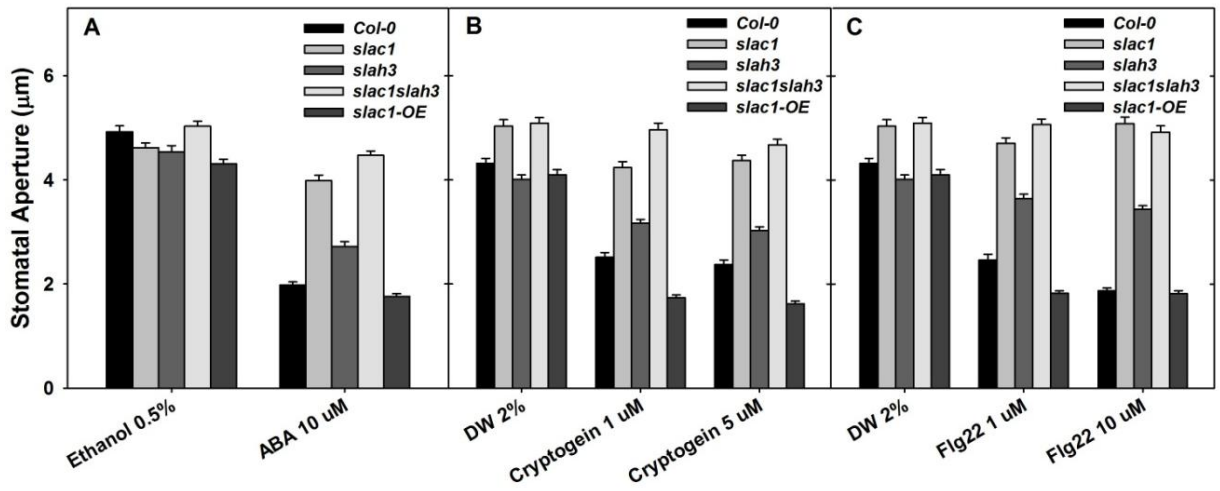

Fig 7.2: Pattern of stomatal closure by ABA (A), cryptogein (B) or Flg22 (C) in *Arabidopsis thaliana* wild type, single and double mutants lacking guard cell expressed anion channel homologues (SLAC1 and/or SLAH3) and SLAC1- OE (overexpressed line). Cryptogein or Flg22 induced stomatal closure was partially impaired in *slac1* and *slah3* single mutants while was completely impaired in *slac1slah3.* Mutation in SLAC1 had more effect on stomatal closure than that of SLAH3. SLAC1 overexpression line *slac1-OE* was hypersensitive to cryptogein or Flg22. Similar patterns of stomatal closure was seen in case of ABA.

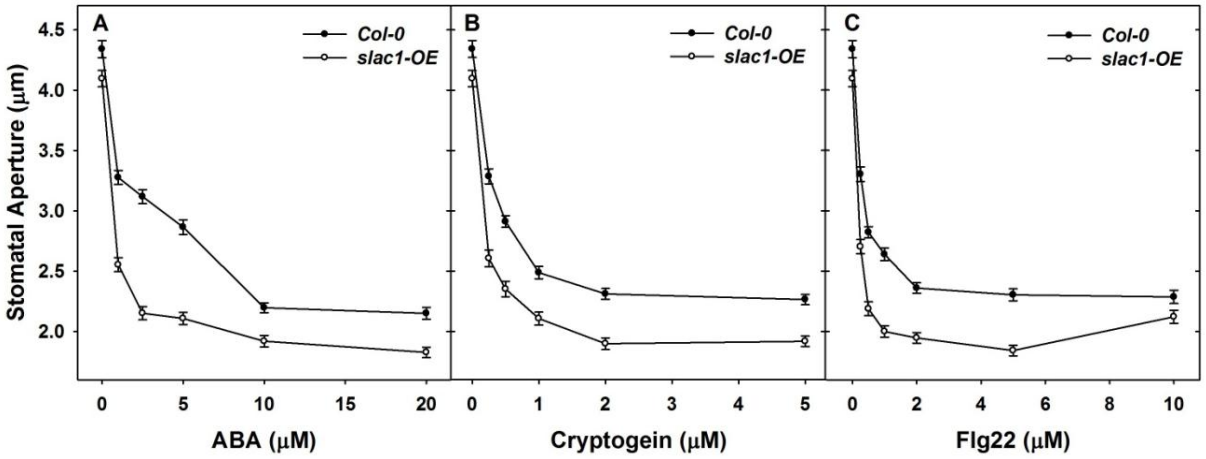

Fig 7.3: Dose dependent stomatal closure by ABA (A), cryptogein (B), Flg22 (C) in *Arabidopsis thaliana* wild type and SLAC1-overexpression line. In dose dependency, *slac1-OE* was hypersensitive to ABA, cryptogein or Flg22 when compared to wild type.

results, the time-dependent ROS production by cryptogein, Flg22 or ABA in guard cells of *slac1slah3* double mutant and *slac1-OE* were studied and compared with wild type. Cryptogein, Flg22 or ABA induced ROS production was neither decreased in *slac1slah3* nor increased in *slac1-OE* (Figs. 7.5 & 7.6).

Only cryptogein or Flg22, and not ABA could induce ROS production in *Arabidopsis* T87 and tobacco BY2 suspension cultured cells

Cryptogein or Flg22 induced an increase of ROS production in both *Arabidopsis* T87 suspension cultured cells (Fig. 7.7) and tobacco BY2 cells (Fig. 7.8). The sensitivity of T87 suspension cultured cells was higher towards Flg22 compared to cryptogein in ROS production (Fig. 7.7). Tobacco BY2 cells have higher sensitivity towards cryptogein compare to Flg22 (Fig. 7.8). However, ABA failed to induce significant increase of ROS in both cell types. Cryptogein or Flg22 induced ROS production was completely prevented in presence of NADPH oxidase inhibitor DPI. Anion channel inhibitors A9C or DIDS largely prevented ROS production by cryptogein or Flg22 in *Arabidopsis* T87 suspension cultured cells (Fig. 7.9).

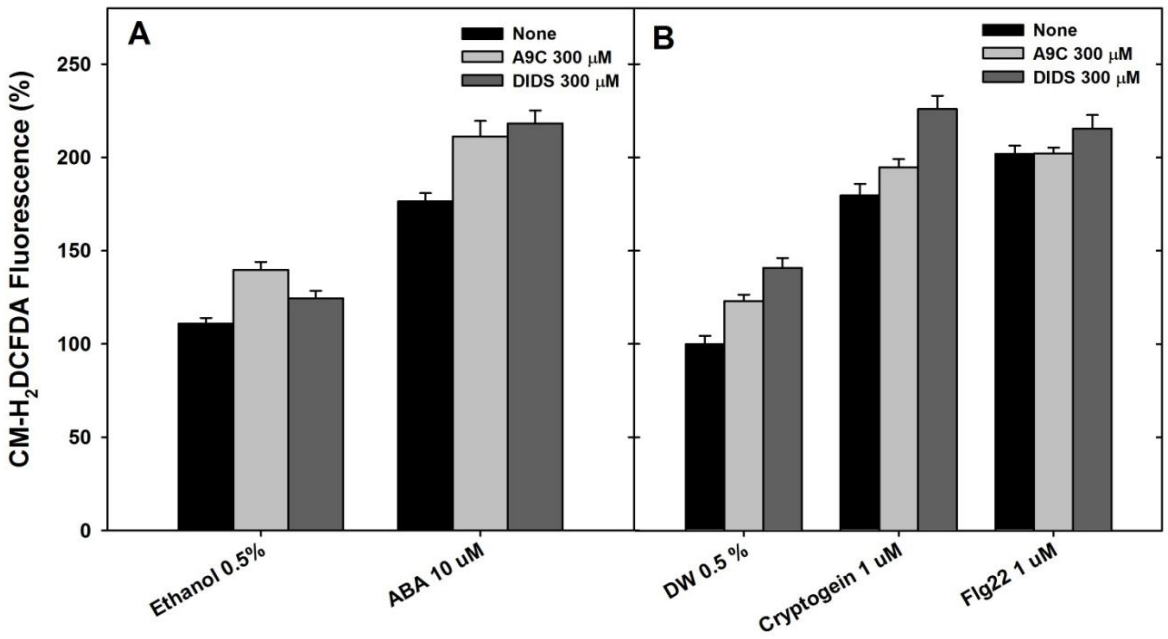

Fig 7.4: Effect of anion channel inhibitors on ROS production by ABA **(A)** or microbial elicitors (B). A9C or DIDS could not inhibit ROS production by cryptogein, Flg22 or ABA.

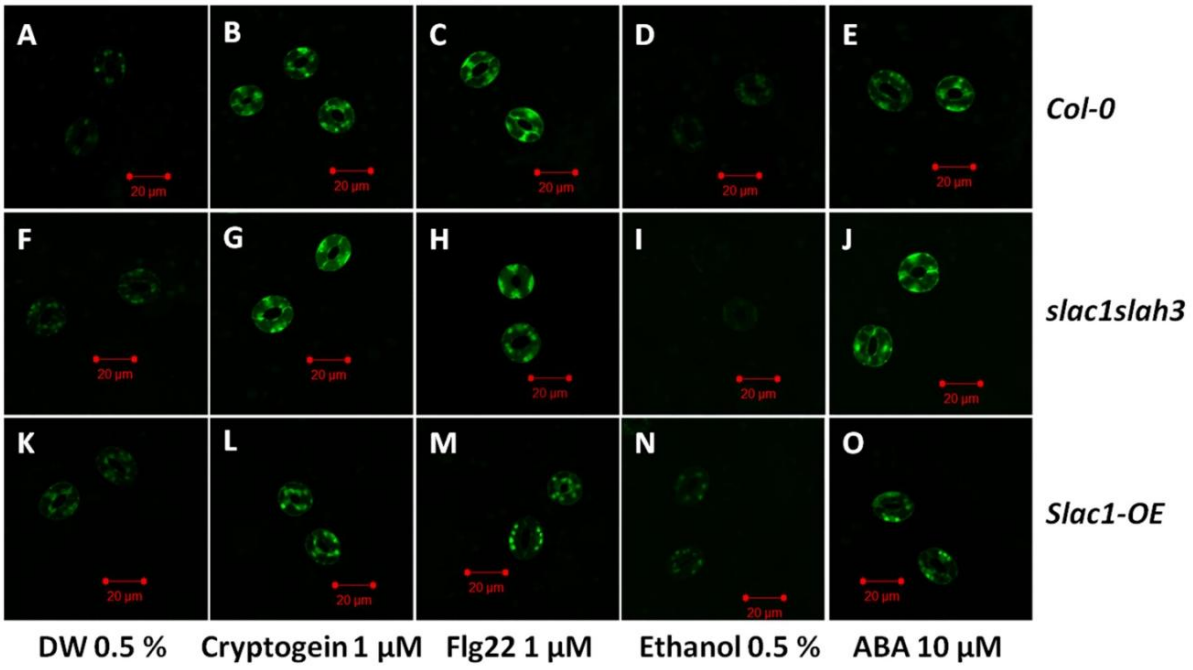

Fig 7.5: Confocal images showing the changes in ROS production by cryptogein, Flg22 or ABA in *Arabidopsis thaliana Col-0* (wild type), *slac1slah3* double mutant and *slac1-OE*. Cryptogein, Flg22 or ABA treated cells exhibited higher ROS production than their respective controls in wild type double mutant and SLAC1-overexpression line.

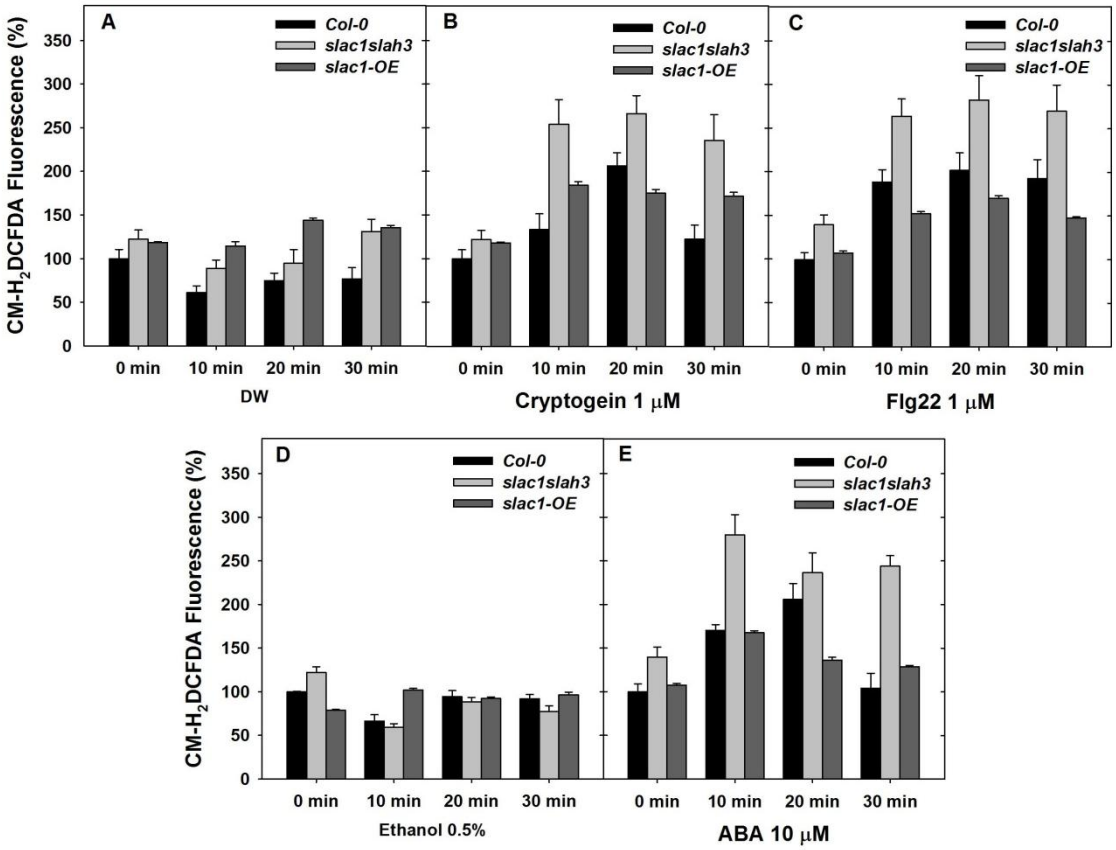

Fig 7.6: Time dependent ROS production by cryptogein, Flg22 or ABA in *Arabidopsis thaliana Col-0, slac1slah3* and *slac1-OE*. Cryptogein, Flg22 or ABA increased ROS production with time in wild type double mutant and SLAC1-overexpression line. ROS production in double mutant is higher than that of wild type, while little less in overexpression line.

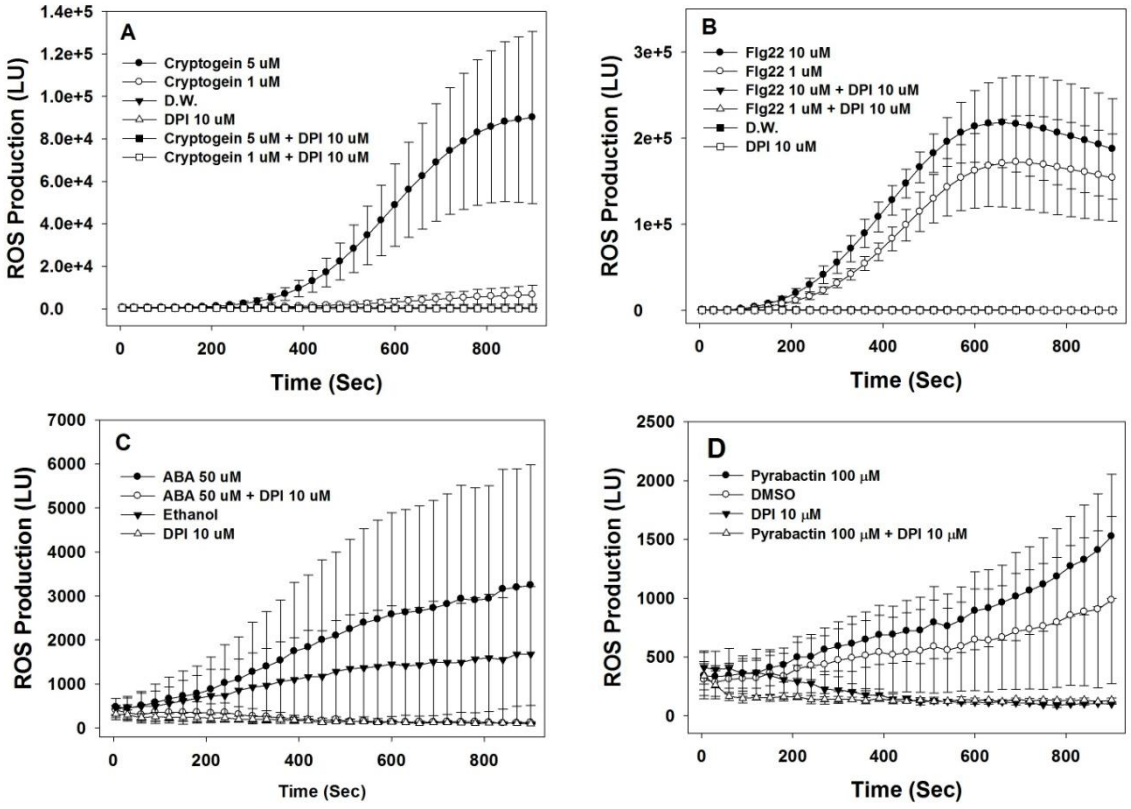

Fig 7.7: ROS production by (A) cryptogein, (B) Flg22, (C) ABA or (D) pyrabactin in T87 cultured cells of *Arabidopsis*. Cryptogein or Flg22 caused rapid ROS production in suspension cultured cells. The magnitude of ROS production by Flg22 was more than that of by cryptogein. ROS production by ABA or its synthetic agonist, pyrabactin, was negligible compared to microbial elicitors. DPI (NADPH oxidase inhibitor) prevented ROS production by both cryptogein and Flg22.

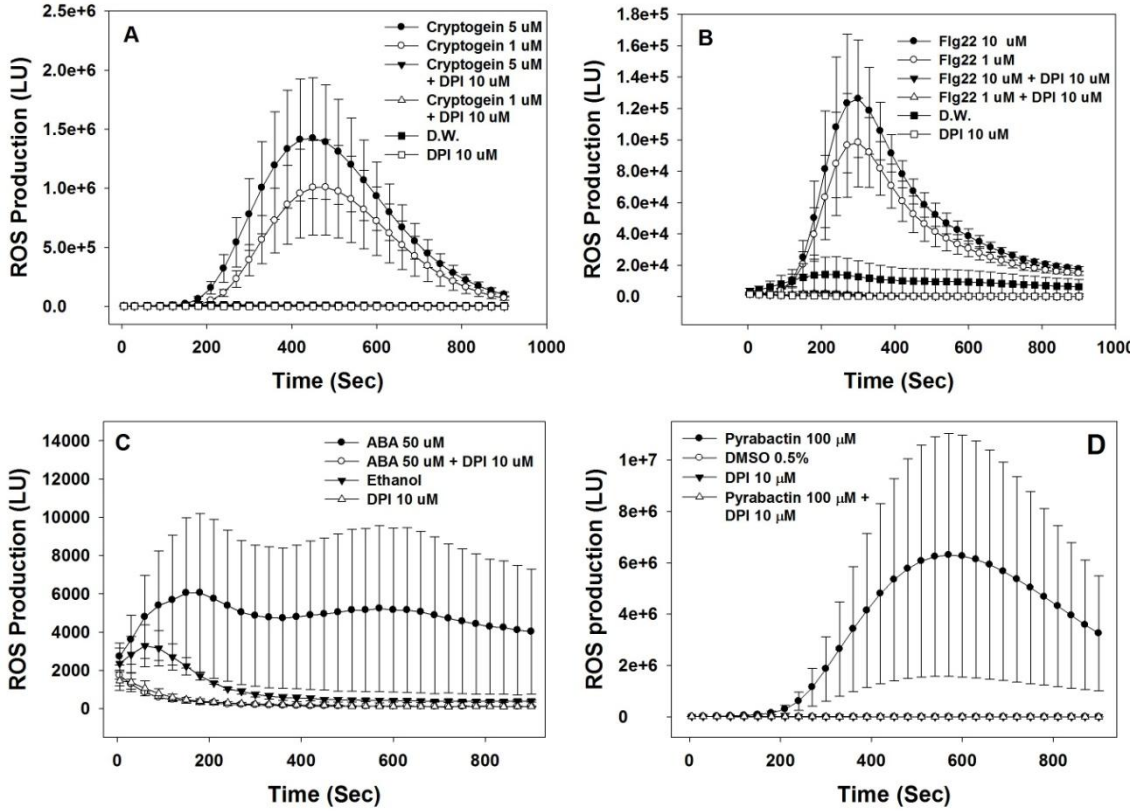

Fig 7.8: ROS production by cryptogein (A), Flg22 (B), ABA (C) or (D) pyrabactin in tobacco BY2 cells. Cryptogein or Flg22 caused rapid ROS production in tobacco BY2 cells. The magnitude of ROS production in presence of cryptogein was more than that in Flg22. The stimulation of ROS production by ABA was negligible compared to microbial elicitors. However, pyrabactin caused rapid ROS production in tobacco BY2 cells similar to cryptogein. DPI prevented ROS production by both cryptogein and Flg22.

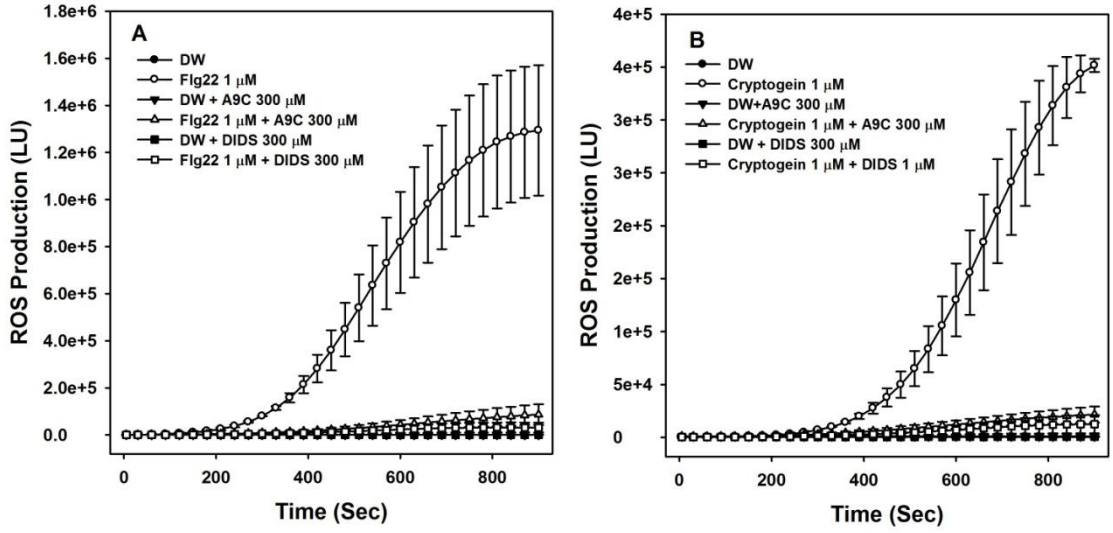

Fig 7.9: Effect of anion channel inhibitors on ROS production by microbial elicitors in *Arabidopsis* T87 suspension cultured cells. A9C or DIDS prevented ROS production by Cryptogein or Flg22.

Discussion

In this study we analyzed the participation and interaction of S-type of anion channels and ROS production by using two different microbial elicitors cryptogein and Flg22. *Arabidopsis* guard cells actively responded to microbial elicitors and rapidly closed after their application (Fig. 7.1).

S-type of anion channels play critical role in elicitor induced stomatal closure

Pharmacological compounds that inhibit anion channels prevented stomatal closure by microbial elicitors (Fig. 7.2). Arabidopsis mutants deficient in guard cell S-type anion channel were impaired in cryptogein or Flg22 induced stomatal closure, while the over-expression line was hypersensitive to microbial elicitors (Figs. 7.4, 7.5). In earlier studies with *Arabidopsis*, mutation in SLAC1 caused an impairment in the stomatal closure by ABA, H_2O_2 or SNP (Vahisalu et al., 2008). The loss of function mutants of *Arabidopsis* SLAC1 gene was impaired in stomatal closure by CO_2, ABA and even dark-light transitions (Negi et al., 2008). There are additional reports on the role of SLAC in other instances. For e.g. cryptogein triggered biphasic (rapid/transient and slow prolonged) Cl^- efflux and H^+ influx in tobacco BY2 cells (Kadota et al., 2004). Growing barley mildew (*Blumeria graminis*) on leaves or infusion of its elicitor chitosan nanoparticles triggered the activation of S-type anion channel currents in *Hordeum vulgare* guard cells (Koers et al., 2011). Our results suggest strongly the participation of S-type of anion channels besides their role in ABA induced stomatal closure, and thus indirectly in plant immunity.

ROS production by NADPH oxidases is a common early event during stomatal closure

Cryptogein or Flg22 triggered ROS production in guard cells prior to stomatal closure (Fig. 7.7). Similarly, both cryptogein and Flg22 induced rapid increase of apoplastic ROS in T87 cultured cells of *Arabidopsis* which was prevented by DPI (Fig. 7.10). Flg22 induced oxidative burst in seedlings and stomatal closure was impaired in *Arabidopsis* mutants lacking NADPH oxidase RBOHD (Mersmann et al., 2010). *Arabidopsis* NADPH oxidases RBOHD and RBOHF play key role in defense-associated metabolism and interplay between oxidative stress and pathogenesis response against *Pseudomonas syringe* (Chaouch et al., 2011). Cryptogein induce rapid ROS production in tobacco BY2 cultured cells which is prevented by the addition of DPI (NADPH oxidase inhibitor) (Kadota et al., 2004). We conclude that ROS production by NADPH oxidases is a common early event during stomatal closure and may reflect plant immune response triggered by various kinds of pathogens or elicitors in different cell types.

The magnitude and characteristics of ROS production differs with PAMP type in different plants

The sensitivity and magnitude of immune response depends on its detection by host plant. Some plants can actively identify and trigger immune response against an array of pathogens but cannot against others. The magnitude of stomatal closure and ROS production by Flg22 is more than that of by cryptogein in both guard cells and T87 cultured cells suggesting that *Arabidopsis* genome offers more resistance towards bacterial elicitor Flg22 but not that much towards fungal elicitor cryptogein. However, tobacco, another plant, actively senses and triggers ROS production against cryptogein even at nanomolar range (Kadota et al., 2004).

Anion channels differentially regulate ROS production in different cell types

Anion channel inhibitor DIDS prevented ROS production by cryptogein in BY2 cells (Kadota et al., 2004). ROS production by Flg22 and cryptogein in *Arabidopsis* T87 cultured cells was prevented by anion channel inhibitors A9C or DIDS (Fig. 7.12). However, neither inhibitors nor mutation in SLAC1 or SLAH3 caused impaired ROS production in guard cells (Figs. 7.7 to 7.9). In addition, overexpression of SLAC1 also caused normal ROS production (Fig. 7.8 & 7.9). H_2O_2 induced stomatal closure was impaired by mutation in SLAC1 (Vahisalu et al., 2008). We suggest that the mechanism of ROS production regulation by anion channels of guard cells could be different from that of by suspension cultured cells. In guard cells, ROS acts upstream of S-type of anion channels, while in suspension cultured cells, anion channels regulate the ROS production. Such variability in cell types is quite surprising and intriguing. This phenomenon needs further detailed studies for confirmation and elucidation of exact reasons.

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Chapter 8

GENERAL DISCUSSION AND CONCLUSIONS

The present work focuses on different aspects of stomatal closure, in response to known modulators such as ABA or its analogue and then elicitors. The studies attempted to understand the signaling events during stomatal closure or changes in cell suspensions. The first chapter describes the ability of pyrabactin, an ABA agonist, to induce stomatal closure. The effects of pyrabactin were studied in detail, in comparison with ABA. The second chapter highlights the importance of SPHK/S1P/Phyto-S1P during stomatal closure by ABA and their modulation of ROS and NO in guard cells. Further, the role of PLD, PI3K and PP2Cs, in S1P/Phyto-S1P modulation of ROS and NO, was checked. The third chapter demonstrates the importance of cytoplasmic pH of guard cells in stomatal closure by S1P/Phyto-S1P and role of PLD, PI3K and Ca^{2+} in S1P/Phyto-S1P modulation of cytoplasmic pH. The last chapter describes the role of S-type of anion channels in plant immunity and their interaction with ROS production in guard cells and points out differences in the patterns with guard cells or suspension cultures.

Pyrabactin is a novel ABA agonist and promotes stomatal closure

The natural modulator of stomatal function, ABA, has major limitations for its usage in agriculture, as an anti-transpirant, due to its instability outdoors in the field and high cost. Synthesis and application of ABA-similar compounds is quite promising for sustaining agriculture, with minimal water. Pyrabactin was considered as one such potential anti-transpirant based on its ability to exert some of the ABA similar functions. Although a synthetic chemical, pyrabactin inhibited seed germination, hypocotyl growth

and stimulated gene expression very similar to ABA in *Arabidopsis thaliana* (Park et al., 2009). However, it was not known, if pyrabactin had the ability to cause stomatal closure. The present work illustrates that pyrabactin modulates strongly stomatal function in *Pisum sativum* abaxial epidermis (Fig. 4.1). This opens up the possibility to test pyrabactin further and exploit the use of similar compounds to use in agriculture, as water-saving chemicals.

Pyrabactin elevated the levels of signaling components, such as ROS, NO and cytoplasmic pH levels in guard cells, with patterns similar to ABA (Fig. 4.4). However, apyrabactin, an inactive analog of ABA, did not affect either stomatal closure or the signaling components of guard cells (Fig. 4.4). The effects of pyrabactin induced changes were reversed by pharmalogical compounds that modulate ROS, NO or cytoplasmic pH levels, quite similar to ABA effects. Fusicoccin, a fungal toxin, could reverse the stomatal closure caused by pyrabactin, as well as in the case of ABA. Hence, pyrabactin (but not apyrabactin) can be considered as full functional agonist of ABA and can be useful tool for dissecting the signaling events in guard cells as well as other cell types (Puli and Raghavendra 2012).

Evidence for the location of pyrabactin binding site in guard cells, close to that of ABA

Our experiments on stomatal closure by varying concentrations of ABA, in presence of fixed concentration of pyrabactin, and *vice versa*, revealed that the actions of ABA and pyrabactin were additive. The apparent K_D of ABA was increased almost 4-fold in presence of pyrabactin, suggesting that pyrabactin and ABA were competing with each other either at the same site or close to the active site (Fig. 4.7). Pyrabactin was the key for illumination of genetically redundant cytosolic ABA receptors i.e.,

PYR/PYL elements (Park et al., 2009). *Arabidopsis thaliana* genome encodes a 14 member family of these proteins, with a (PYR1 and PYL1-13) redundant function in ABA signaling (Park et al., 2009). Pyrabactin was shown to bind PYR1, PYL1 and PYL2 receptors during *in vitro* reconstitution assays, and inhibit the PP2C-ABI1 activity when added together with PYR1, PYL1, PYL3, PYL5 or PYL6 (Hao et al., 2010; Yuan et al., 2010; Melcher et al., 2010). Microarray analysis revealed that PYR/PYL elements were highly expressed in guard cells (Park et al., 2009). We therefore conclude that pyrabactin could trigger stomatal closure and modulate ABA-related signal-transduction components through PYR/PYL receptors in stomatal guard cells.

Bioactive phospho/sphingolipids as key modulators of ABA signaling in guard cells

The dissection of plant signaling in recent past revealed the pivotal role of PA, PI3P and S1P/Phyto-S1P in ABA signaling. The diversity in molecular species and non specificity of their metabolizing enzymes towards their substrates enhanced the complexity of understanding lipid signaling in plants. ABA enhanced the levels of Phyto-S1P besides d18:0-P, d18:1-P and t18:1-P in *Arabidopsis thaliana* leaves (Guo et al., 2012). Similarly, ABA triggered the elevation of different PA molecular species in leaves of *Arabidopsis thaliana*, of which the major difference occurred in 16:0/18:2, 16:0/18:3, 18:2/18:2 and 18:2/18:3 (Guo et al., 2012). The levels of PI3P were increased by ABA in *Vicia faba* epidermis (Jung et al., 2002). S1P or Phyto-S1P triggered stomatal closure in *Commelina communis* and *Arabidopsis thaliana* (Ng et al., 2001, Coursol et al., 2003). Stomatal closure was induced by also PA in *Arabidopsis thaliana* by inhibiting PP2C ABI1 (Mishra et al., 2006). In our experiments too, S1P/Phyto-S1P and PA triggered the stomatal closure in *Pisum sativum* both in concentration and time

dependent manner (Fig. 5.1, 5.9, 6.1, 6.7). Lipid metabolizing enzymes such as PLD, PI3K and SPHKs are known to modulate ABA signaling during stomatal closure (Jung et al., 2002, Guo and Wang 2012). Impaired stomatal closure by ABA in *Arabidopsis thaliana* mutants lacking PLDα1, SPHK1 and SPHK2 confirmed the participation of these enzymes in ABA signaling (Guo and Wang 2012). PI3K inhibitors prevented stomatal closure in both *Arabidopsis thaliana* and *Vicia faba* leaves. In addition, ABA enhanced the levels of PI3P in *Vicia faba* epidermal tissues (Jung et al., 2002, Park et al., 2002). Our results with the inhibitors of SPHK, PLD and PI3K preventing stomatal closure by ABA in *Pisum sativum* guard cells (Fig 5.4, 5.8, 5.10) confirm their similar role in *Pisum sativum* too.

NO is a key target of S1P/Phyto-S1P action on guard cells during stomatal closure

Earlier literature revealed the role of Ca^{2+}, G-proteins and ion channels during stomatal closure by S1P or Phyto-S1P in guard cells of *Commulina communis* and *Arabidopsis thaliana* (Ng et al., 2001, Coursol et al., 2003 &2005). The present study brought out the importance of ROS and NO during S1P or Phyto-S1P action in guard cells of *Pisum sativum* and *Arabidopsis thaliana*. Pharmacological studies and *in vivo* fluorescence imaging by the usage of ROS and NO specific inhibitors and fluorophores revealed that the participation of NO is critical for stomatal closure by S1P/Phyto-S1P (Figs. 5.2, 5.3, 5.6, 5.7). The use of SPHK inhibitors further confirmed the participation of SPHK during the generation of NO but not ROS by ABA in guard cells of *Pisum sativum* (Fig. 5.5).

ABA induced nitric oxide production in guard cells possibly via two routes: either by nitrate reductases (NRs) or by nitric oxide synthase (NOS) like activity.

However, the existence of NOS in plants was under debate as functional characterization of NOS in higher plants had not been successful. Earlier report of AtNOS as plant NOS failed to stand criticism and the enzyme was renamed as AtNOA1 (Guo et al., 2003, Besson-Bard et al., 2008, Neill et al., 2008). In *Arabidopsis thaliana*, NR played major role in production of NO by ABA compared to AtNOA1 (Besson-Bard et al., 2008, Neill et al., 2008). As per our results, L-NAME, but not tungstate, completely relieved the S1P/Phyto-S1P effects (Figs. 5.6, 5.7). In contrast, both L-NAME and tungstate displayed almost equal effect on stomatal closure and NO production by ABA in *Pisum sativum* guard cells. We therefore suggest that S1P/Phyto-S1P induced stomatal closure and NO production is possibly dependent more on L-NAME sensitive NOS pathway, but not on NR.

The prevention of stomatal closure due to ABA as well as S1P/Phyto-S1P by PLD and PI3K inhibitors suggest that both act downstream of SPHK/S1P/Phyto-S1P during ABA induced stomatal closure. These inhibitors prevented also the elevation of NO by ABA as well as S1P/Phyto-S1P (Figs. 5.8, 5.10). These observations demonstrated that S1P/Phyto-S1P triggered the elevation of NO through PLD, PI3K pathways. In *Arabidopsis thaliana* even though Phyto-S1P induced stomatal closure was impaired in *pldα1* mutant, Phyto-S1P could not bind directly to the PLDα1 (Guo and Wang 2012). In addition, PA alone could not induce NO production in guard cells of *Arabidopsis thaliana* (Uraji et al., 2012). With current knowledge, the mechanism of NO production by S1P/Phyto-S1P was not predictable and further studies required for confirmation.

Importance of cytoplasmic alkalization during S1P/Phyto-S1P induced stomatal closure

Cytoplasmic alkalization is an early signal during stomatal closure by ABA in guard cells of *Paphiopedilum tonsum*, *Arabidopsis thaliana* and *Pisum sativum* (Irving et al., 1992, Suhita et al., 2004, Gonugunta et al., 2008). There have been no earlier reports on the relation-ship between stomatal closure and guard cell pH-changes by S1P/Phyto-S1P. In our experiments, S1P/Phyto-S1P also triggered a marked rise in the cytoplasmic pH of guard cells, while SPHK inhibitors prevented the stomatal closure as well as the increase in pH by ABA (Figs. 6.2 to 6.5). These point out at the important role of SPHK and S1P/Phyto-S1P during ABA elevation of cytoplasmic pH.

Previous reports on the mode of S1P/Phyto-S1P promotion of stomatal closure revealed that heterotrimeric G-protein α sub unit GPA1, K^+ inward channels, PLDα1, and Ca^{2+} act downstream of SPHK and S1P/Phyto-S1P (Ng et al., 2001, Coursol et al., 2005, Guo et al., 2012). Inhibition of PLDα1 prevented both the stomatal closure and cytoplasmic pH rise by S1P/Phyto-S1P as in case of ABA (Fig. 6.6), while, PA triggered the pH increase of guard cells in *Pisum sativum* (Fig. 6.7). These results suggest that, PLD or PA acts downstream of SPHK/S1P/Phyto-S1P and upstream of cytoplasmic pH. In addition, PI3K inhibition too prevented the alkalization of cytoplasm suggested the participation of PI3P in regulation of cytoplasmic pH increase by S1P/Phyto-S1P (Fig. 6.8). In contrast, Ca^{2+} removal did not altered the cytoplasmic pH increase by S1P/Phyto-S1P (Fig. 6.9). Hence, the S1P/Phyto-S1P elevation of cytoplasmic pH depends on PLD/PA and PI3K but not Ca^{2+}. S1P inhibited the K^+ inward channels and promoted the S-type of anion channels in guard cells of *Arabidopsis thaliana* via GPA1. Alkalization of cytoplasmic pH inactivates the K^+

inward channels in guard cells of *Vicia faba* (Blatt 1992). We conclude that the stomatal closure by ABA operates via SPHK/S1P/Phyto-S1P, and further mediated through GPA1, PLD/PA, PI3K, cytoplasmic alkalization. Possibly, these events integrate at the anion channel activation and/or cytoplasmic Ca^{2+} changes.

Stomatal closure by Flg22 or cryptogein in *Arabidopsis thaliana* leaves and possible mode of action

Besides their importance in control of transpiration and gaseous exchange open stomata facilitate the entry of pathogens into the leaf intracellular spaces (Melotto et al., 2006). Plants try to close their stomata, when they sense or recognize the pathogenic microbes to restrict their entry as part of the plant immunity. Elicitors or PAMPs appear to be key players in mediating such stomatal closure and may form an important component of plant innate immunity (Melotto et al., 2006). Application of Flg22 or cryptogein to *Arabidopsis thaliana* leaves caused stomatal closure, similar to ABA (Fig. 7.1) suggesting that guard cells of *Arabidopsis thaliana* can sense these molecules. Recognition of elicitors is critical to defend the entry of microbes into the plant tissues. For e.g. *Arabidopsis* mutants lacking FLS2 receptor did not show stomatal closure, when exposed to Flg22 (Melotto et al., 2006). In present study, the presence of Flg22 or cryptogein in the medium induced stomatal closure while triggering marked changes in signaling components within the guard cells.

Participation of S-type of anion channels and ROS production during stomatal closure by elicitors as in case of ABA

Participation of S-type of anion channels and ROS production during ABA

induced stomatal closure has been well established in *Arabidopsis thaliana* (Vahisalu et al., 2008, Negi et al., 2008, Kwak et al., 2003). However their role and interaction during elicitor induced stomatal closure are not known. The failure of stomatal closure by cryptogein or flg22 in presence of anion channel inhibitors (Fig. 7.1) or in SLAC/SLAH mutants (Fig. 7.2) and elevated ROS of guard cells (Fig. 7.4) revealed their versatile role in stomatal closure by elicitors as well as ABA. ABA elevates ROS production in *Arabidopsis* by the activation of guard cell expressed NADPH oxidases RBOHD and RBOHF (Kwak et al., 2003). Flg22 could not induce oxidative burst in seedlings and stomatal closure in *Arabidopsis* mutants lacking RBOHD (Mersmann et al., 2010). RBOHD and RBOHF are reported to play important role in defense-associated metabolism and interplay between oxidative stress and pathogenesis response in *Arabidopsis* against *Pseudomonas syringe* (Chaouch et al., 2011). Such similarities in ABA and elicitor signaling in plant tissues are not surprising. For example, OST1 and NO (two key ABA signaling elements in guard cells) were reported to participate in Flg22 or LPS induced stomatal closure as well (Melotto et al., 2006). In addition, chitosan also triggered the elevation of ROS and NO in guard cells of *Pisum sativum* as in case of ABA (Srivastava et al., 2009). One possible reason for these similarities was the stimulation by elicitors of ABA synthesis in guard cells during stomatal closure.

Elicitors are expected to induce stomatal closure after their perception by specific receptors. Flg22 triggers stomatal closure in *Arabidopsis thaliana* by the activation of FLS2 receptor (Melotto et al., 2006). The nature of cryptogein binding proteins is not yet known. Flg22 induced stomatal closure was impaired in *Arabidopsis* mutants that participate in ABA and ethylene biosynthesis (Melotto et al., 2006, Mersmann et al.,

2010). LPS induced stomatal closure was compromised in salicylic acid deficient mutants (Melotto et al., 2006). Elicitor induced stomatal closure involves the participation of protein kinase OST1, NO, heterotrimeric G-proteins, K^+ fluxes (Nicaise et al., 2008) besides ROS and S-type of anion channels. Based on our observations (Figs. 7.4 to 7.6), we suggested that ROS act upstream of SLAC1/SLAH3 in guard cells during stomatal closure.

Promotion of ROS production by elicitors in cell suspension cultures and the involvement of anion channels

The two different cell suspensions used in present experiments, differ in their sensitivity towards elicitors, ABA and pyrabactin. Cryptogein and Flg22 induced ROS production in *Arabidopsis* T87 and tobacco BY2 suspension cultured cells whereas ABA failed to induce ROS in both cell types. Synthetic ABA agonist pyrabactin triggered ROS in tobacco BY2 cells but not in *Arabidopsis* T87 cells (Fig. 7.7, 7.8). The magnitude of ROS induced by cryptogein or Flg22 was also variable in different cell types. Such variation may partly be due to the difference in the recognition of particular elicitor by given species. DPI prevention of ROS production by elicitors in suspension cultures () pointed at the role of NADPH oxidases, as in case of stomatal guard cells. Further detailed evaluation is required to elucidate the exact mechanisms for these variations.

The impaired stomatal closure by H_2O_2 in SLAC1 (Vahisalu et al., 2008) and the normal ROS production in SLAC1/SLAH3 mutants (Fig. 7.6) suggested that ROS production might act upstream of anion channel activation in guard cells. However, DIDS prevention of ROS production by cryptogein in BY2 cells (Kadota et al., 2004) as

well as A9C/DIDS prevention of ROS production by Flg22 and cryptogein in *Arabidopsis* T87 cells (Fig. 7.9) revealed another possibility of anion channel action upstream of ROS production in cell suspensions.

Conclusions

Major findings from the present work as follows.

1. Pyrabactin, an analogue of ABA, was as powerful as ABA in inducing stomatal closure and elevation of intracellular messengers, such as ROS, NO and cytoplasmic pH. The kinetics of rise in ROS, NO and pH by pyrabactin was quite similar to the response induced by ABA. Pyrabactin competed with ABA for induction of stomatal closure.
2. S1P/Phyto-S1P induced stomatal closure, by raising remarkably the levels of NO, but with only marginal increase in ROS. PLD and PI3K appeared to act downstream of S1P/Phyto-S1P during ABA induced stomatal closure and elevation of NO and ROS. ABI1, and RbohD/F appeared to require for S1P/Phyto-S1P induced NO and ROS increase but not sufficient for stomatal closure.
3. Cytoplasmic pH rise was an important component during stomatal closure by S1P/Phyto-S1P and appeared to be at downstream of SPHK, PLD, and PI3K during ABA induced stomatal closure. Ca^{2+} required for the induction of stomatal closure but not increase of cytoplasmic pH by S1P/Phyto-S1P.
4. S-type of anion channels played critical role in stomatal closure induced by microbial elicitors. There seems to be differences in the charecteristics of such elicitor induced ROS production in guard cells and cell suspension cultures. For

example, anion channels could differentially regulate ROS production in different cell types, i.e. downstream of ROS production in guard cells and upstream of ROS in suspension cultured cells.

Further work is needed to characterize the pyrabactin regulation of intracellular Ca^{2+} levels and the integration of different signals towards ion fluxes in guard cells leading to stomatal closure. The present study demonstrates the potential role of S1P/Phyto-S1P in elevating NO of guard cells and cause stomatal closure, but the aspect of such NO production, apparently independent of ROS production, needs further examination. The molecular mechanism of NO production by S1P/Phyto-S1P is not completely clear and warrants further experiments.

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Papers published in peer-reviewed international journals:

1. **Mallikarjuna Rao Puli** and Agepati S. Raghavendra (2011) Pyrabactin, an ABA agonist induced stomatal closure and signaling components of guard cells in abaxial epidermis of *Pisum sativum*. *Journal of Experimental Botany*. **63:** 1349-1356.
2. Nupur Srivastava, Vijay K. Gonugunta, **Mallikarjuna R. Puli** and Agepati S. Raghavendra (2009) Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of Pisum sativum. *Planta.* **229:** 757-765.
3. Vijay K. Gonugunta, Nupur Srivastava, **Mallikarjuna R. Puli** and Agepati S. Raghavendra (2008) Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid. *Plant, Cell and Environment*. **31:** 1717-1724.

Presentations at conferences:

a. **Mallikarjuna R. Puli,** Masaaki Okada, Takamitsu Kurusu, Katsunori Siato, Agepati S. Raghavendra and Kazuyuki Kuchitsu (2012). Involvement of reactive oxygen species, nitric oxide and intracellular pH change in stress signal transduction of plants. **"RNA Research Symposium"** organised by Tokyo University of Science, Noda, Chiba, Japan. June 18.

b. **Mallikarjuna R. Puli** & Agepati S. Raghavendra. (2011). Participation of sphingosine-1-phosphate and phosphoinositide-3-kinase during abscisic acid induced stomatal closure and NO production in *Pisum sativum* guard cells. **"Keystone Symposia on Plant Abiotic Stress Tolerance Mechanisms, Water and Global Agriculture"** organised by Keystone Symposia on Cellular and Molecular Biology, Keystone, USA. January, 17-22.

c. Agepati S. Raghavendra & **Mallikarjuna R. Puli**. (2011). Changes in patterns of signaling components in guard cells leading to stomatal closure by pyrabactin, an analogue of ABA, and evidence for competition between ABA and pyrabactin in vivo. **"Keystone Symposia on Plant Abiotic Stress Tolerance Mechanisms,**

Water and Global Agriculture" organised by Keystone Symposia on Cellular and Molecular Biology, Keystone, USA. January, 17-22.

d. **Mallikarjuna R. Puli** & Agepati S. Raghavendra (2011). Phosphoinositide-3-kinase is an important component during NO production and stomatal closure caused by sphingosine-1-phosphate and phytosphingosine-1-phosphate in *Pisum sativum* guard cells. **" International Conference on Plant Science in Post Genomic Era"** organised by School of Life Sciences, Sambalpur University, Sambalpur, India in association with the Society for Plant Physiology and Biochemistry, New Delhi, India. February, 17-19.

e. **Mallikarjuna R. Puli** & Agepati S. Raghavendra. (2010). Sphingosine-1 Phosphate is an important signaling component and increases NO levels in *Pisum sativum* guard cells during stomatal closure. ***Bioquest-2010*** organised by School of Life Sciences, University of Hyderabad, Hyderabad, India. March 20.

f. Vijay K. Gonugunta, Nupur Srivastava, **Mallikarjuna R. Puli** & Agepati S. Raghavendra. (2008). Signaling components during stomatal closure induced by methyl jasmonate: Role of protein kinases and protein phosphatases ***"2nd International Conference on Trends in Cellular and Molecular Biology"*** Organised by School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. January 5-7.

g. Vijay K. Gonugunta, Nupur Srivastava, **Mallikarjuna R. Puli** & Agepati S. Raghavendra. (2008). Necessity of cytosolic alkalinization in nitric oxide production during stomatal closure induced by abscisic acid. ***"4th Asia Oceania Conference on Photobiology 2008***" Organized by department of Zoology, Banaras Hindu University, Varanasi, India. November 24-26.

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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).

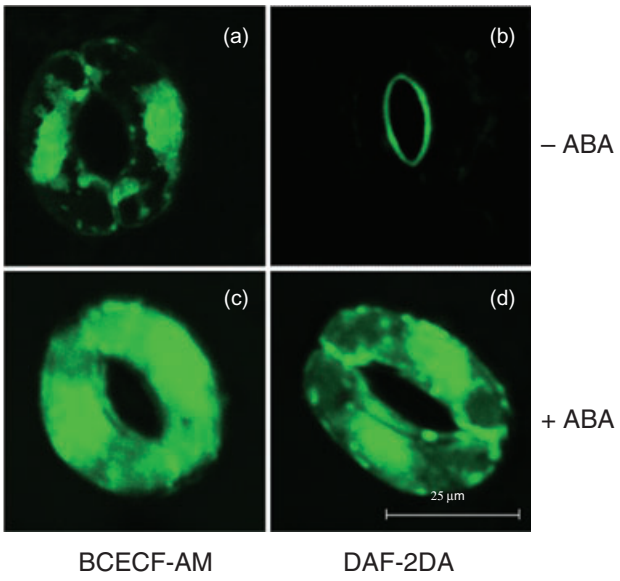

Figure 1. Confocal fluorescence images of stomata stained with 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) (a,c) or 4,5-diaminofluorescein diacetate (DAF-2DA) (b,d). These were taken after 12 min for BCECF-AM and 18 min for DAF-2DA treatment with 10 μM abscisic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

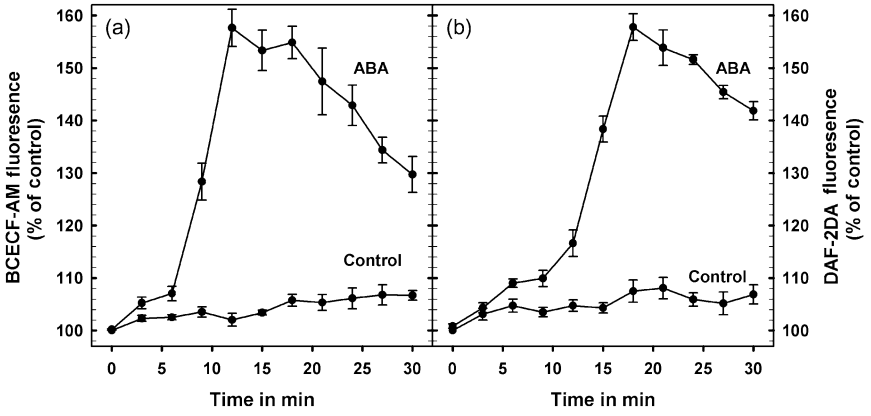

Figure 2. Kinetics of increase in pH (a) or nitric oxide (NO) (b) in epidermal strips of *Pisum sativum* in response 10 μM abscisic acid (ABA). Epidermal strips were loaded with either 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) (to monitor pH) or 4,5-diaminofluorescein 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.

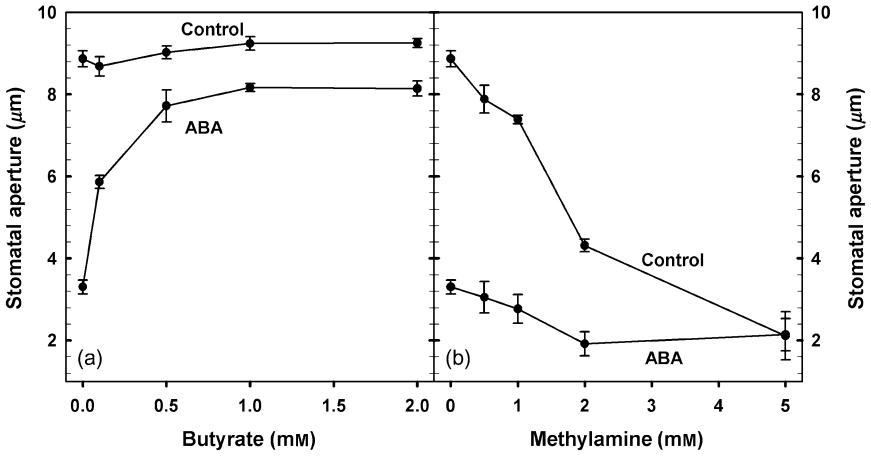

Figure 3. Effect of butyrate, a weak acid (a), or methylamine, an alkalinizing agent (b), on stomatal closure induced by 10 μM abscisic acid (ABA) in epidermal strips of *Pisum sativum*. Butyrate prevented stomatal closure by ABA, while methylamine further enhanced such stomatal closure. Butyrate alone 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

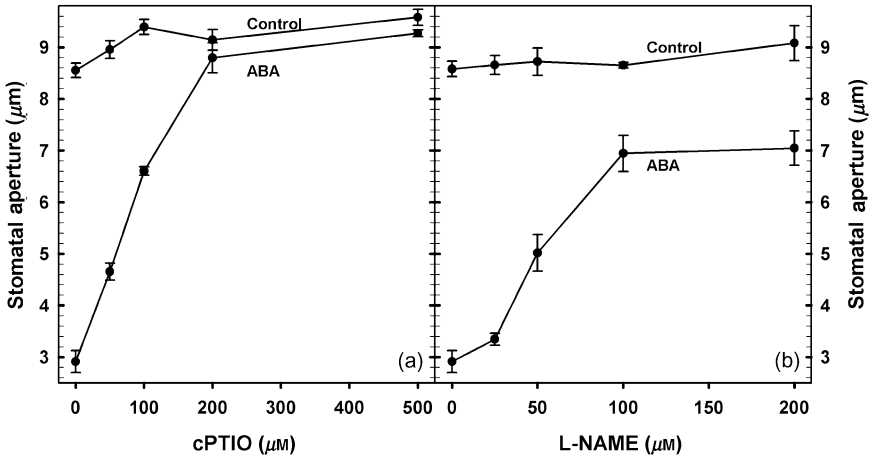

Figure 4. Prevention of abscisic acid (ABA)-induced stomatal closure in epidermal strips of *Pisum sativum* by either cPTIO, a nitric oxide (NO) scavenger (a), or L-NAME, an inhibitor of nitric oxide synthase (NOS) (b). The presence of 0.2 mM or above cPTIO prevented ABA-induced stomatal closure almost 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.

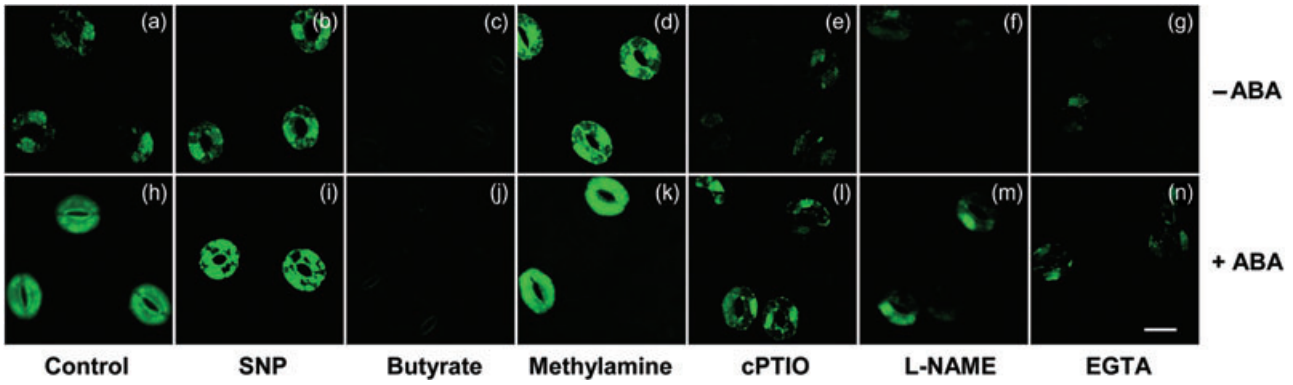

Figure 5. Effect of different modulators on 10 μM abscisic acid (ABA)-induced increase in pH, as indicated by 2′,7′-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM) fluorescence in stomatal guard cells of *Pisum sativum*. (a) to (g) are the controls: treated with water (a), 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.

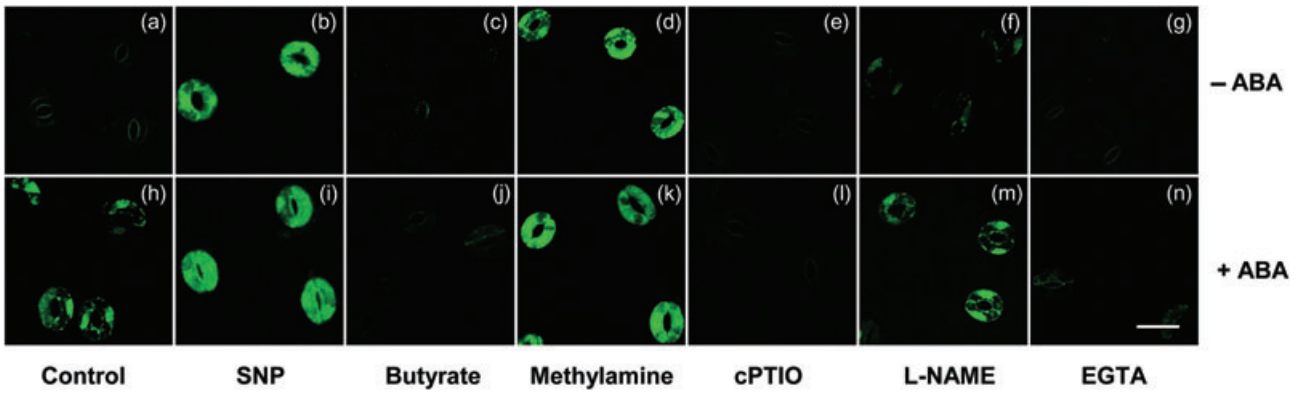

Figure 6. Effect of different modulators on 10 μM abscisic acid (ABA)-induced nitric oxide (NO) production, as indicated by 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence in stomatal guard cells of *Pisum sativum*. (a) to (g) are the controls: treated with water (a), 0.1 mM sodium nitroprusside (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*

	−ABA 10 μM			+ABA 10 μM		
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 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.

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RESEARCH PAPER

Pyrabactin, an ABA agonist, induced stomatal closure and changes in signalling components of guard cells in abaxial epidermis of *Pisum sativum*

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Abstract

Pyrabactin, a synthetic agonist of abscisic acid (ABA), inhibits seed germination and hypocotyl growth and stimulates gene expression in a very similar way to ABA, implying the possible modulation of stomatal function by pyrabactin as well. The effect of pyrabactin on stomatal closure and secondary messengers was therefore studied in guard cells of *Pisum sativum* abaxial epidermis. Pyrabactin caused marked stomatal closure in a pattern similar to ABA. In addition, pyrabactin elevated the levels of reactive oxygen species (ROS), nitric oxide (NO), and cytoplasmic pH levels in guard cells, as indicated by the respective fluorophores. However, apyrabactin, an inactive analogue of ABA, did not affect either stomatal closure or the signalling components of guard cells. The effects of pyrabactin-induced changes were reversed by pharmalogical compounds that modulate ROS, NO or cytoplasmic pH levels, quite similar to ABA effects. Fusicoccin, a fungal toxin, could reverse the stomatal closure caused by pyrabactin, as well as that caused by ABA. Experiments on stomatal closure by varying concentrations of ABA, in the presence of fixed concentration of pyrabactin, and vice versa, revealed that the actions of ABA and pyrabactin were additive. Further kinetic analysis of data revealed that the apparent K_D of ABA was increased almost 4-fold in the presence of ABA, suggesting that pyrabactin and ABA were competing with each other either at the same site or close to the active site. It is proposed that pyrabactin could be used to examine the ABA-related signal-transduction components in stomatal guard cells as well as in other plant tissues. It is also suggested that pyrabactin can be used as an antitranspirant or as a priming agent for improving the drought tolerance of crop plants.

Key words: Abscisic acid, cytoplasmic pH, fusicoccin, guard cell, nitric oxide, *Pisum sativum*, pyrabactin, reactive oxygen species, stomatal closure.

Introduction

Stomatal closure is an adaptation to conserve water loss during drought/water stress conditions. During the stress conditions, the synthesis and mobilization of abscisic acid (ABA) form key physiological events, facilitating stomatal closure by ABA (Seo and Koshiba, 2002; Christmann *et al.*, 2006). In view of the powerful effects of ABA, the signalling components during ABA-induced stomatal closure have been examined extensively (Hetherington, 2001; Wasilewska *et al.*, 2008; Acharya and Assmann, 2009; Kim *et al.*, 2010). Protein phosphatases such as ABI1, ABI2, and HAB1 are negative regulators during ABA-induced stomatal closure, while protein kinases such as SnRK2s (including OST1) are positive regulators (Mustilli *et al.*, 2002; Li *et al.*, 2006; Hubbard *et al.*, 2010; Kim *et al.*, 2010). Other protein kinases such as CBLs and CIPKs also play crucial roles in ABA-induced stomatal closure. In addition, ABA promotes the activity of anion channels (e.g. SLAK1, AtALMT12) and down-regulates the activity of inward K^+ channels (KAT1and KAT2) in stomatal guard cells (Geiger *et al.*, 2009; Lee *et al.*, 2009; Sirichandra *et al.*, 2009; Kim *et al.*, 2010). Besides the above signalling components, participation of several small molecules like reactive oxygen species

(ROS), nitric oxide (NO), and ions like Ca^{2+}, besides a rise in guard cell pH are all essential during ABA-mediated stomatal closure (Neill *et al.*, 2002; Suhita *et al.*, 2004; Gonugunta *et al.*, 2008, 2009).

Despite repeated attempts, the identity of ABA putative receptors was not established for a long time. In 2009, two independent groups identified and established that PYR/PYL/RCAR proteins, that belong to the cyclase subfamily of the START/Bet v I protein superfamily, acted as ABA receptors in *Arabidopsis* (Ma *et al.*, 2009; Park *et al.*, 2009). Soon after, the crystallization, molecular modelling, and simulation of the structure of PYR/PYL/RCAR proteins unravelled the novel mechanisms of their function (Nishimura *et al.*, 2009; Yin *et al.*, 2009; Melcher *et al.*, 2010*a*). In the absence of ABA, PP2Cs keep the pool of SnRK2s dephosphorylated and limit the phosphorylation of transcription factors involved in ABA-induced gene expression. When present, ABA binds to PYR/PYL and then to PP2C making a functional complex, and blocks the normal function of PP2C. As a result, the SnRK2s stay in a phosphorylated state and activate the transcription factors and induce ABA-activated gene expression (Cutler *et al.*, 2010; Melcher *et al.*, 2010*b*; Raghavendra *et al.*, 2010).

The identification of PYR/PYL proteins as ABA receptors was made possible by the discovery of pyrabactin (4-bromo-N-(pyridine-2-yl methyl) naphthalene-1-sulfonamide), a synthetic compound. Pyrabactin was found to suppress markedly seed germination and hypocotyl growth, besides the promotion of gene expression, very similar to the pattern with ABA (Park *et al.*, 2009). Pyrabactin was considered as a potential anti-transpirant/stress adaptor, with possible uses in agriculture. It became clear that pyrabactin was acting as an agonist during ABA action. The expression of *pyr/pyl* mRNA was quite high not only in the seeds, but also in guard cells. In addition, the *Arabidopsis* quadruple mutants lacking *pyr1pyl1pyl2pyl4* were impaired in ABA-induced stomatal closure and the ABA-inhibition of stomatal opening (Nishimura *et al.*, 2010). All these studies imply that pyrabactin must affect guard cell function and stomatal closure. However, there have been no direct detailed experiments on stomatal closure in response to pyrabactin. In this report, the response of *Pisum sativum* guard cells to pyrabactin during stomatal closure was studied and the effects with ABA were compared. The effect of ABA on stomatal closure was examined in detail as well as changes in the signalling components, including pH, ROS, and NO. The influence of pyrabactin on stomata was then examined in the absence/presence of ABA and vice versa. Attempts were made to determine the apparent K_D for pyrabactin and ABA.

Materials and methods

Chemicals

Pyrabactin and apyrabactin were from Sigma-Aldrich and Chembridge Corporation (San Diego, CA), respectively. DAF-2DA was from Calbiochem (Rockland, MA). BCECF-AM was from Invitrogen (Molecular Probes). The remaining chemicals were from Sigma-Aldrich. The stock solutions of ABA and fusicoccin were prepared in ethanol and methanol, respectively. The stocks of pyrabactin, apyrabactin, fluorescent probes, and DPI were in DMSO and all the remaining chemicals were in milli Q water. Stock solutions were prepared in such a way that the final concentration of solvent was <0.2% in the final medium.

Plant materials and growth conditions

Plants of pea (*Pisum sativum* L., cv. Arkel) were raised from seeds, procured from Pocha Seeds, Pune, India. The plants were grown outdoors under a natural photoperiod of approximately 12 h and an average temperature of 30/20 °C day/night. The second pair of fully unfolded leaves was picked at about 09.00 h from 9–15-d-old plants for subsequent use.

Stomatal closure in epidermal strips

The abaxial epidermis was peeled from the leaves and cut into pieces of *c.* 0.4 cm^2. Twenty-five epidermal strips were transferred to 3 cm diameter Petri dishes containing 3 ml of opening medium (10 mM MES-KOH, pH 7.0, and 50 mM KCl). The epidermal strips were exposed to a bank of tungsten lamps, whose light was filtered through water jacket white light of 200–-250 μmol m^{-2} s^{-1}, for 150 min, to get maximum stomatal opening. 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. After 150 min of illumination, three epidermal strips were transferred to each of 24 well plates, containing medium and the required concentrations of ABA, pyrabactin or test compounds (inhibitors or scavengers). Illumination was continued for the next 120 min, before measuring stomatal apertures. When used together, the test compounds were added 15 min prior to the addition of ABA or pyrabactin.

The width of the stomatal apertures was measured under a research microscope with the help of a precalibrated ocular micrometer. 10–-15 apertures were monitored at random in each of three different epidermal strips, from each treatment. The experiments were repeated on at least three different days, making each measurement of stomatal aperture an average of a minimum of 90 stomata.

Fluorescent probes to monitor ROS, NO or cytoplasmic pH changes

Changes in ROS, NO or cytoplasmic pH levels in guard cells were monitored by using respective fluorescent probes, 2′,7′-dichlorofluorescien diacetate (H_2DCF-DA); 4, 5-diaminofluorescein diacetate (DAF-2DA); or 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein), acetoxymethyl ester (BCECF-AM) (Murata *et al.*, 2001; Neill *et al.*, 2002; Gonugunta *et al.*, 2008). Epidermal peels were mounted on a microscope slide with medical adhesive Telesis V (Premiere Products Inc., Pacaima, California, USA). Stomata were allowed to open by incubating epidermal tissues under 200–250 μmol m^{-2} s^{-1} white light for 150 min, in a medium of 10 mM MES-KOH, pH 7.0, and 50 mM KCl. After 150 min, the epidermal tissues were loaded with 30 μM H_2DCF-DA, 10 μM DAF-2DA or 5 μM BCECF-AM (30 min in dark), respectively, at 25±1 °C. The strips were rinsed with incubation buffer, to wash off excessive fluorophore. For studying time-course changes in ROS/NO/pH levels, the epidermal tissues were treated with 20 μM ABA or 20 μM pyrabactin, at zero-time and changes in fluorescence levels were measured at 3 min intervals. In the control sets, an equal and appropriate volume of ethanol or DMSO was added. Modulators were added 10 min prior to the addition of 20 μM ABA or 20 μM pyrabactin. The data are representative of the averages ±SE of three independent experiments, with measurements on a minimum of 60 individual stomata.

For time-course measurements, guard cells were observed under an inverted fluorescence microscope (Optiphot-2, Nikon, Japan) fitted with a monochrome high-resolution digital cooled CD camera (Cool snap FX) that enabled the quick capture of images, for further analysis later on. 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). Fluorescence intensity was measured in pixels in a scale of 0 (darkest) to 250 (brightest). The fluorescence intensity in the guard cells, without ABA, pyrabactin or any effectors (at the beginning of the experiment), was taken as 100% (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008). In some of the experiments (as indicated in the figure legends), a confocal microscope (TCSSP-2, AOBS 4 channel UV and visible; Leica, Heidelberg, Germany) was used to observe the changes in fluorescence indicating ROS, NO or cytoplasmic pH.

Results

Pyrabactin induced stomatal closure and changes in ROS, NO, and cytoplasmic pH levels in guard cells

ABA or its analogue pyrabactin caused stomatal closure in a concentration-dependent manner. The concentrations of ABA or pyrabactin, required for maximal stomatal closure, were quite similar (Fig. 1a, b). By contrast, apyrabactin, an inactive analogue of ABA, did not have any significant effect on stomata (Fig. 1c). The presence of fusicoccin (FC, a fungal toxin) prevented the stomatal closure caused by either ABA or pyrabactin (Fig. 2).

Pyrabactin increased ROS, NO, and cytoplasmic pH levels in guard cells within a few minutes after treatment, when compared with their respective controls (Fig. 3). Apyrabactin, an inactive analogue of ABA, did not cause any noticeable changes in ROS/NO/pH of guard cells (Fig. 3d, h, l). The initial rise of H_2DCF-DA fluorescence (indicating ROS levels) was seen at 6 min after treatment and fluorescence peaked between 18–24 min (Fig. 4a). Similarly, NO-specific DAF-2DA fluorescence (indicating NO) showed an initial rise at 9 min after treatment and fluorescence peaked after 18 min of treatment (Fig. 4b). The BCECF-AM fluorescence (reflecting the pH) initially increased within 3 min and peaked after 24 min (Fig. 4c). A similar pattern of changes were observed with ABA.

Modulators of ROS/NO/pH can relieve pyrabactin-induced stomatal closure and dampen the rise in ROS, NO or pH levels of guard cells

ROS modulators: DPI (NADPH oxidase inhibitor) or catalase (H_2O_2 scavenging enzyme), partially relieved stomatal closure by ABA or pyrabactin. Similarly, stomatal closure by ABA or pyrabactin was compromised in the presence of either cPTIO (NO scavenger), or L-NAME (nitric oxide synthase inhibitor) or tungstate (nitrate reductase inhibitor). Butyrate (a weak acid), relieved stomatal closure induced by ABA or pyrabactin (Fig. 5).

Catalase completely relieved the increase in H_2DCF-DA fluorescence by pyrabactin or ABA, while DPI had a partial effect, conforming the increase in fluorescence due to ROS (Fig. 6). DAF-2DA fluorescence increase by ABA or pyrabactin was abolished by cPTIO (NO scavenger). Similarly, L-NAME or tungstate (inhibitors of NO synthase or nitrate reductase) restricted the DAF-2DA fluorescence increase by ABA or pyrabactin. Butyrate restricted the rise in BCECF-AM fluorescence by ABA or pyrabactin.

Pyrabactin competes with ABA during stomatal closure

Experiments using varied concentrations of pyrabactin (0–100 μM), in the absence or presence of 5 μM ABA and vice-versa (varied concentrations of ABA in the absence or presence of 5 μM pyrabactin), revealed that the effects of ABA and pyrabactin were additive (Fig. 7). Kinetic analyses of these data indicated that the apparent IC_{50} of pyrabactin or ABA did not change much in the presence of ABA or pyrabactin (Fig. 7a, b). By contrast, the double reciprocal

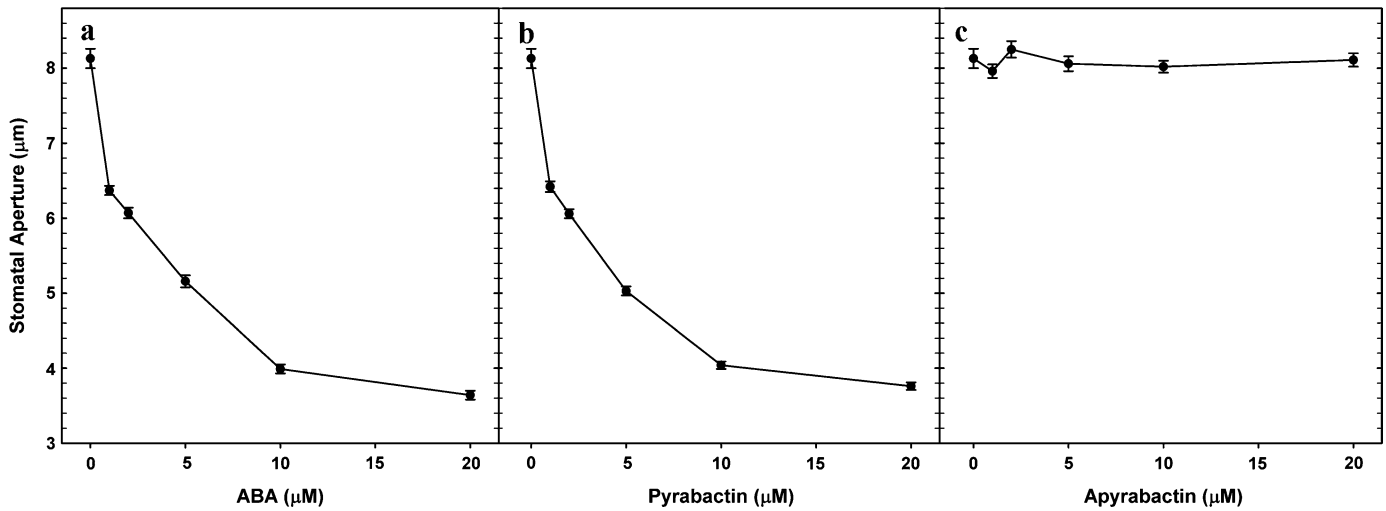

Fig. 1. Effect of ABA, pyrabactin or apyrabactin concentrations on stomata in abaxial epidermis of *Pisum sativum*. ABA or pyrabactin caused marked stomatal closure in a similar pattern, while apyrabactin did not have any significant effect. The data are averages of three independent experiments ±SE.

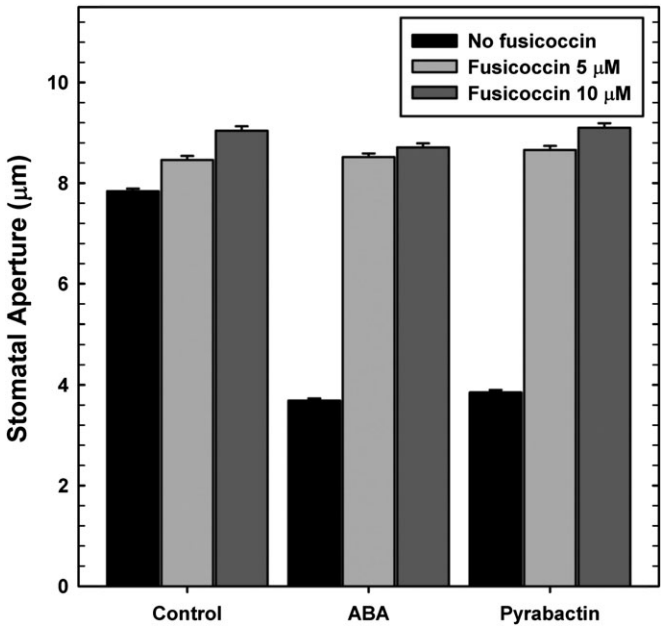

Fig. 2. Effect of fungal toxin, fusicoccin (FC), on stomatal closure caused by ABA or pyrabactin. Fusicoccin completely relieved stomatal closure by ABA or pyrabactin. The data are means of three experiments ±SE.

plots using low concentrations of varied concentrations of ABA or pyrabactin (0.5–5 μM) in the absence or presence of 5 μM pyrabactin or ABA demonstrated that the K_D of ABA increased by almost 4-fold in the presence of pyrabactin and the K_D of pyrabactin increased by nearly 3-fold in the presence of ABA (Fig. 7c, d).

Discussion

Pyrabactin, a synthetic ABA analogue, is considered to be a potential tool in future agriculture. Initially identified as a seed germination inhibitor, pyrabactin led the way for the identification, purification, and characterization of ABA receptors. Most of the earlier experiments with pyrabactin were done on either germinating seeds or *in vitro* reconstituted systems. Our results present an unequivocal and direct demonstration that pyrabactin is as powerful as ABA in promoting stomatal closure in abaxial epidermis.

Pyrabactin is as powerful as ABA in inducing stomatal closure

Pyrabactin caused a marked reduction in stomatal aperture. The effective concentrations, as well as the effect of pyrabactin on stomatal closure, were quite similar to ABA (Fig. 1). In contrast, apyrabactin did not induce closure of

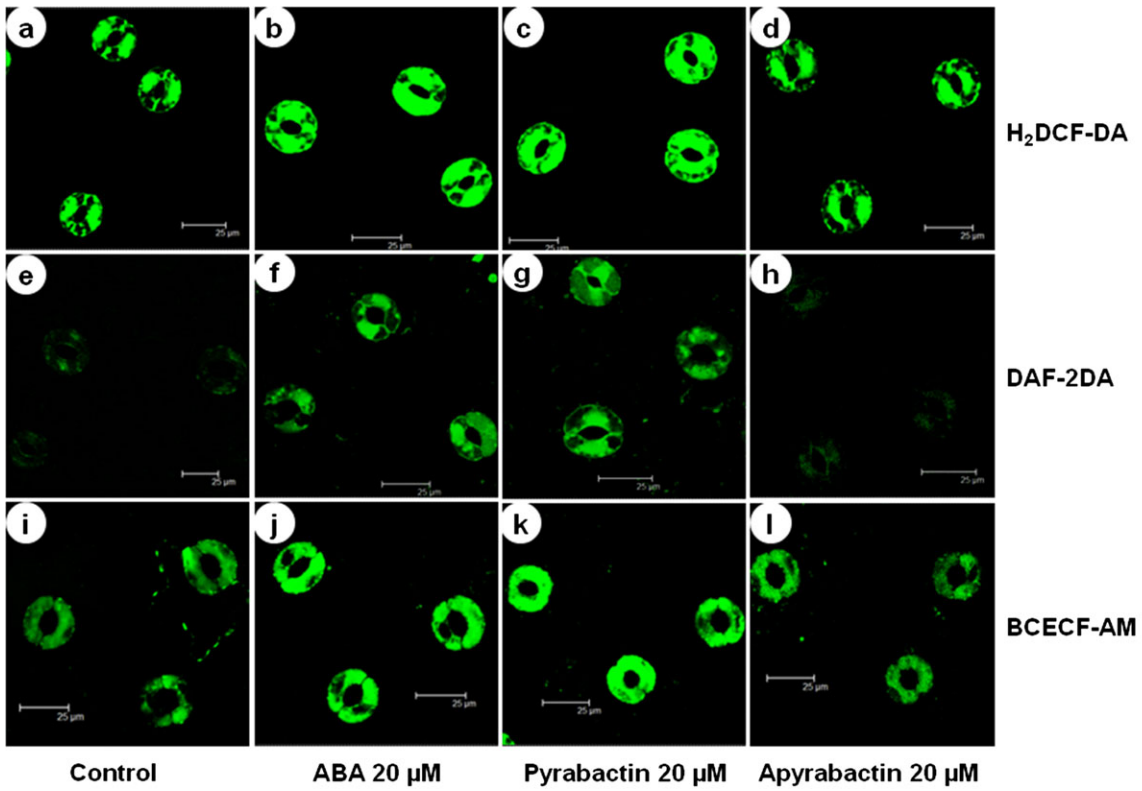

Fig. 3. Representative confocal images showing the changes in ROS, NO, and cytoplasmic pH changes in *Pisum sativum* guard cells in the presence or absence of ABA, pyrabactin or apyrabactin. (a–d) Changes in ROS levels as indicated by H_2DCF-DA fluorescence. (e–h) Changes in NO levels as indicated by DAF2-DA fluorescence. (i–l) Changes in cytoplasmic pH levels as indicated by BCECF-AM fluorescence. ABA or pyrabactin increased the levels of ROS, NO, and cytoplasmic pH, compared to respective controls.

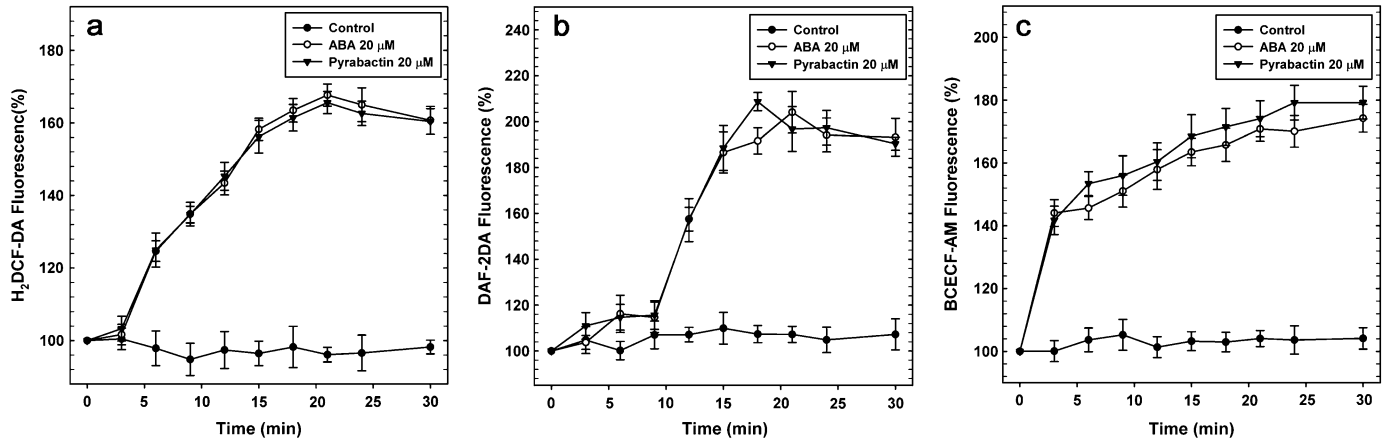

Fig. 4. Changes with time in fluorescence of guard cells, loaded with fluorescent probes specific for ROS, NO or pH. The fluorescence was monitored at different times after exposure to pyrabactin or ABA, using an inverted fluorescence microscope. The details are described in the Materials and methods. ABA or pyrabactin increased with time in the fluorescence intensities of H_2DCF-DA, DAF2-DA, and BCECF-AM reflecting the rise in ROS, NO, and pH of guard cells. The effects of pyrabactin and ABA were quite similar. The data are averages ±SE of three independent experiments, each representing a minimum of 60 individual stomata.

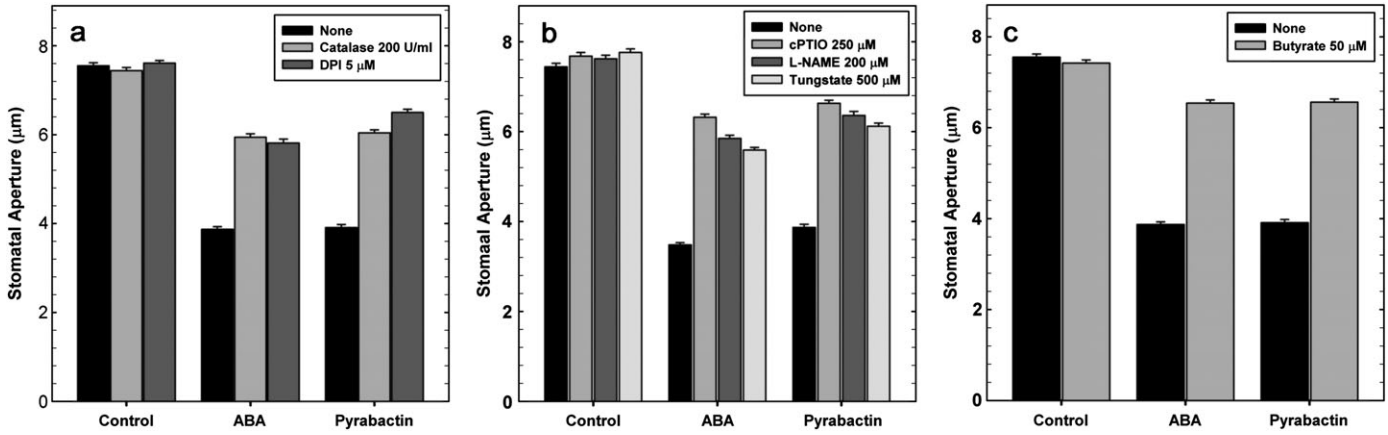

Fig. 5. The effect of ROS, NO or pH modulators on stomatal closure caused by ABA or pyrabactin. The decrease in stomatal aperture by ABA or pyrabactin was relieved by ROS modulators, catalase or DPI (a), NO modulators cPTIO and L-NAME or tungstate (b), and pH modulator, butyrate (c). The data are means ±SE of three independent experiments, each representing a minimum of 90 individual stomata.

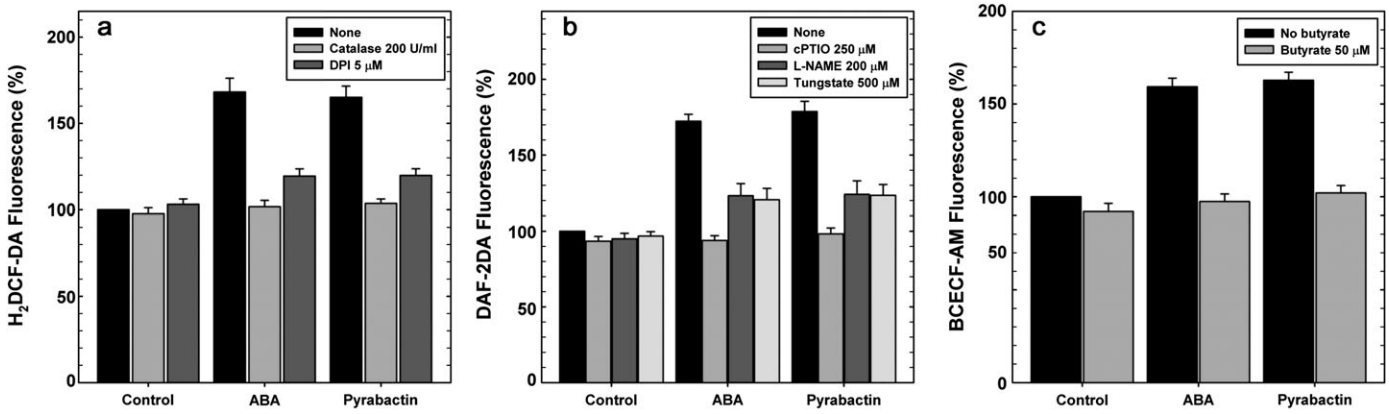

Fig. 6. Effect of ROS, NO or pH modulators on H_2DCF-DA, DAF2-DA, or BCECF-AM fluorescence levels respectively. Changes in fluorescence levels were monitored by using an inverted fluorescence microscope. (a) ROS modulators, catalase or DPI, prevented ABA or pyrabactin-induced increase of H_2DCF-DA fluorescence. (b) NO modulators, cPTIO and L-NAME or tungstate, prevented ABA- or pyrabactin-induced increase of DAF2-DA fluorescence. (c) Butyrate, a pH modulator, restricted the ABA- or pyrabactin-induced increase in BCECF-AM fluorescence. The data are averages ±SE of three independent experiments, each with a minimum of 60 individual stomata.

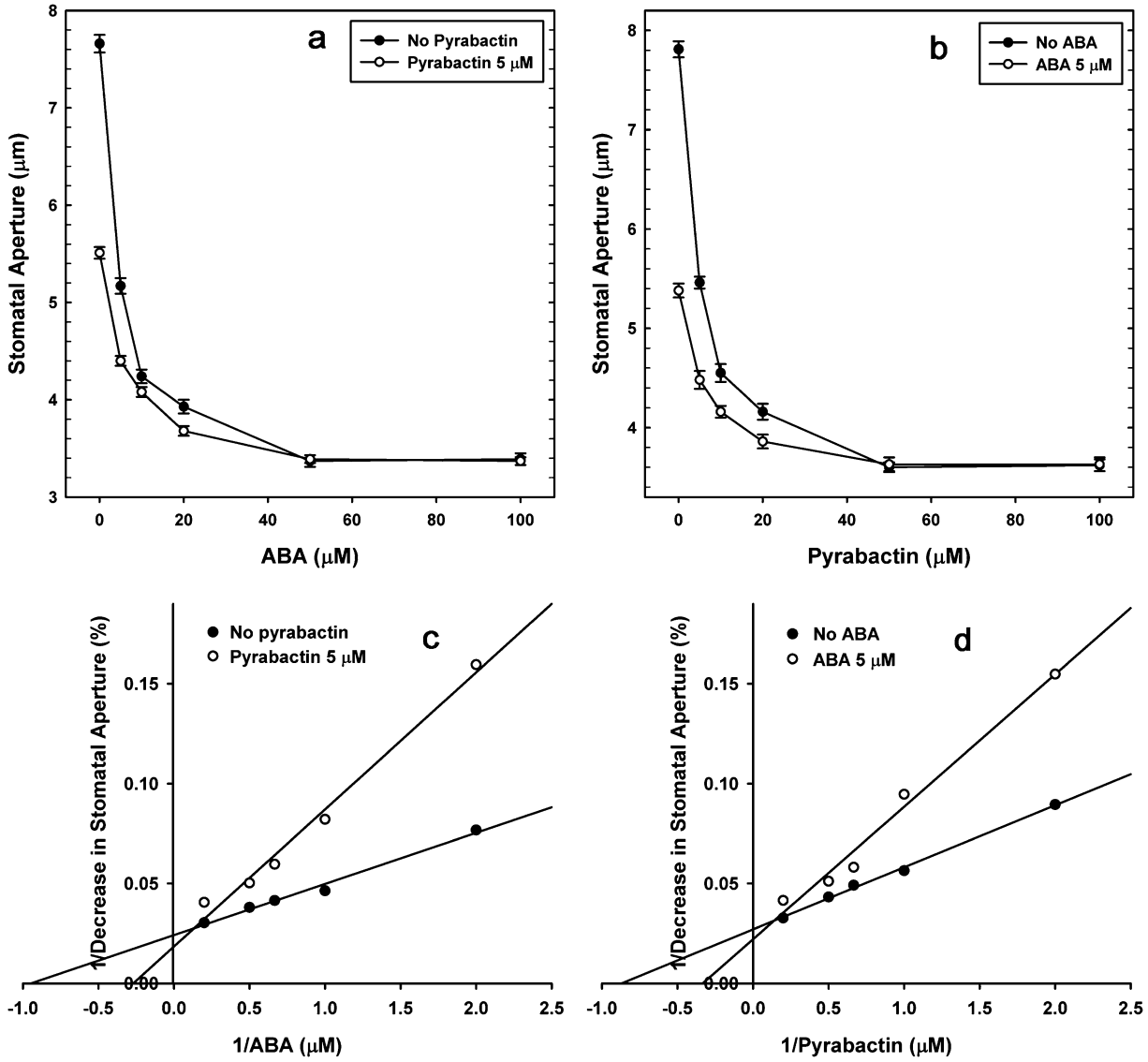

Fig. 7. Additive effect of ABA and pyrabactin during stomatal closure. (a) The apparent IC_{50} of ABA changed from 4.26 μM to 4.88 μM in the presence of 5 μM pyrabactin, when the change in stomatal closure was plotted as a function of concentration of ABA. (b) The apparent IC_{50} of pyrabactin changed from 4.47 μM to 5.05 μM in the presence of 5 μM ABA, when the change in stomatal closure was plotted as a function of concentration of pyrabactin. (c) The double reciprocal plot showing the increase of the apparent K_D of ABA, almost 4-fold, from 1.08 μM to 3.82 μM in the presence of 5 μM pyrabactin. (d) The double reciprocal plot demonstrated that the apparent K_D of pyrabactin increased by almost 3-fold from 1.17 μM to 3.05 μM in the presence of 5 μM ABA. The marked increase in K_D of ABA/pyrabactin in the presence of pyrabactin/ABA suggested their competition at or near their binding site during stomatal closure. The data are the average means of three independent experiments.

stomata suggesting that only pyrabactin can mimic ABA during stomatal closure. Park *et al.* (2009) have reported that pyrabactin caused the inhibition of seed germination like ABA, and facilitated the binding of PP2C with PYR1 during seed germination, while apyrabactin did not. These observations demonstrate that pyrabactin is an agonist of ABA, while apyrabactin is not an agonist. Several effects of ABA, such as the inhibition of seed germination or the promotion of stomatal closure are reversed by FC (She *et al.*, 2010; Zeng *et al.*, 2010). The stomatal closure caused by pyrabactin was also reversed completely by FC (Fig. 2), reconfirming our observations that pyrabactin is a strong mimic of ABA, in its effect on stomata.

Signalling components during pyrabactin-induced stomatal closure

The signalling components involved during ABA-induced stomatal closure have been extensively studied. After the initial recognition of ABA signal, through the ABA-PYR/PYL/RCAR-PP2C complex, the ABA-responsive kinases are activated. Subsequently, the guard cell pH becomes alkaline, the membrane-bound NADPH oxidase becomes active, the ROS levels are elevated, followed by a rise in NO

levels (Suhita *et al.*, 2004; Bright *et al.*, 2006; Gonugunta *et al.*, 2008; Kim *et al.*, 2010). On exposure to pyrabactin too, there were marked increases in pH, ROS, and NO levels (Fig. 3). Again, apyrabactin, an inactive analogue, did not cause any significant changes in the signalling components of guard cells (Fig. 3). It was therefore concluded that pyrabactin is an active analogue of ABA, in relation to its influence on stomatal function and signal transduction in guard cells. The present study illustrated that pyrabactin could successfully induce stomatal closure and generate small intracellular components, ROS, and NO during stomatal closure besides increasing the cytoplasmic pH. However, the exact mechanism of the induction of these signalling events is not yet known. It is quite possible that pyrabactin induces ROS and NO production in guard cells by the mediation of PYR/PYL/RCARs, PP2Cs, and OST1/SnRK2.6.

The kinetics of the rise in pH/ROS/NO as indicated by the respective fluorophores (Fig. 4) suggested that there was marked similarity in the sequence of changes due to pyrabactin or ABA. The ability of DPI and L-NAME to dampen the pyrabactin-induced rise in ROS/NO indicates that NADPH oxidase and putative NOS play an important role during pyrabactin effects. That the action of either pyrabactin or ABA required the alkalinization of guard cells was evident by the ability of butyrate to prevent the rise in pH as well as closure (Figs 5, 6). The effect of pyrabactin on stomatal closure and its dependence on rise in pH/ROS/NO of guard cells strikes a strong similarity with the action of ABA as well as methyl jasmonate (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008, 2009).

ABA and pyrabactin compete during stomatal closure

Further experiments on stomatal closure in response to varying concentrations of pyrabactin, in the presence of fixed concentration of ABA and vice versa, revealed interesting information on the apparent IC_{50} and K_D values of pyrabactin in relation to ABA (Fig. 7). The method followed here is similar to that used for examining the ethylene effects on bud and flower drop of *Begonia* in the presence of the gaseous ethylene-binding inhibitor, silver thiosulphate (Serek *et al.*, 1994). The apparent IC_{50} for pyrabactin did not change much in the presence of ABA or vice versa (Fig. 7a, b). By contrast, the K_D of pyrabactin or ABA (about 4–5 mM) was elevated in the presence of ABA or pyrabactin (Fig. 7c, d). These results suggested that pyrabactin was competing with ABA during the induction of stomatal closure, either at the active site or very close to the active site on ABA receptors. These values of IC_{50} or K_D values for pyrabactin or ABA (4–5 μM) appear high compared with the IC_{50} values reported for ABA (60–125 μM) during interaction with PP2C *in vivo* using a reconstituted system (Ma *et al.*, 2009; Park *et al.*, 2009). However, it has already been noted that the IC_{50} values of ABA to interact *in vivo* with PP2C (60 nM) can vary with that for suppressing root growth (3 μM), as observed by Ma *et al.* (2009). Similarly, IC_{50} values of 2–4 μM were reported for pyrabactin during the inhibition of seed germination and hypocotyl elongation (Park *et al.*, 2009).

The limitations of our experiments are acknowledged. For example, only the amount of pyrabactin or ABA in the external medium is known. The actual concentrations of pyrabactin or ABA within the cells (at the ABA receptor level) would be much less. Further, the rate of movement of pyrabactin or ABA across the guard cell could also vary. These factors can explain the differences in the observed K_D for pyrabactin/ABA in our experiments (done *in vivo*) and the values obtained during reconstitution attempts (*in vitro*) by Park *et al.* (2009) and Hao *et al.* (2010). In *Arabidopsis* seed germination assays, the pyrabactin concentration (100 μM) required to get an effect similar to ABA (10 μM) was almost 10 times higher (Park *et al.*, 2009; Melcher *et al.*, 2010*b*). However, our major point, that pyrabactin and ABA are competing with each other, seems to be certain.

Concluding remarks

The ability of pyrabactin, an analogue of ABA, to induce stomatal closure in *Pisum sativum* leaf abaxial epidermis was as powerful as ABA. This observation opens up an exciting possibility of using pyrabactin as an anti-transpirant. However, it is necessary to explore the possibility of synthesizing pyrabactin and/or analogues at an affordable price for suitable application in agriculture. Since the pattern of signalling components in response to pyrabactin and the reversal of pyrabactin effects by modulators was quite similar to that of ABA, it is suggested that pyrabactin and similar synthetic compounds could be quite useful in studying the signal transduction mechanisms in guard cells as well as in other plant tissues. Being quite similar to ABA in its mode of action, pyrabactin offers a promising potential for use in improving the plant adaptation to drought or other stress conditions.

Acknowledgements

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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.

Nupur Srivastava and Vijay K. Gonugunta have contributed equally.

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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

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

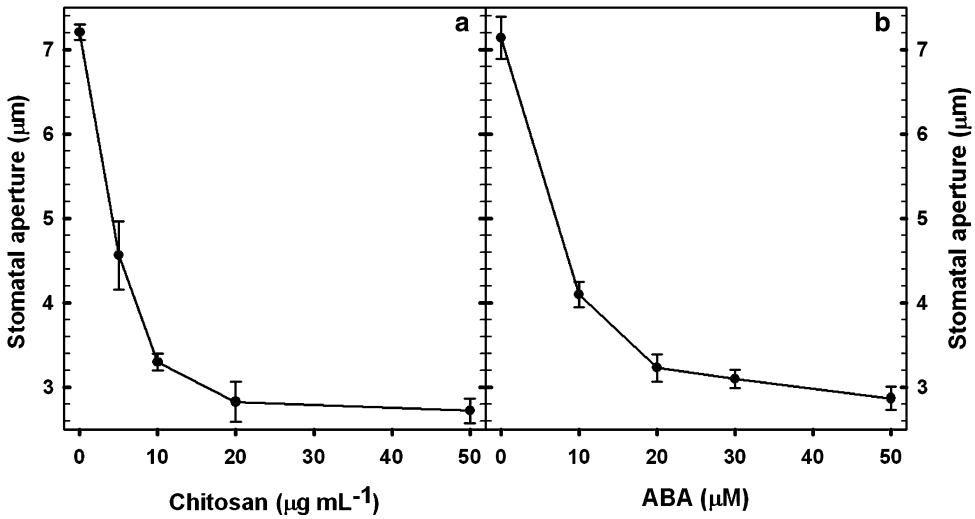

Fig. 1 Concentration dependent stomatal closure in epidermal strips of *Pisum sativum* by chitosan (**a**) or ABA (**b**). Results are the average ± SE of three to four independent experiments. Further details are given in "Materials and methods"

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

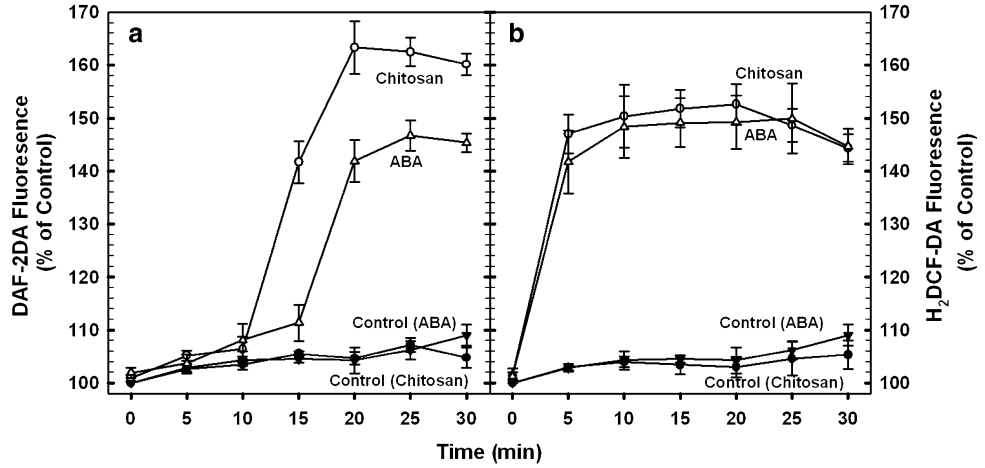

Fig. 3 Kinetics of increase in NO (**a**) or ROS (**b**) of guard cells in response to 5 μg ml^{-1} chitosan or 10 μM ABA. The epidermal strips were loaded with 40 μM DAF-2DA to monitor NO or 30 μM H_2DCFDA for ROS and incubated with or without chitosan. The levels of NO reached maximum at 20 min and 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"

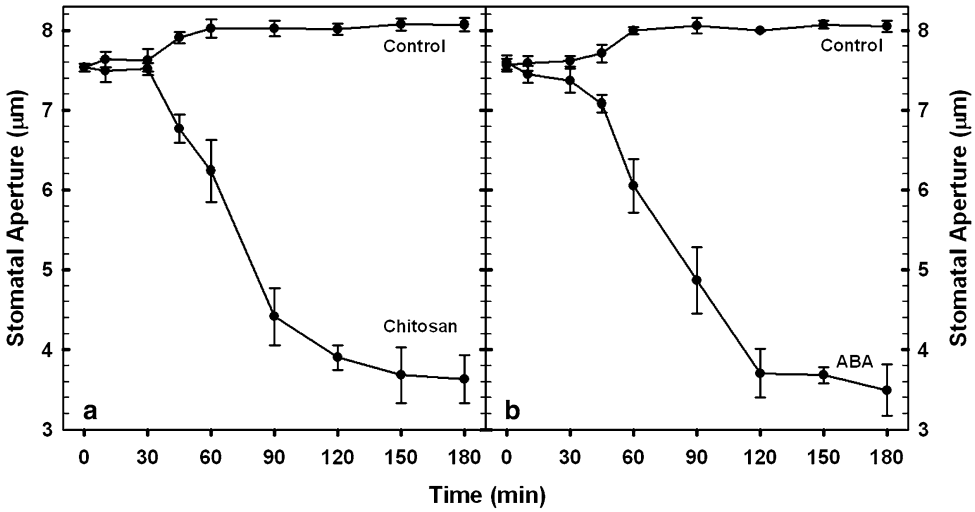

Fig. 4 Kinetics of stomatal closure by 5 μg ml^{-1} chitosan (**a**) or 10 μM ABA (**b**) in abaxial epidermis of *Pisum sativum*. The patterns may be compared to those of NO and ROS in Fig. 3. Results are the average ± SE of three to four independent experiments. Further details are given in "Materials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

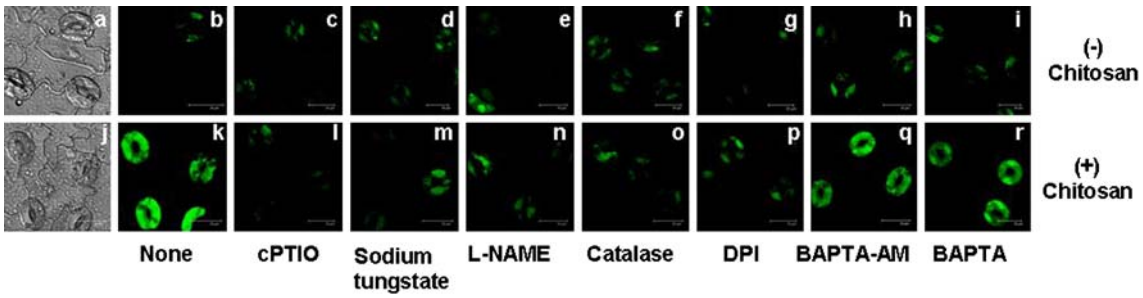

Fig. 5 The effect of NO and ROS modulators on the extent of NO production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe DAF-2DA. **b–i** Guard cells which are not exposed to chitosan. **k–r** Guard cells exposed to 5 μg ml^{-1} chitosan. **b**, **k** No modulators added. Treated 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

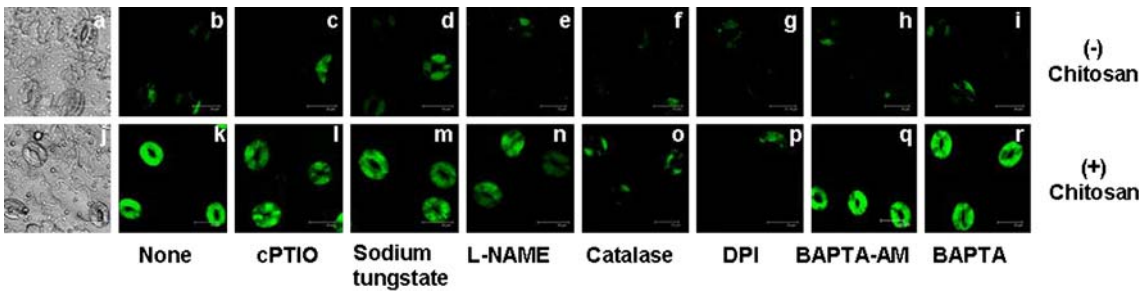

Fig. 6 The effect of NO/ROS modulators on the extent of ROS production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe H_2DCFDA. **b–i** Guard cells which are not exposed to chitosan. **k–r** Guard cells exposed to 5 μg ml^{-1} chitosan. **b**, **k** No modulators added. Treated 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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