Importance of reactive oxygen species and nitric oxide during chitosan-induced stomatal closure in *Pisum sativum* and *Arabidopsis thaliana*

A thesis submitted to the University of Hyderabad
for the award of Ph.D. degree in
Plant Sciences

By

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August 2012

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DECLARATION

I, Nupur, hereby declare that this thesis entitled "**Importance of reactive oxygen species and nitric oxide during chitosan-induced stomatal closure in *Pisum sativum* and *Arabidopsis thaliana***" 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. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**Importance of reactive oxygen species and nitric oxide during chitosan-induced stomatal closure in *Pisum sativum* and *Arabidopsis thaliana***" is a record of bonafide work done by Mrs. Nupur, a research scholar for Ph.D. programme in Plant Sciences, Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

Prof. A. S. Raghavendra
(Supervisor)

(Head of the Department) (Dean of the School)

Acknowledgements

I owe my gratitude to my supervisor, **Prof. A.S. Raghavendra**, for his constant encouragement, guidance and support.

I would like to thank **Prof. Atipalli R. Reddy**, Head, Department of Plant Sciences and **Prof. M. Ramanathan**, Dean, School of Life Sciences, for providing necessary facilities for my research. I extend my thanks to former Heads of Department **Prof. P.B. Kirti** and **Prof. Appa Rao Podile** and the former Dean, **Prof. A.S. Raghavendra**.

I am also highly thankful to my doctoral committee members, **Prof. A.R. Reddy** and **Dr. K.P.M.S.V. Padmasree** for their helpful suggestions.

I would like to express my gratitude to **Prof. Steven Neill** and **Dr. Ian Wilson (**University of West of England, Bristol, UK) for allowing me to work in their lab and for useful discussions and suggestions throughout my stay.

I extend my sincere thanks to **C.S. Murthy,** Senior Scientific Officer and **Nalini** (technical assistant) CIL, for their kind help to use central facilities during my research.

I am also thankful to my former labmates **Dr. Riazunnissa**, **Dr. Bakshu**, **Dr. Uday** and present labmates **Dr. Sunil**, **Dr. Poonam**, **Dr. Bindu**, **Sai, Rajsheel**, **Gayatri, Srinivas, Aswani** and project students **Pushpanjali**, **Purvi, Suma, Pratyusha and Raheem** for creating a friendly and lively environment in the lab.

It gives me immense pleasure to extend my special thanks to my senior **Dr. Vijay** and my junior **Mallikarjun** for being friendly and also for their constant support and encouragement.

I am also highly thankful to **Mr. Venu** and **Narasimha** for their assistance in the lab and monitoring the plants for my research work. I am also thankful to the non-teaching staff members of School of Life Sciences.

I am heartily thankful to all my friends and colleagues in **School/ Department of Life Sciences** for their help and support.

I am indebted to my labmates **Dr. Leon Civale,** research scholars, **Ms. Kuiama Lewandowski** and **Mr. Kannan Vembu** in Bristol, UK for their helping

and friendly support. I am also thankful to the technical staffs **Mrs. Alison** for arranging lab requirements and **Mr. David Corry** for helping me to handle the confocal microscopy in UK.

I gratefully acknowledge the financial support from **Commonwealth Split-Site Scholarship** for providing me a wonderful opportunity to carry out part of my research work in UK. Funding from **CSIR**, **DBT and UoH-DBT CREBB**, **DST-JC Bose** to **Prof. A. S. Raghavendra** as well as the entire umbrella of funding from **DST-FIST, DBT-CREBB and UGC-SAP** to the Department and School are highly acknowledged.

Last, but most important are the love and gratitude for my family members. I owe my deep respect for my father **Mr. Umesh Chandra Srivastava** for his constant affection and encouragement. I am heartily thankful to my husband **Abhinav** who stood by my side in both good and bad circumstances and for his unconditional love and support. I would like to express my love and affection for my daughter **Aditri** whose smile always made me to move forward during my research.

I also want to express deep sense of respect to my father-in-law **Mr. Amrendra Kumar** and mother-in-law **Mrs. Mira Sinha** and **Mrs. Anju Sinha** for their constant love and support during my studies.

Finally, I dedicate my entire research work to my mother **Mrs. Saroj Bala.**

Nupur

Dedicated to my beloved and respected Mother……..

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 acetoxy methyl ester
cerk1	=	chitin elicitor receptor kinase 1
cPTIO	=	carboxy-2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide
DAF-2DA	=	4, 5-diamino fluorescein diacetate
DPI	=	diphenyleneiodonium chloride
H_2DCFDA	=	2', 7'-dichlorofluorescein diacetate
L-NAME	=	N-nitro-L-Arg-methyl ester
MAPK	=	mitogen-activated protein kinase
MJ	=	methyl jasmonate
nia1	=	nitrate reductase isoform 1
nia2	=	nitrate reductase isoform 2
NO	=	nitric oxide
NOS	=	nitric oxide synthase
NR	=	nitrate reductase
PI3K	=	phosphatidylinositol 3 kinase
rbohD/F	=	respiratory burst oxidase homologue D/F
ROS	=	reactive oxygen species
SNP	=	sodium nitroprusside

All the remaining abbreviations are all standard ones and as per *Plant Physiology* issue, 2010, Instructions for contributors, website: http://www.aspb.org

Contents

Chapter 1

Introduction and Review of Literature

Chapter 1
Introduction and Review of Literature

Global climate change and lack of availability of water in several parts of the world increase the need for understanding how plants regulate stomatal closure, and exploite such knowledge to sustain crop yields. The stomata play a major role in controlling gas exchange between plant and the surrounding atmosphere. The stomatal pore is formed by two specialized guard cells, which in some plant species are surrounded by also a set of subsidiary cells (Willmer and Fricker 1996). The opening and closing of stomatal pores are brought about by changes in turgidity of guard cells, stomata being open when guard cells are turgid and closed when guard cells are flaccid. The changes in turgor and guard cell volume are facilitated by the transport of ions and water through protein channels across the plasma and vacuolar membranes (Schroeder et al. 2001; Pandey et al. 2007). When solutes accumulate, the water potential of guard cells is lowered. As a result, a steep water potential gradient, drives water into the guard cells from the neighbouring cells. The guard cells become turgid and swell in size, opening stomata. In a reversal of these events, guard cells lose solutes, water moves out of the guard cells, making them flaccid leading to stomatal closure. The unique structure of the cell wall including plasma membrane, tonoplast and cytoskeleton of guard cells are also involved in the regulation of stomatal movement (Gao et al. 2009).

Guard cells have become a model system to dissect the dynamics and mechanisms of plant-cell signaling because they regulate stomatal apertures by responding quickly to both endogenous hormonal stimuli and environmental signals. These signals typically include light, CO_2, plant pathogens, the hormones and water stress (Schroeder et al. 2001;

Kim et al. 2010; Zeng et al. 2010). Further, guard cells are ideal to study ion transport and signal transduction, as they lack functional plasmodesmata and are physiologically isolated from the rest of the plant body (Willmer and Fricker 1996). Considerable research has been carried out to understand the mechanism of stomatal movement and guard cell signaling. Some of the reviews in last five years related to guard cell signaling are: Shimazaki et al. (2007), Lee and Lee (2008), Neill et al. (2008), Wang and Song (2008), Acharya and Assmann (2009), Mott (2009), Sirichandra et al. (2009), Inoue et al. (2010), Kim et al. (2010), Kinoshita and Hayashi (2011), Mori and Murata (2011), Araújo et al. (2011).

Abiotic factors affecting stomatal movements

Light

Stomatal opening is induced by both blue and red lights. Blue light activates the plasma membrane H^+-ATPase and its activation is mediated via the phosphorylation of threonine residues in the C-terminus with a subsequent binding of a 14-3-3 protein (Kinoshita and Hayashi 2011). The activated pump drives K^+ accumulation through the voltage-gated inward-rectifying K^+ channels and finally results in stomatal opening. Blue-light-induced stomatal opening is mediated via phototropins that transduce light signals into diverse responses including stomatal opening (Inoue et al. 2010). The stomatal opening response to red light requires a high light intensity, and has mostly been studied with either leaves or isolated epidermis. The guard cell protoplasts, also show, but small responses in red light (Mott 2009).

Carbon dioxide

Stomata close in response to high CO_2 concentrations and open at low CO_2. The physiology of CO_2 control of stomatal apertures has been discussed in a few reviews (Vavasseur and Raghavendra 2005; Israelsson et al. 2006; Kim et al 2010; Araújo et al. 2011). High atmospheric CO_2 concentrations activate plasma membrane anion channels and K^+ efflux channels in guard cells, and promote chloride release from guard cells and membrane depolarization. Plant mutants with affected CO_2stomatal response have also been identified (Young et al. 2006).

Humidity and temperature

Stomatal closure occurs rapidly in response to a reduction in relative humidity, affecting the turgor pressure in guard cells, as well as in epidermal cells. The ability of guard cells to sense changes in water status can be improved by ABA-dependent signaling (Roelfsema and Hedrich 2005). Stomata close rapidly under drought conditions. In warm and moist environments, stomata open wide, while at low temperatures they tend to close (Reynolds-Henne et al. 2010).

Plant hormones

Depending on their concentration and type, plant hormones promote either opening or closure effects on stomatal guard cells (Acharya and Assmann 2009). Exogenous GA application appears to have little or no effect on stomatal apertures in *Arabidopsis*. Low auxin concentrations promote stomatal opening, particularly in adaxial epidermis. Exogenous cytokinins can restrict abscisic acid (ABA) induced stomatal closure in

diverse species (Acharya and Assmann 2009). Ethylene alone promotes stomatal closure but antagonizes the effects of ABA-induced stomatal closure.

The stress hormone, ABA, is a classic example for hormone-induced stomatal closure and decrease in transpiration. The signaling components in guard cells during ABA-induced stomatal closure have been extensively studied. The presence of ABA promotes activation of G-proteins, production of reactive oxygen species (ROS), generation of NO, elevation of cytosolic pH or cytosolic Ca^{2+}, proteinphosphorylation/dephosphorylation (Wang and Song 2008; Neill et al. 2008; Gonugunta et al. 2008; Umezawa et al. 2010; Raghavendra et al. 2010). Exogenous application of ethylene gas, ethephon (an ethylene-releasing compound), or ACC (the immediate precursor of ethylene) all promote stomatal closure in *Arabidopsis* leaves. Brassinolide (BL) also promotes stomatal closure and inhibits stomatal opening in epidermal peels of *Vicia faba* (Acharya and Assmann 2009). Methyl jasmonate (MeJA)-mediated stomatal closure has been associated with cytoplasmic alkalinization in guard cells, production of ROS (via *AtrbohD/F*) and NO, and activation of K^+ efflux channels and slow anion channels (Suhita et al. 2004; Munemasa et al. 2011).

Biotic factors affecting stomatal movement

Elicitors

Among the most important biotic factors that affect stomatal movement are elicitors. The elicitors may be cell wall components (such as chitosan), pathogen secretions, or substances released by hydrolytic enzymes of pathogens and plants. Elicitors stimulate several plant defense responses, including increase in calcium fluxes, generation of ROS

and NO, activation of signaling enzymes, stomatal closure and expression of several defense-related proteins (Agrawal et al. 2002; Klüsener et al. 2002; Hu et al. 2004; Garcia-Brugger et al. 2006; Srivastava et al. 2009; Khokon et al. 2010). Stomatal closure in response to elicitors limits the entry of various pathogens including fungi (Hamel and Beaudoin 2010). Some examples of these elicitors include oligosaccharide elicitors as chitooligosaccharides/chitosan, proteinaceous elicitors (cryptogein, flagellin as Flg22, xylanase), glycoprotein elicitors, lipid elicitors (sphingolipids, ergosterols) (Gauthier et al. 2007; Garcia-Brugger et al. 2006).

Chitosan is a biopolymer (polymer of β-1,4 linked glucosamine residues) derived from chitin that elicits a wide range of defense responses in plants, one of them being stomatal closure (Table 1.1). The biological activity of chitosan, besides the plant model, strictly depends on its physicochemical properties (deacetylation degree, molecular weight and viscosity). Unlike vast literature on the responses of guard cells to hormones such as ABA, studies on the physiological and biochemical effects of elicitors, like chitosan, on guard cells are quite limited (Lee et al. 1999; Srivastava et al. 2009; Khokon et al. 2010). Chitosan effects on plant cells are reviewed by Shibuya and Minami (2001), Iriti and Faoro (2009), Hamel and Beaudoin (2010).

Signal transduction in guard cells

The signaling pathway in guard cells involves the binding of a signaling factor (internal or external) to a receptor, which causes a change in the concentrations of certain molecules, described as second messengers, in the plasma membrane or cytosol. The concerted influence of secondary messengers and other signaling components brings out

the cellular response. The role of a few components during chitosan signaling in guard cells is known, but the exact sequence of their participation is still being studied.

Inward and outward rectifying K^+ channels carrying K^+ ions across the membrane play a critical role in turgor regulation of guard cells. Cells in the depolarized state will have a net efflux of K^+ leading to stomatal closure, while cells in the hyperpolarized state have a net K^+ influx, which in turn lead to stomatal closure (Szczerba et al. 2009; Sirichandra et al. 2009).

An increase in cytosolic free Ca^{2+} activates anion channels and inactivates the plasma membrane H^+-ATPase, causing membrane depolarization, which results in turgor reduction and stomatal closure (Roelfsema and Hedrich 2010). Some studies have indicated possible roles of Ca^{2+} in CO_2-regulated stomatal aperture, auxin-induced stomatal opening and light-dependent stomatal opening (Israelsson et al. 2006; Harada and Shimazaki 2009). However, the importance of the Ca^{2+} signal in guard cells is still debated. Cytosolic alkalization can activate outward K^+ currents and inactivate inward K^+ currents in guard cells to promote net efflux of K^+, leading to stomatal closure (Irving et al. 1992; Suhita et al. 2004; Gonugunta et al. 2008).

Protein kinases and phosphatases that are already identified to regulate guard cell ABA signaling are: (AAPK) ABA-activated Protein Kinase, OPEN STOMATA (OST)1/Srk2e/SnRK2.6, and protein phosphatase type 2C (PP2C). Some other kinases are calcium dependent protein kinases CPK3, CPK4, CPK6 and CPK11, mitogen-activated protein kinases MPK3, MPK9 and MPK12. At least four of the PP2C genes (*ABI1*, *ABI2*, *AtPP2CA*, *HAB1*) are identified as negative regulators of ABA (Wasilewska et al. 2008; Lee and Luan 2012).

Table 1.1. Cellular responses elicited by chitosan in plants

Plant Responses	References
Induction of calcium transients	Zuppini et al. 2004
Plasma membrane H^+-ATPase inhibition	Amborabé et al. 2008
MAP-kinase activation	Hamel and Beaudoin 2010
Callose apposition	Malerba et al. 2012
Production of ROS and NO	Srivastava et al. 2009
Hypersensitive response/programmed cell death	Vasil'ev et al. 2009
Induction of PR-Pathogenesis related proteins	Povero et al. 2011
Secondary metabolite production	Ahmed and Se-Kwon 2010

H_2O_2 produced by NADPH oxidases play an important role in signaling and development in plants (Fig. 1.1). In guard cells of several species (*Arabidopsis*, *Vicia*, *Lycopersicon*, *Commelina* and *Pisum*) production of ROS occurs in response to ABA, MJ, bicarbonate or even by elicitors (Suhita et al. 2004; Kwak et al. 2006; Kolla et al. 2007; Srivastava et al. 2009; Khokon et al. 2010). NADPH oxidase participates during hormonal responses in plants, and is named respiratory burst oxidase homologs (Rbohs). Among the 10 Rbohs (*Atrboh A-J*) in *Arabidopsis* plants, *rbohD* and *rbohF* are required for various plant processes including ABA guard cell signaling and also for oxidative burst in response to pathogens (Desikan et al. 2004; Marino et al. 2012).

Nitric oxide (NO) is a reactive nitrogen species and controls a diverse set of processes (Fig. 1.1). Two possible plant enzymes capable of NO biosynthesis in plants are nitric oxide synthase (NOS) like enzyme and nitrate reductase (NR). However, the occurrence of NOS-like enzymes in plants, is questioned (Zemojtel *et al.* 2006). NR can mediate NO generation from nitrate in an NAD(P)H-dependent manner (Neill et al. 2008). In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2*. The involvement of NR-mediated NO production during ABA-induced stomatal closure has been demonstrated using *Arabidopsis* mutants defective in NR activity (Desikan et al. 2004; Bright et al. 2006). Guard cells also generate NO in response to elicitor challenge and MeJA (Melotto *et al.* 2006; Srivastava et al. 2009), treatments that both induce stomatal closure. As with the effect of ABA, in these cases the prevention of NO accumulation also inhibited the associated stomatal closure.

Chitosan-mediated changes in signalling components

Addition of chitin or chitosan to plant cells elicits typical defense responses, which include changes in ion fluxes, plasma membrane depolarization by inhibiting the activity of plasma membrane H^+-ATPase generation of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), induction of defense gene expression, synthesis of phytoalexins, cell wall strengthening and in some cases, induction of cell death (Shibuya and Minami 2001; Lin et al. 2005). Chitosan treatments also increase intracellular calcium (Ca^{2+}) level and accelerate production of ROS via NADPH oxidase activity (Shibuya and Minami 2001; Iriti and Faoro2009). Chitin/chito-oligosaccharides treatment also induces changes in protein phosphorylation status implicating both protein kinases and phosphatases in chito-oligosaccharide mediated responses. Various studies have demonstrated AtMPK3/6 are activated within minutes following chitin/chito-oligosaccharide perception (Hamel and Beaudoin 2010). Involvement of other MAPK signaling components are yet to be examined.

The identification and isolation of a chitosan-binding protein from cabbage leaves (Chen and Xu 2005) suggest that specific receptors may detect chitosan. However, the binding of the lysin motif (LysM)-containing chitin elicitor receptor kinase 1 (CERK1) to chitosan appeared to be physiologically relevant in *Arabidopsis* (Petutschnig et al. 2010). The signaling pathway for chitooligosaccharides as chitin are broadly studied (Hamel and Beaudoin 2010) but chitosan induced modulation of signaling components need to be further examined. The role and interactions of some of the secondary messengers as ROS, NO, $Ca^{2+}$and pH during chitosan-induced guard cell signaling need to be studied. It

would be interesting to characterize the genes induced by chitosan during defense responses.

Use of *Arabidopsis* mutants for studying guard cell signaling

Arabidopsis mutants have been quite useful to understand the plant function, including mechanisms of signaling cascades and various aspects of plant development (Koornneef and Meinke 2010). Such studies with *Arabidopsis* mutants have demonstrated the essential role of signaling components involved during ABA-induced stomatal closure or ABA-mediated inhibition of stomatal opening: for example, protein phosphatase type 2C or PP2C (*abi1* and *abi2* mutants); serine/threonine protein kinases (*ost1-1* and *ost1-2* mutants); (Pei et al. 1997; Murata et al. 2001; Mustili et al. 2002; Umezawa et al. 2009); a heterotrimeric GTP binding protein α-subunit (*GPA1* mutants) (Zhang et al. 2011).

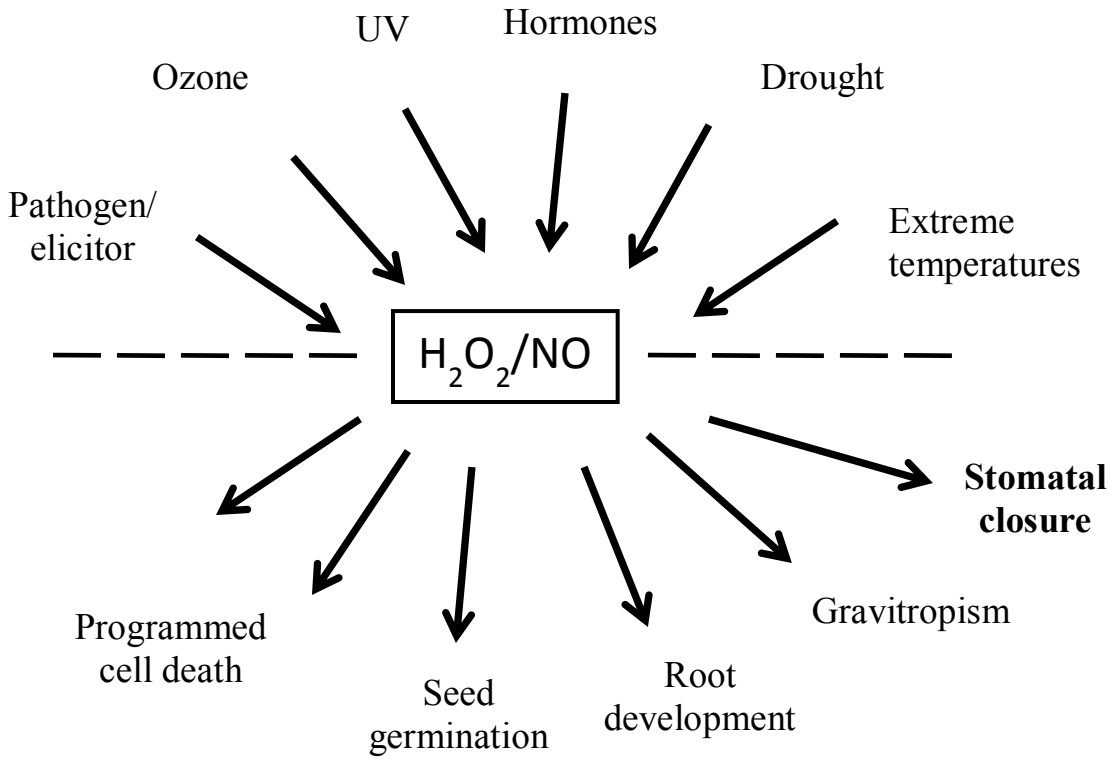

Figure 1.1. The central role of H_2O_2 and NO as signalling components during cellular responses to various stresses and stimuli

NADPH oxidase, particularly RBOHD/RBOHF (*AtrbohD/F* mutants) (Khokon et al. 2010); nitrate reductase (NR-deficient) (*nia1* and *nia2* mutants) (Desikan et al. 2002; Neill et al. 2008); chitin elicitor receptor kinase (*cerk1* mutants) (Miya et al. 2007) were studied during both ABA or chitosan-induced responses in guard cells.

The *Arabidopsis* mutants, *abi1* and *abi2*, are mutants deficient in type 2C protein phosphatases, which are normally negative regulators of ABA mediated responses (Merlot et al. 2001). Some additional negative regulators of ABA signaling in guard cells include the farnesyltransferase β-subunit ERA1 (Pei et al. 1998), the mRNA cap binding protein ABH1 and the Sm-like snRNP protein SAD1 (Xiong et al. 2001). The recessive *gca2* mutation inhibits ABA-induced stomatal closure, indicating that GCA2 is a positive regulator of ABA signaling. The disruption of the *GPA1* gene, which encodes a heterotrimeric GTP binding protein α-subunit, impairs the ABA inhibition of stomatal opening (Wang et al. 2001).

Infrared thermal imaging system was used often to screen for *Arabidopsis* mutants that displayed a reduced ability to close their stomata in response to drought stress and hence appeared colder than the wild type (Merlot et al. 2002). Two of the mutants recovered correspond to a novel locus designated *Open Stomata1* (*OST1*). The stomata of the *ost1* mutants were insensitive to ABA but were responsive to light and CO_2. OST1 appear to act in between ABA perception and the production of ROS (Mustilli et al. 2002).

Need for further studies

Physiological, cell biological and molecular genetic approaches have identified numerous components and regulatory mechanisms during ABA-induced signaling in guard cells. However, the signaling mechanisms involved during chitosan elicitation are quite limited. Various pharmacological, genetic, proteomic and metabolomic studies are still required for predicting the signaling interactions and novel regulatory mechanisms in chitosan/stress signaling. The interactions among the secondary messengers as ROS, NO, Ca^{2+} or pH during chitosan signaling need further studies. The receptors and protein-protein interactions during chitosan perception have to be elucidated. Also, real-time measurements of signaling components in guard cells during the responses to chitosan are also needed.

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

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Chapter 2
Objectives and Approach

Chapter 2
Objectives and Approach

Guard cells are highly specialized with unique structural, physiological and biochemical features. These cells have evolved to be model systems to examine the dynamics and mechanisms of signal transduction in plant cells. The guard cells are metabolically active and respond quickly and reversibly to diverse environmental signals. For e.g. these cells respond to plant hormones such as abscisic acid (ABA), methyl jasmonate (MJ) or auxin and elicitors, such as chitosan. The signal transduction occurs through several secondary messengers including reactive oxygen species (ROS), nitric oxide (NO), G-proteins, calcium and protein kinases/protein phosphatases, ultimately exerting effects on ion channels.

Extensive research has been carried out, on the effects of ABA on plant tissues, with focus on the elucidation of secondary messengers involved and regulation of gene expression during the transduction of ABA signal. Chitosan, as an elicitor modulates stomatal movement, as effectively as ABA. Unlike vast literature on the responses of guard cells to ABA, studies on the mechanism of chitosan action on guard cells are quite limited. Taking cue from the existing literature on signaling components during ABA-induced stomatal closure, the following objectives were framed for studying the role and importance of secondary messengers involved during chitosan-induced stomatal closure, as well as the patterns of gene expression in leaves.

Objectives

1. Study the role and importance of ROS and NO during chitosan-induced stomatal closure in abaxial epidermis of *Pisum sativum*.

2. Examine the role and importance of ROS, NO and Ca^{2+} during chitosan-mediated inhibition of light induced stomatal opening in abaxial epidermis of *Pisum sativum*
3. Validate the role of nitrate reductase, NAD(P)H oxidase and chitin elicitor receptor kinase during chitosan-induced stomatal closure using suitable *Arabidopsis* mutants.
4. Characterize the chitosan-induced expression of nitrate reductase and NAD(P)H oxidase genes in leaves of *Arabidopsis* wild types and mutants.

Pisum sativum (pea) and *Arabidopsis thaliana* plants were used for the experiments because the leaves of these species offers an excellent experimental material, which can be in the form of either intact leaves or epidermal peels for the study of physiological features. The advantage of *Pisum sativum* is that the epidermal strips of these plants is very easy to peel and the strips are quite pure. These can be used for monitoring stomatal movements and for studying the levels of ROS and NO production along with fluorescent probes. The images can be acquired and later analysed using the image analysis system.

The role and importance of ROS and NO were established using different modulators. ROS modulators include catalase (ROS scavenger) and DPI (NADPH oxidase inhibitor) while NO modulators used are cPTIO (NO scavenger), L-NAME (nitric oxide synthase inhibitor) and tungstate (nitrate reductase inhibitor). The real time monitoring of ROS and NO production was carried out using fluorescent probes: for NO production and ROS production as DAF-2DA (for NO) and H_2DCFDA (for ROS) respectively. Such monitoring gave a fair idea of sequence of signaling events that occur during stomatal closure by chitosan.

The role of ROS and NO during chitosan-induced stomatal closure, was validated by using *Arabidopsis* mutant plants deficient in NADPH oxidase (*rbohD/F*), nitrate reductase (*nia1* or *nia2*) or chitin elicitor receptor kinase (*cerk1*). *Arabidopsis* plants were raised in controlled growth chambers and light regimes. *Arabidopsis* mutants were used also for experiments to characterize the role of genes related to biosynthesis of ROS/NO.

The variation in the experimental plants used during the present studies is because part of my experiments have been carried out under the umbrella of Commonwealth Split-Site Scholarship Programme, in the laboratory of Professor Steven Neill, Centre for Research in Plant Sciences, University of West of England, Bristol, UK.

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Chapter 3
Materials and Methods

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Plant material and growth conditions

Plants of pea (*Pisum sativum* L., cv. Arkel) were raised from seeds, procured from Pocha Seeds company, Pune, India. The pea seeds were soaked in water overnight and then surface sterilized with 0.2% (v/v) sodium hypochlorite solution. The seeds were kept covered in moist black cloth at 25 °C until they germinated, usually for 3 days. The germinating seeds were then sown in plastic trays filled with soil and farmyard manure (3:1, v/v) and were watered twice daily. The plants were grown in green house, under natural photoperiod of approximately 12 h and average daily temperatures of 30 °C day / 20 °C night. The second pair of fully unfolded leaves were picked from 2-3-weeks old plants and used for epidermal peels (Fig. 3.1 A).

Wild-type and various mutants of the Columbia ecotype of *Arabidopsis thaliana* were sown in Levington's F2 compost (Avoncrop, Bristol, UK) and grown (16-h photo-period, 100 μE m^{-2} s^{-1}, 22 °C, 60% relative humidity) in controlled environment growth chambers (Sanyo-Gallenkamp, Loughborough, UK). Fully expanded young leaves were harvested at 4-5 weeks after germination (Fig. 3.1 C-H). The *nia1:ds* seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) with the Ler wild type background. *nia2:5* mutant seeds were obtained from N. Crawford (University of California, San Diego, CA, USA), *atrbohD/F* double mutant seeds were obtained from J. Jones (The Sainsbury Laboratory, Norwich, UK) and *cerk1* seeds were obtained from NASC (Nottingham, UK) with Col-0 wild type respectively as the background (Fig. 3.1).

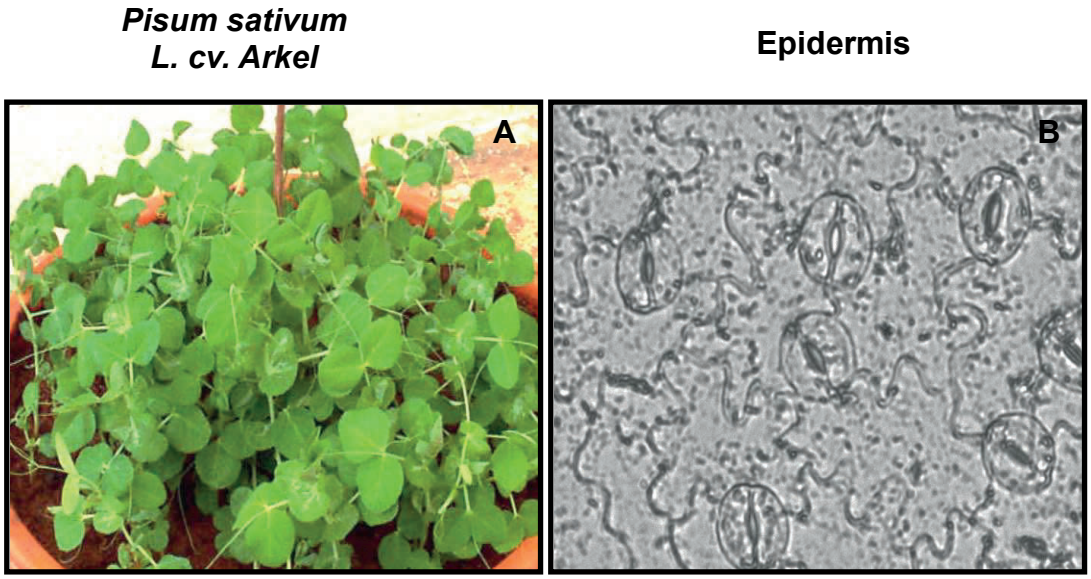

Figure 3.1. A view of *Pisum sativum* plants used in the experiments. (A) The plants of *Pisum sativum* were grown in a green house. The epidermal strips (B) were used for bioassays.

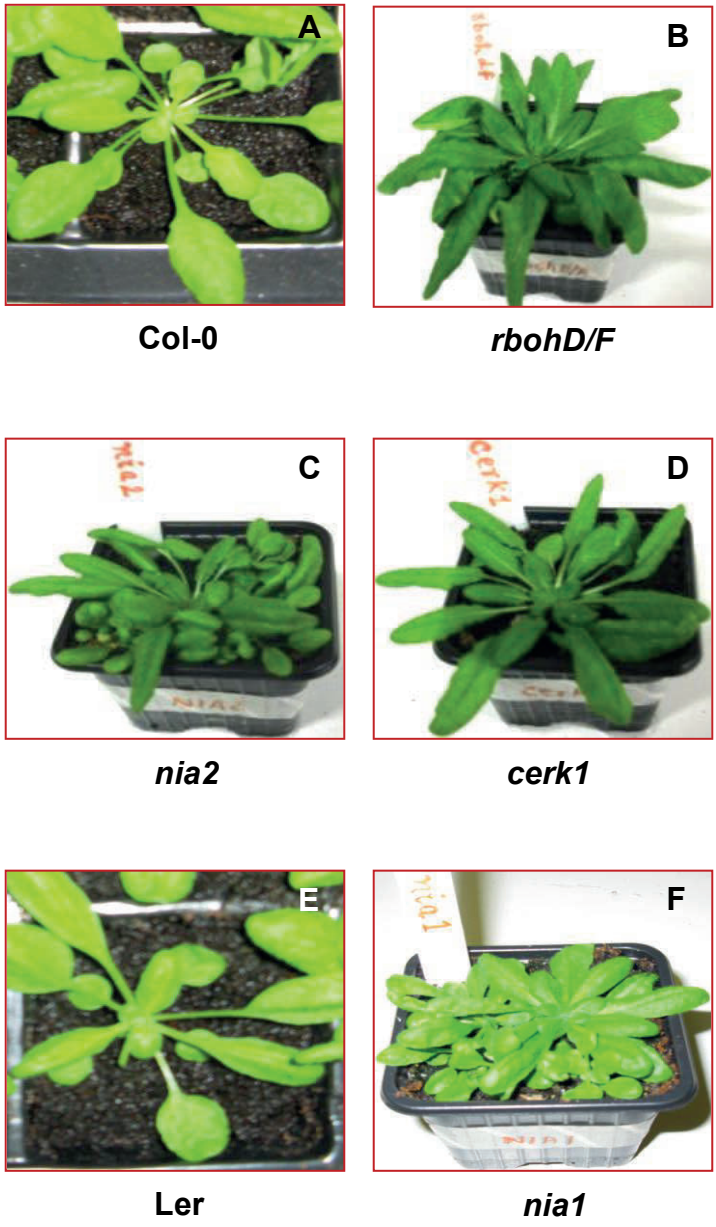

Figure 3.2. A view of *Arabidopsis thaliana* plants used in the experiments. The plants of *Arabidopsis* were used for epidermal bioassays as well as for RNA isolation. The upper panel shows (A) Col-0 wild type ecotype plants, (B) *rbohD/F* mutant plants. The middle panel shows the mutants (C) *nia2* and (D) *cerk1* plants respectively. The lower panel shows (E) Ler wild type plants with its (F) *nia1* mutant plants.

Stomatal bioassays

The abaxial (lower) epidermis was peeled off from the leaves of *Pisum* and *Arabidopsis* and cut into strips of ca. 0.16 cm^2 (Fig. 3.1 and 3.2). 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 pre-incubated for 3 h under white light (250 µmol m^{-2} s^{-1}), provided by a bank of tungsten lamps and filtered through water jacket. In case of *Arabidopsis*, the light intensity was (100–150 µE m^{-2} s^{-1}). 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.

The width of stomatal aperture of *Pisum*were measured under a research microscope (DMIL, Leica, Germany) with the help of a precalibrated ocular micrometer in India. In case of *Arabidopsis* (done in UK), stomatal aperture were measured using a light microscope (40x) and imaging camera with LEICA QWIN image processing and analysis software (Leica Microsystems and Imaging Solutions, Cambridge, UK). 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.

When used, the test compounds (inhibitors or scavengers) as NO modulators: carboxy-2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (cPTIO), *N*-nitro-L-Arg-methyl ester (L-NAME) and sodium tungstate; ROS modulators: catalase and diphenyleneiodonium chloride (DPI); Ca^{2+}chelators: BAPTA and BAPTA-AM were added after the 3 h light period, followed by chitosan or ABA after 10 min. The control sets were added with an equal volume of solvents used for their stocks. Ethanol was the

solvent used for ABA. Stocks of chitosan were made in 0.1 M glacial acetic acid and dilutions in the buffered incubation medium. When used alone in the incubation medium, only chitosan or ABA were present, without modulators. Incubation of epidermal strips was then continued for another 3 h in same light, before measuring the stomatal apertures.

Measurement of H_2O_2 and NO using confocal fluorescent microscopy

The production of NO in guard cells of *Pisum sativum* was examined by using DAF-2DA and changes in ROS were monitored with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Further details are described in our earlier articles (Kolla and Raghavendra2007; Kolla et al. 2007; Srivastava et al. 2009).

The strips of abaxial epidermis of *Pisum* for fluorescence studies were prepared by cutting and sticking the epidermal sections on glass cover slips with the help of medical adhesive, Telesis V (Premiere Products Inc., Pacoima, CA, USA). The mesophyll cells were then removed by flushing with water, to have clear epidermal sections sticking to the cover slip. The epidermal strips (sticking to cover slips) were then transferred and allowed to float in stomatal opening buffer (10 mM MES-KOH pH 7.0, 50 mM KCl) and were pre-incubated for 2.5 h under white light (250 µmol m^{-2} s^{-1}) before the subsequent addition of various compounds. The strips were then loaded with the required dye: 40 µM DAF-2DA (20 min) or 30 µM H_2DCFDA (20 min), in dark at 25 ± 1°C, and 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 case of *Arabidopsis*, the epidermal strips were cut into sections of ca. 0.16 cm^2 and were incubated under white light (100–150 μE m^{-2} s^{-1}) for 2.5 h in stomatal opening buffer (10 mM MES-KOH pH 7.0, 50 mM KCl). The strips were then loaded with the required dye: 40 μM DAF-2DA (20 min) or 30 μM H_2DCFDA (20 min), in dark at 25 ± 1°C, and 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 (Nikon PCM2000, Kingston-upon-Thames, UK).

In experiments involving time-course monitoring of signaling components the guard cells of *Pisum sativum*, in epidermal strips were examined under an inverted fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a monochrome high-resolution digital cooled CD camera (Cool- SNAP *cf*, Photometrics, Roper Scientific) that enabled to capture the images with DAF-2DA or H_2DCFDA fluorescence (filter: excitation 465–495 nm, emission 515–555 nm).

Quantitation of images

The levels of the fluorescence in the acquired images (using either the inverted fluorescence microscope or confocal microscope) were determined by using NIH Image for windows. The images were imported to the NIH Image software and opened as TIFF files. A square box was drawn on the image window using the cursor and the intensity of

fluorescence were calculated by analyzing the pixels of the square box in the fluorescent image. The mean value of square area box were obtained by taking the pixels within the given fluorescence image window. After taking 'n' different pixel intensities of the square box of the same size in the non-fluorescent area was taken as the control (background).

The pixel intensity of fluorescent guard cells were recorded as (X) and the background of the fluorescence images as (Y). The difference of the background and area of interest was calculated and Y-X gives the actual intensity of the fluorescent image. The intensity of fluorescence was obtained, as intensity of pixels in the control/beginning of the experiment and taken as 100%. The rise in fluorescence, as indicated by pixel values, was calculated as % of controls (Suhita et al. 2004; Kolla et al. 2007; Gonugunta et al. 2008).

Extraction of total RNA from *Arabidopsis* leaves

Arabidopsis thaliana leaves (0.1 g) were ground with liquid nitrogen in a mortar and pestle and the resulting powder was mixed with 1 ml of TRIzol® Reagent to isolate total RNA according to the manufacturer's instructions. Residual DNA was removed from the resulting total RNA samples by their treatment with RNase-free DNase 1 using a TURBO DNA-free kit, according to the manufacturer's instructions. RNA concentrations were measured spectrophotometrically and RNA integrity was confirmed by the presence of discrete ribosomal bands observed under UV illumination in 5 µg samples after their electrophoresis through a 1.2% (w/v) agarose 1X TAE gel. Total RNA samples were stored at -80^{o}C, until use.

Primer design and Quantitative RT-PCR

Oligonucleotide PCR primer pairs were designed with either the online primer design tool "Primer-BLAST" (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or with Primer Express Software (Applied Biosystems) to find primers specific for the relevant *Arabidopsis* gene sequences. The primer sequences of the genes used for QRT-PCR are:

nia1-2 (AT1G77760)

Forward primer: 5'-aatcgcaaaggaaggttgg-3'

Reverse primer: 5'-ctctagatttggctgcaacg-3'

nia2:5 (AT1G37130)

Forward primer: 5'-tgtacgtcatttatgctaaccg-3'

Reverse primer: 5'-cctgatggattagatggctca-3'

AtrbohD (AT5G47910)

Forward primer: 5'-ggacgagatttcggagttagacg-3'

Reverse primer: 5'-ttgcaacgactgaagcatgg-3'

AtrbohF (AT1G64060)

Forward primer: 5'-tcagaatacagcacaggaagc-3'

Reverse primer: 5'-cgttcatgacacctttgaacc-3'

Two-step real-time, quantitative, reverse transcription polymerase chain reaction (Q-RTPCR) experiments were then performed using an ABI 7300 real time PCR machine. Filtered pipette tips (Greiner Bioone), sterile Combi-plus-tips (Eppendorf), Multipipetter (Eppendorf) and an ultraviolet illuminator hood were used routinely to ease handling and limit cross-contamination. Reactions were carried out in either duplicate (for standard curves) or triplicates, using skirted microamp optical 96 well plates which

were sealed with clear plastic optical adhesive covers. Total RNA samples (5 µg), spiked with 3 ng μl^{-1} polyadenylated tumour necrosis factor receptor associating factor 1 (TRAF1) mRNA transcript were used to synthesize cDNA as described by Wilson et al. (2004). After completion, the reactions were heat terminated and diluted tenfold with RNase free water.

Synthesis of TRAF1 mRNA transcript was carried out as described by Wilson et al. (2004). A full length, polyadenylated, 2403 bp cDNA corresponding to a mammalian-specifically expressed mRNA transcript encoding human tumour necrosis factor receptor associating factor 1 (BC024145) was obtained as the plasmid pTRAF1. After *Xho*1-digestion of pTRAF1 to linearise the plasmid, mRNA transcripts for this cDNA were produced using T3 RNA polymerase in the presence of rNTPs. After synthesis, the plasmid template was removed by digestion with RNase-free DNase 1 and the mRNA transcripts were purified by phenol/$CHCl_3$ extraction and ethanol precipitation and were dissolved in 15 to 20 µl of single distilled water. Transcript concentrations were determined spectrophotometrically and their purity and full length assessed by agarose gel electrophoresis. Before use the TRAF1 transcripts were dissolved in RNase free water at 0.15 ng μl^{-1}.

Aliquots of each of the diluted reverse transcribed (RT) reaction products were subjected to PCR in the presence of above mentioned gene-specific forward and reverse primer sets and using a QuantiTect SYBR Green PCR kit (Qiagen, UK) as instructed. Each PCR reaction was performed in three or four parallel independent experiments. Thermocycling conditions were activation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s (denaturation), and combined annealing/elongation at 60°C for 1 min. The

SYBR green fluorescence was measured after combined annealing/elongation step. The methodology followed here is as described by Winfield et al. (2009), with slight modifications in the cycling conditions. For any given primer set, amplicon product size was determined by melting curve analysis step of 1 cycle each of 95^{o}C for 15 s, 60^{o}C for 20 s and 95^{o}C for 15 s where the melting temperature (T_m) was confirmed to be equivalent across all reactions. Additionally, amplification of negative controls as –RT, -RNA and RNase free water control samples was confirmed to be within acceptable levels for each primer set. The data collection was carried out during the extension step. On completion of the PCR reactions, melting curve and melting point data for individual PCR products were obtained by monitoring the level of fluorescence after each of a series of incremental temperature rises.

Progress of the PCR reactions was monitored using the ABI 7300 software. The concentrations of PCR product concentrations were determined by comparison with standard reactions performed using M13 forward and reverse primers and various quantities of pBSII SK+ (Stratagene) plasmid DNA. The efficiency of the reverse transcription step of the process was ascertained by monitoring the accumulation of the PCR product related to the TRAF1 mRNA spiked into each of the total RNA samples and normalising the results accordingly. The PCR reactions used to amplify any of the genes studied produced only single amplification products as determined by agarose gel electrophoresis or by observation of the melting curve data produced by the Q-RT-PCR machine for any of those products at the end of the PCR programme. The purity of the resulting PCR products was assessed by agarose gel electrophoresis.

Solvents, Chemicals and Materials

Wherever relevant, the control sets/media contained 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.

Medium molecular weight chitosan and abscisic acid (ABA) was from Sigma (St Louis, MO, USA), carboxy-2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide; (cPTIO), *N*-nitro-L-Arg-methyl ester; (L-NAME), sodium tungstate and nuclease free water (Molecular biology reagent) were obtained from Sigma-Aldrich, UK. 4, 5 diamino fluoresceindiacetate (DAF-2DA), 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), TRIzol®Reagent were from Invitrogen, Paisley, UK. Diphenyleneiodonium chloride (DPI) were from Life Technologies (Eugene, OR, USA) and catalase from Roche Chemicals (Basel, Switzerland). TURBO DNA-free kit from (Ambion, UK), HotStarTaq polymerase and QuantiTect SYBR Green PCR kit was from Qiagen, UK. DNA ladders were purchased from Bioline, UK. Primers were ordered from MWG Operon, UK. All other chemicals were of analytical grade and were from following companies: Sisco Research Laboratories, Spectrochem, LobaChemie, Himedia laboratories and Qualigens.

Filtered pipette tips were purchased from Greiner Bioone, UK, sterile Combi-plus-tips and Multipipetter were from Eppendorf, UK. Microamp optical 96 well plates and optical adhesive covers were purchased from Ambion, UK.

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Chapter 4

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

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; 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). Several recent reports emphasize the key function of NO in the fine-tuned regulation of stomatal closure (Bright et al. 2006; Neill et al. 2008).

Cell wall fragments of plants or pathogens can serve as elicitors in many plant species. 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, like chitosan, are quite limited. 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.

This chapter describes the experiments 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.

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. 4.1 A), while >40% stomatal closure occurred in presence of 10 µM ABA (Fig. 4.1 B). 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. 4.2B) and could be seen only at 20 min (Fig. 4.2C) after exposure to chitosan. In contrast, the increase in ROS was visible already by 5 min (Fig. 4.2G) and did not rise much thereafter (Fig. 4.2H). 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. 4.3A), whereas most of the increase in ROS occurred by 5 min (Fig. 4.3B).

Effects 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 closure induced by chitosan (Table 4.1). These inhibitorsalone 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.

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. 4.4, 4.5). cPTIO or sodium

tungstate or L-NAME alone had no effect but restricted the rise in NO induced by chitosan (Fig. 4.4 L–N). These compounds did not prevent the ROS production (Fig. 4.5 L–N). In contrast, catalase or DPI prevented the NO (Fig. 4.4 O, P) as well as ROS production (Fig. 4.5 O, 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 4.1) but NO and ROS levels remained high (Figs.4.4 Q, R 4.5 Q, R).

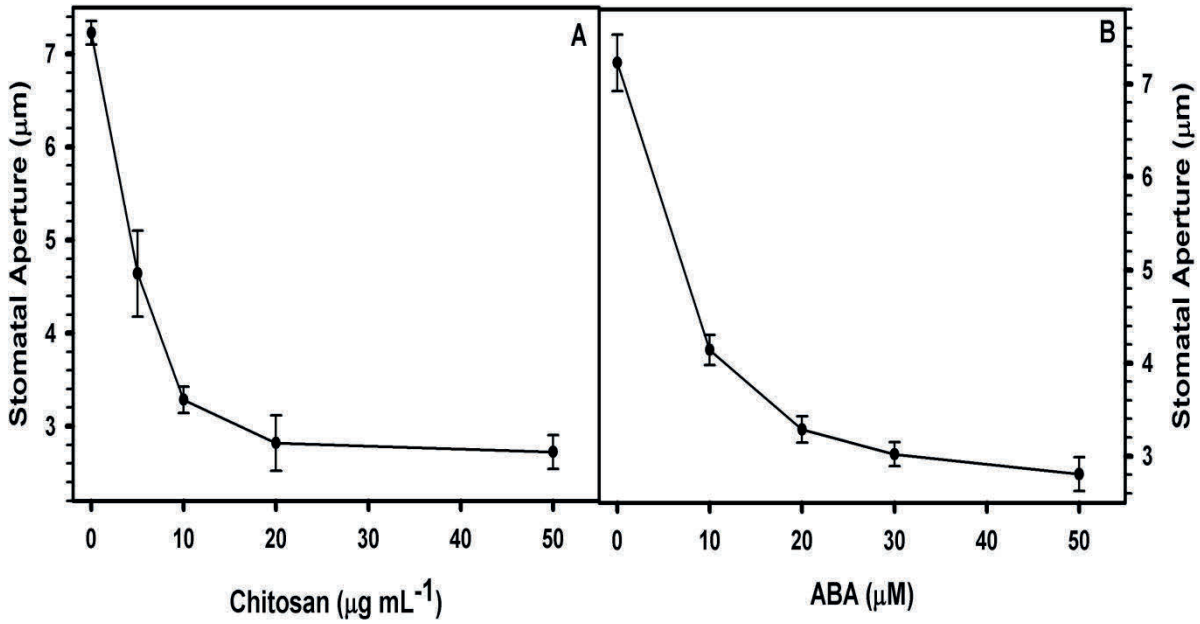

Figure 4.1. Concentration dependent stomatal closure in epidermal strips of *Pisum sativum* by chitosan (A) or ABA (B). Results are the average ± SE of three to four independent experiments. Further details are given in Materials and Methods.

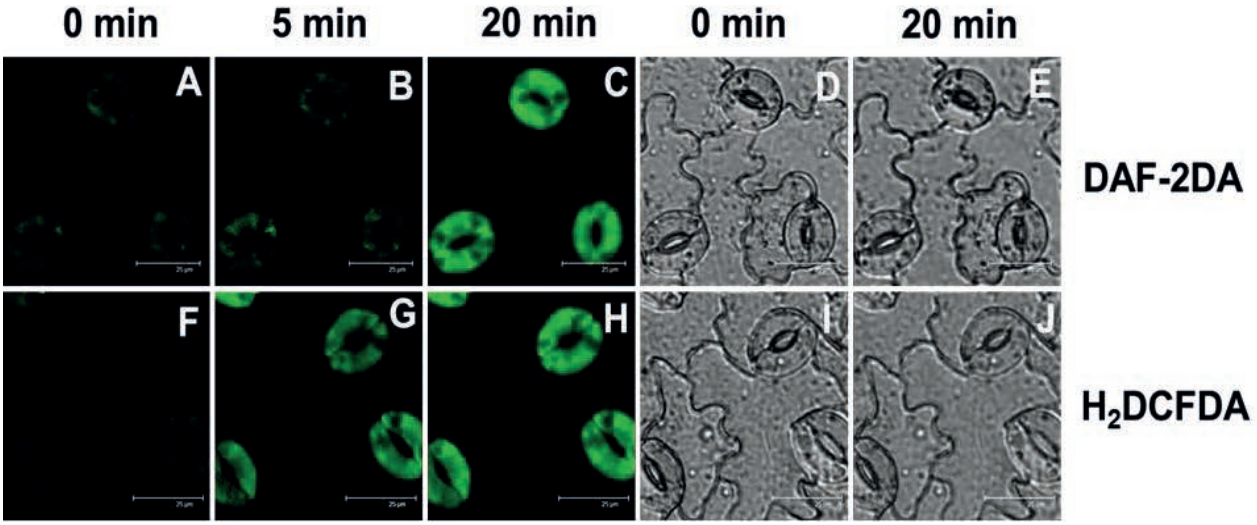

Figure 4.2. Increase in the levels of NO or ROS in guard cells of *Pisum sativum* on exposure to chitosan, as indicated by the fluorescent probes. The panels a to c are fluorescence images of guard cells loaded with 40 µM DAF-2DA reflecting the levels of NO, while F to H represent changes in ROS as indicated by 30 µM H_2DCFDA. The panels A and F are at the beginning of experiment. The panels B and G are the images at 5 min after treatment with 5 µg mL^{-1} chitosan, while C and H are at 20 min after treatment. The bright field images of stomata at 0 and 20 min after exposure to chitosan are in panels D/I and E/J respectively. Further details are given in Materials and Methods. Bar = 25µm.

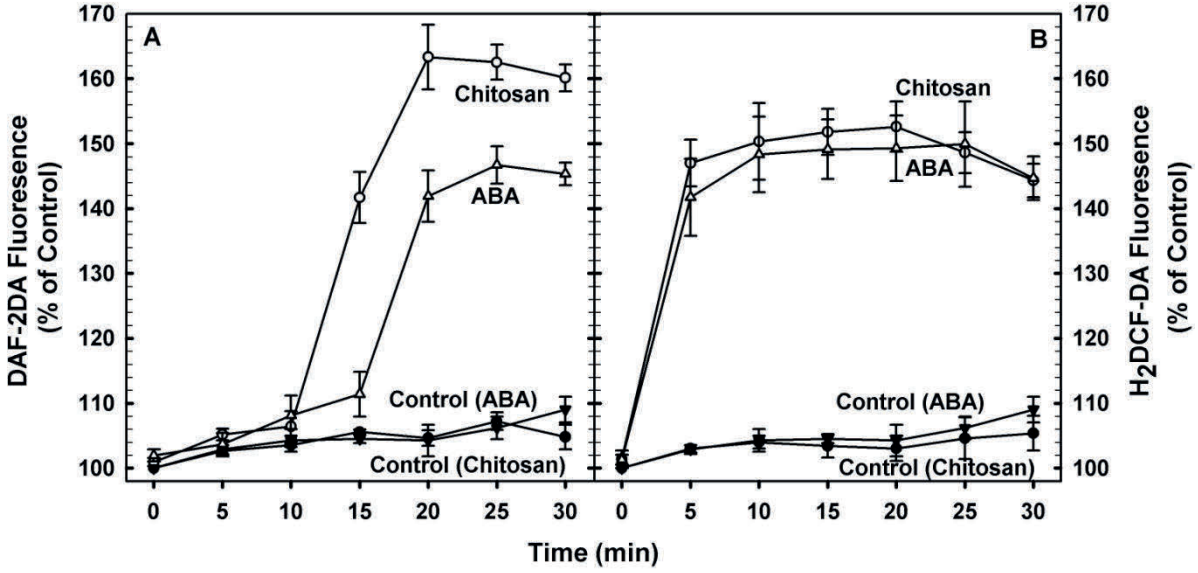

Figure 4.3. Kinetics of increase in NO (A) or ROS (B) of guard cells in response to 5 µg mL^{-1} chitosan. The epidermal strips were loaded with 40 µM DAF-2DA to monitor NO or 30 µM H_2DCFDA for ROS and incubated with or without chitosan. The levels of NO reached maximum at 20 min and those of ROS by about 5 min. The extent of NO or ROS production in the guard cells without chitosan is taken as 100%.Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

Table 4.1. 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.* 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 averages ± SE of 3 to 4 independent experiments. For comparisons between different treatments, one way ANOVA was used. Means denoted with different letters differed significantly at $P < 0.05$ according to one-way ANOVA.

	No Chitosan			**5 µg mL^{-1} Chitosan**		
Modulator	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}±0.1	100^{b}±0	100^{c}±0	3.3^{ad}±0.1	160^{be} ±5	155^{cf}± 6
0.2 mM cPTIO	7.6^{a}± 0.1	95^{b}±4	107^{c}± 4	7.3^{a}± 0.1	97^{b}± 4	143^{cf}± 7
0.1 mM Sodium tungstate	7.7^{a}±0.1	103^{b}± 1.2	107^{c}± 2	6.3^{a}± 0.5	111^{b}± 2	154^{cf}± 3
0.1 mM L-NAME	6.9^{a}± 0.1	97^{b}±5	107^{c}± 4	6.8^{a}± 0.2	116^{b}± 5	147^{cf}± 7
100 U mL^{-1} Catalase	8.0^{a}± 0.1	99^{b}± 6	99^{c}± 3	7.5^{a}± 0.1	109^{b}± 6	109^{c}± 2
5 µM DPI	7.4^{a}± 0.1	97^{b}± 3	97^{c}± 4	7.3^{a}± 0.1	109^{b}± 2	108^{c}± 2
10 µM BAPTA-AM	7.8^{a}±0.1	102^{b}±3	100^{c}±2	6.2^{a}±0.8	140^{be}±4	138^{cf} ±2
20 µM BAPTA	7.5^{a}± 0.2	106^{b}± 2	102^{c}±2	7.0^{a}±0.1	133^{be} ±3	138^{cf} ±2

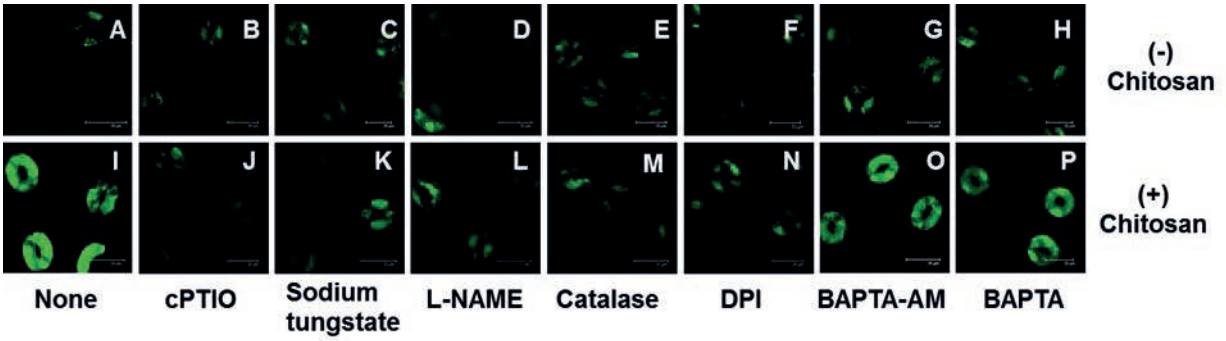

Figure 4.4 The effect of NO/ROS modulators on the extent of NO production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe DAF-2DA. The panels A to H represent guard cells which are not exposed to chitosan, while I to Pare of those exposed to 5 μg mL^{-1} chitosan. Panels A & I: no modulators, B & J: treated with 0.2 mM cPTIO, C & K: 0.1 mM sodium tungstate, D & L: 0.1 mM L-NAME, E & M: 100 U mL^{-1} Catalase, F & N: 5 μM DPI, G & O: 10 μM BAPTA-AM and H & P: 20 μM BAPTA. Images were taken 20 min after addition of chitosan. Further details are given in Materials and Methods. *Bar* = 25 μm.

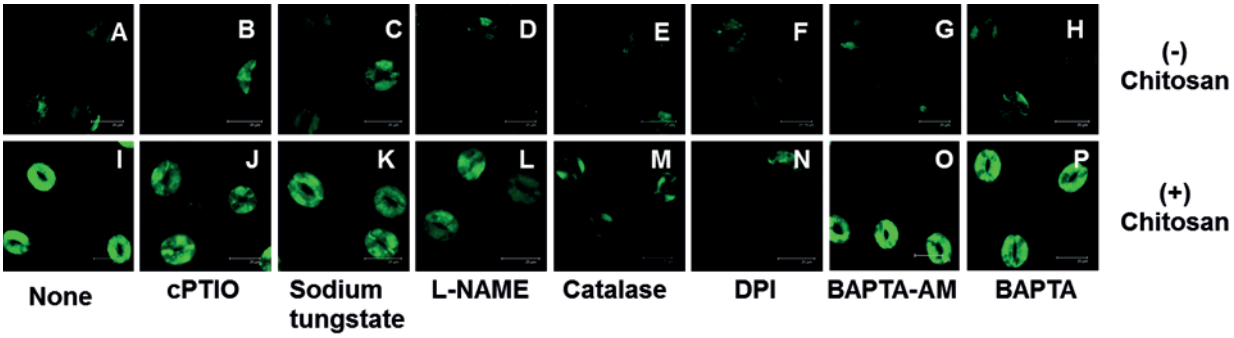

Figure 4.5. Theeffect of NO/ROS modulators on the extent of ROS production in guard cells of *Pisum sativum*, as indicated by the fluorescent probe H_2DCFDA.The panels A to H represent guard cells which are not exposed to chitosan, while I to P are of those exposed to 5 µg mL^{-1} chitosan. Panels A&I: no modulators, B & J: treated with 0.2 mM cPTIO, C & K: 0.1 mM sodium tungstate, D & L: 0.1 mM L-NAME, E & M: 100 U mL^{-1} Catalase, F & N: 5 µM DPI, G & O: 10 µM BAPTA-AM and H & P: 20 µM BAPTA. Images were taken 20 min after addition of chitosan. Further details are given in Materials and Methods. *Bar*= 25 µm.

Discussion

Rise and essentiality of NO during chitosan induced stomatal closure

NO, ROS and calcium are essential signaling components during stomatal closure induced by not only ABA but also MJ and bicarbonate (Neill et al. 2002; Yang et al. 2003; Suhita et al. 2004; Kolla et al. 2007; Kwak et al. 2006). 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 levels in guard cells (Figs. 4.2 and 4.4), prevention of stomatal closure along with a decrease in NO levels by cPTIO or sodium tungstate or L-NAME (Fig.4.4 L, M, N; Table 1) and initiation of stomatal closure after the rise in NO/ROS (Figs. 4.1 and 4.3). Thus, the effect of chitosan on guard cells were quite similar to that of ABA (Desikan et al. 2004; Bright et al. 2006; Gonugunta et al. 2008, 2009). 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; Genkins 2009).

Chitosan raised the levels of ROS and calcium in guard cells during stomatal closure in tomato and *Commelina* (Lee et al. 1999; Klüsener et al. 2002). The marked enhancement in the levels of both NO and ROS by chitosan even at 5 µg mL^{-1} (Fig. 4.2), 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 (Foissner et al. 2000). Kinetic studies using DAF-2DA revealed that chitosan induced increase in NO reached maximum by 20 min (Fig. 4.3 A), compared to 5 min required for ROS elevation (Fig. 4.3 B). 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 ROS as well as NO production in guard cells (Figs. 4.5 O, P and 4.4 O, P) and the inability of NO modulators to restrict the ROS levels (Fig. 4.5 L, M, N; Table 1), but NO (Fig.4.4 L, M, 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 *Arabidopsisnia1, 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 4.1) 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 & Lamattina 2003; del Río et al. 2004). A clear picture may emerge only after further studies in future.

Although several investigators used DPI as an inhibitor of NAD(P)H oxidase (Murata et al. 2001; Kwak et al. 2006; 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 4.1) but also the ROS (Fig. 4.5 P) 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 (McAinsh et al. 1997). Externally applied H_2O_2induced 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 4.1) 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 to prevent the stomatal closure, despite the high levels of NO/ROS in guard cells (Table 4.1), demonstrate 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.

Conclusions

1. NO is an important secondary messenger, besides ROS and calcium during chitosan induced stomatal closure in *Pisum sativum* epidermis.
2. Time course experiments with fluorescent probes demonstrated that NO-production occurred after that of ROS.
3. The ability of catalase or DPI to restrict the production of ROS as well as NO, and the ability of NO-modulators to prevent the rise in only NO levels but not the ROS in guard cells, indicated that ROS production was necessary for NO production.

4. 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.
5. Calcium may act either downstream of NO and ROS or independent of NO/ROS.

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Chapter 5

Role and importance of ROS, NO and Ca^{2+} during chitosan-mediated inhibition of stomatal opening in abaxial epidermis of *Pisum sativum*

Chapter 5

Role and importance of ROS, NO and Ca^{2+} during chitosan-mediated inhibition of stomatal opening in abaxial epidermis of *Pisum sativum*

Chitosan not only induces stomatal closure (Srivastava et al. 2009) but also inhibits light-induced stomatal opening (Lee et al. 1999; Allègre et al. 2009; Ördög et al. 2011). The mechanism for inhibition of stomatal opening is believed to be different from that of stomatal closure (Allen et al. 1999; Li et al. 2000; Wang et al.2001; Mishra et al.2006). Although it is clear that NO, ROS and Ca^{2+} are important secondary messengers in the signaling cascade during chitosan-induced stomatal closure, the processes during chitosan inhibition of stomatal opening are not completely understood. The upstream steps of the chitosan-induced ROS generation also remain largely unknown.

In contrast to the limited work with chitosan, there are extensive studies on the signaling molecules involved during ABA-mediated inhibition of stomatal opening (Garcia-Mata and Lamattina 2007; Yan et al. 2007). For example, in *Vicia faba* guard cells, there is a critical Ca^{2+}-dependent NO increase during the ABA-mediated inhibition of stomatal opening (Garcia-Mata and Lamattina 2007). There are reports suggesting an important role for PI3K during ABA-induced stomatal closure (Jung et al. 2002; Kwak et al. 2003). PI3K is involved in signal transduction events mediating chitosan stimulation of anthraquinone synthesis in *Rubia tinctorum* (Vasconsuelo et al. 2004). In the present work, we studied the role of selected signaling components, particularly ROS, NO, Ca^{2+} and PI3K and their importance during chitosan-mediated inhibition of light-induced stomatal opening in *Pisum sativum* guard cells. Attempts were made to monitor changes in ROS, NO and Ca^{2+} during chitosan effects on stomatal opening. In some of the

experiments, the effects of chitosan were compared with those of ABA as well as added Ca^{2+}.

Results

Concentration-dependent inhibition of light-induced stomatal opening by chitosan

When the epidermal strips of *Pisum sativum* were exposed to different concentrations of chitosan, ranging from 0 to 100 μg mL^{-1}, maximal inhibition of stomatal opening occurred at 10 μg mL^{-1} chitosan (Fig. 5.1). Stomatal aperture was measured before dark/light shift and after light shift.

ROS and NO production during chitosan-dependent inhibition of stomatal opening

Chitosan increases DAF-2DA fluorescence (represents NO levels) and H_2DCFDA fluorescence (represents ROS levels) within few minutes with respect to their control (Fig. 5.2 A and E), in *Pisum sativum* epidermal strips (Figs. 5.2 B and F). Similar increase in NO and ROS levels were seen when epidermal strips were treated with ABA (Fig. 5.2 C and G) and $CaCl_2$ (Fig. 5.2 D and H).

Requirement of NO and ROS during chitosan-mediated inhibition of stomatal opening

The epidermal strips were incubated with chitosan or ABA in the presence or absence of cPTIO (a specific NO scavenger), L-NAME (a specific NOS inhibitor) and tungstate (a specific NR inhibitor). cPTIO blocked the chitosan-mediated inhibition of stomatal opening (Fig. 5.3). Similarly, L-NAME inhibited both chitosan and ABA-mediated inhibition of stomatal opening. Further, tungstate were not able to prevent chitosan-

mediated inhibition of light-induced stomatal opening (Fig. 5.3). In another experiment, the epidermal strips were incubated with chitosan or ABA in the presence or absence of catalase (ROS scavenger) and DPI (an NADPH oxidase inhibitor). Both these compounds prevented the inhibition of light-induced stomatal opening by both chitosan and ABA (Fig. 5.4).

Requirements for Ca^{2+}during chitosan dependent ROS and NO production

The presence of BAPTAand BAPTA-AM prevented the chitosan- and $CaCl_2$-mediated inhibition of stomatal opening as well as the rise in NO production (Fig. 5.5). cPTIO decreased the chitosan as well as $CaCl_2$-mediated increase in NO production and inhibition of stomatal opening. In a similar way, catalase decreased the chitosan as well as $CaCl_2$-mediated increase in ROS production in addition to inhibition of stomatal opening (Fig. 5.6). In contrast, BAPTA decreased the $CaCl_2$-mediated ROS production, but was not able to prevent the chitosan-mediated increase in ROS production (Fig. 5.6). However, BAPTA-AM, prevented the rise in the ROS levels during chitosan and $CaCl_2$-mediated inhibition of stomatal opening (Fig. 5.6).

Ca^{2+} inhibited stomatal opening through a NADPH oxidase dependent ROS production and NR-dependent NO production

In order to investigate the interactions observed between ROS, NO and Ca^{2+}, pre-darkened epidermal strips were treated with $CaCl_2$ in presence or absence of modulators of ROS as well as NO. The Ca^{2+}-mediated inhibition of stomatal opening was blocked by 5 µM DPI and 1 mM tungstate (Fig. 5.7 A, B). Even the addition of 0.1 mM $CaCl_2$,

induced a marked rise in ROS or NO production (Fig. 5.7 A, B). Such Ca^{2+}-dependent increase of ROS or NO level was prevented by DPI and tungstate, respectively.

ROS and NO production during chitosan-mediated inhibition of stomatal opening requires also PI3K

The epidermal strips were incubated with chitosan in the presence or absence of WM (Wortmannin) and LY294002 (PI3K inhibitor). Both WM and LY294002 prevented chitosan-mediated inhibition of light-induced stomatal opening (Fig. 5.8 A and B) as well as the rise in NO and ROS levels (Fig. 5.8 A and B) caused by chitosan.

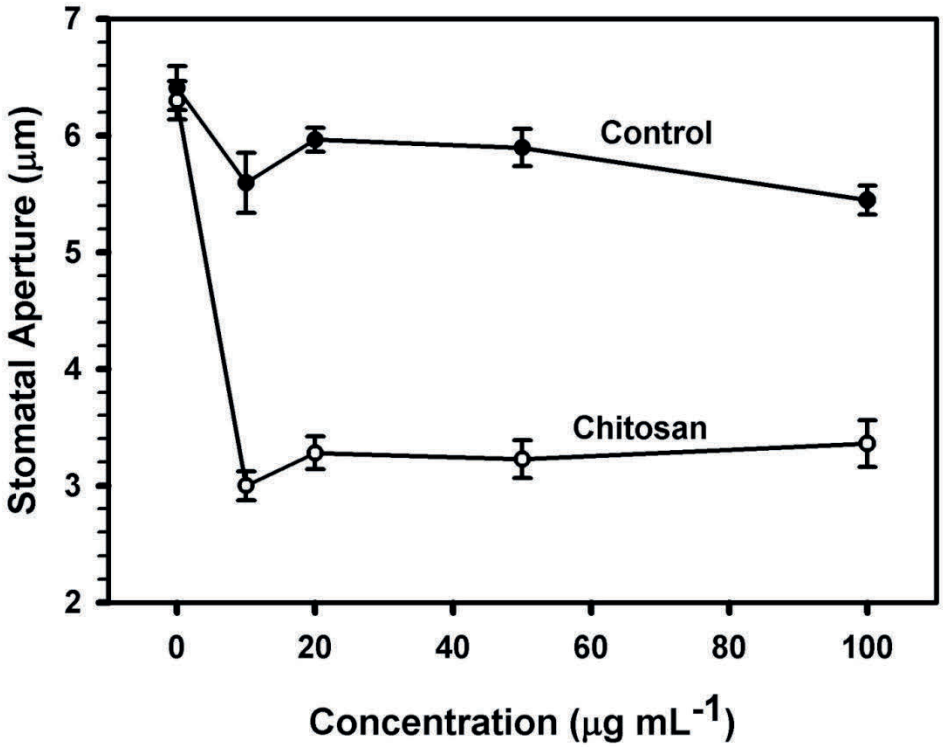

Figure 5.1. Concentration dependent inhibition by chitosan of light induced stomatal opening. *Pisum sativum* epidermal strips were pre-incubated in the dark for 2 h and 30 min and then treated with different chitosan concentrations for 30 min in darkness and subsequently incubated in white light for 90 min. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

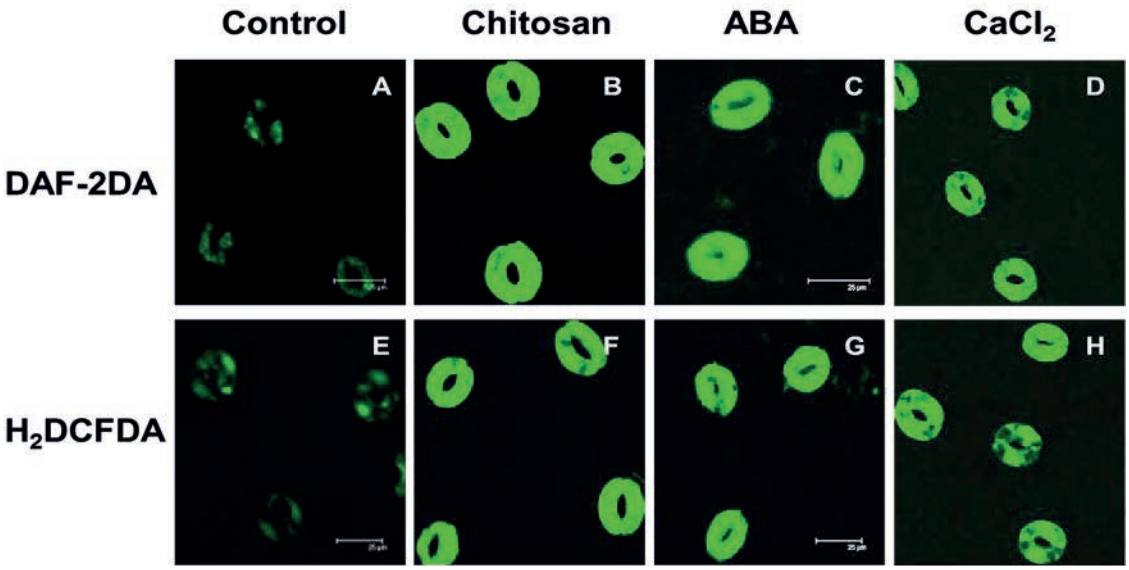

Figure 5.2. Representative confocal images showing the changes in NO and ROS changes in *Pisum sativum* guard cells in the presence or absence of chitosan (10 µg mL^{-1}), ABA (20 µM) and $CaCl_2$ (0.1 mM). (A–D) Changes in NO levels as indicated by DAF2-DA fluorescence. (E–H) Changes in ROS levels as indicated by H_2DCFDA fluorescence. Chitosan, ABA and $CaCl_2$ increased the levels of NO and ROS, compared to respective controls.

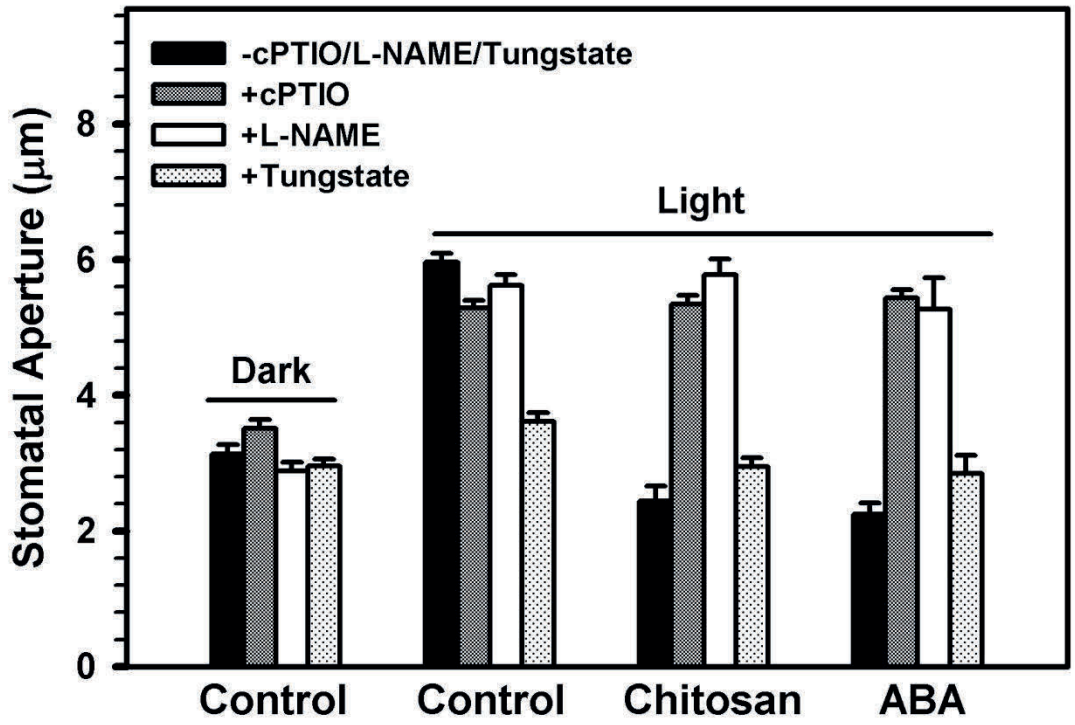

Figure 5.3. Chitosan and ABA inhibition of stomatal opening requires NO through NOS and NR. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min, and then treated with or without 200 μM cPTIO, 0.1 mM L-NAME, 0.1 mM tungstate for 30 min. Strips were then treated for 30 min without (control), with 10 μg mL^{-1} chitosan or with 20 μM ABA. Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

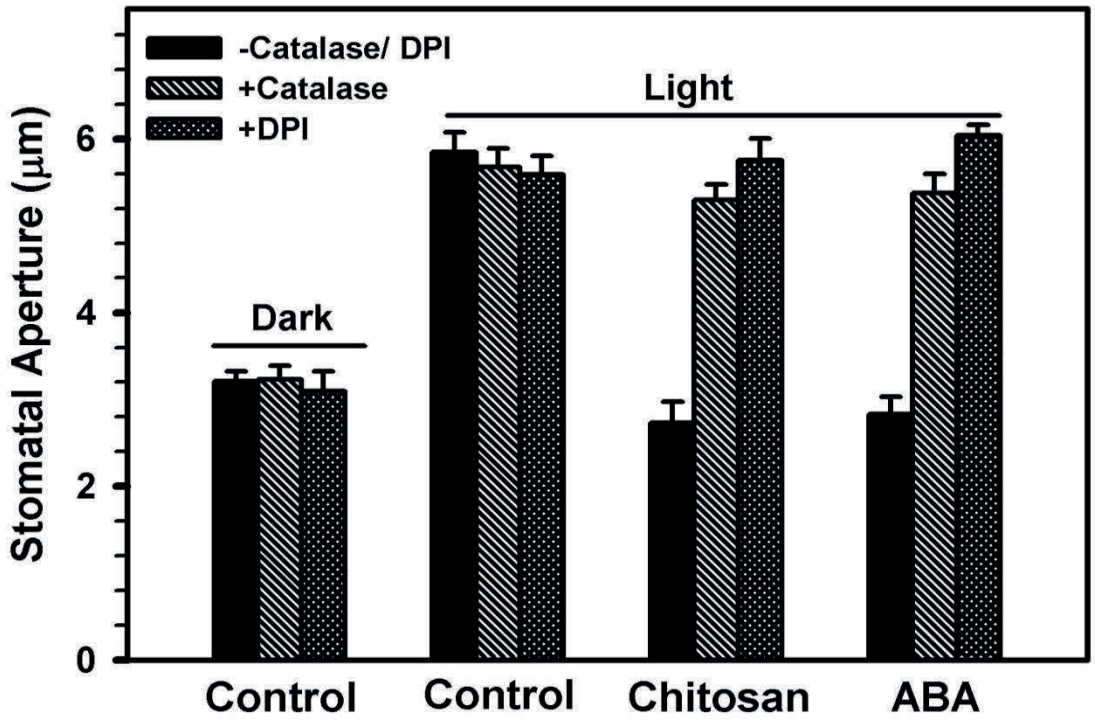

Figure 5.4. Chitosan and ABA inhibition of stomatal opening requires ROS through NADPH oxidase. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min and then treated with or without 100 U mL^{-1} catalase, 5 μM DPI for 30 min. Strips were then treated for 30 min without (control), with 10 μg mL^{-1} chitosan or with 20 μM ABA. Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

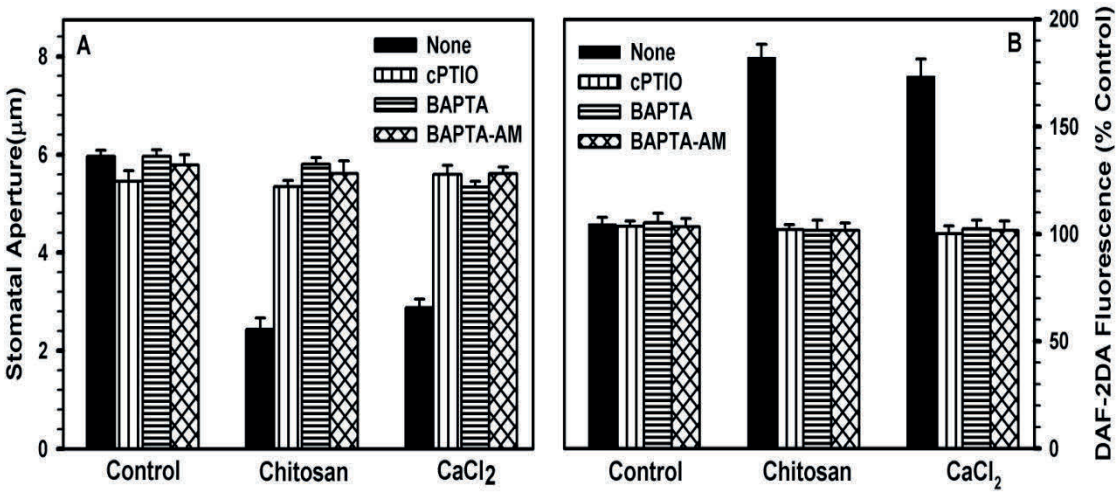

Figure 5.5. Ca^{2+} is required for chitosan dependent NO production in the inhibition of stomatal opening. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min and then treated with 200 μM cPTIO, 20 μM BAPTA, 10 μM BAPTA-AM for 30 min. Strips were then treated for 30 min without control, 10 μg mL^{-1} chitosan or 0.1 mM $CaCl_2$. (A) Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. (B) NO generation was determined by DAF-2DA fluorescence either without (control) or with chitosan (10 μg mL^{-1}) or ABA (20 μM) in the presence of cPTIO (200 μM), BAPTA (20 μM), BAPTA-AM (10μM). Fluorescence intensities were determined 30 minutes after treatment. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

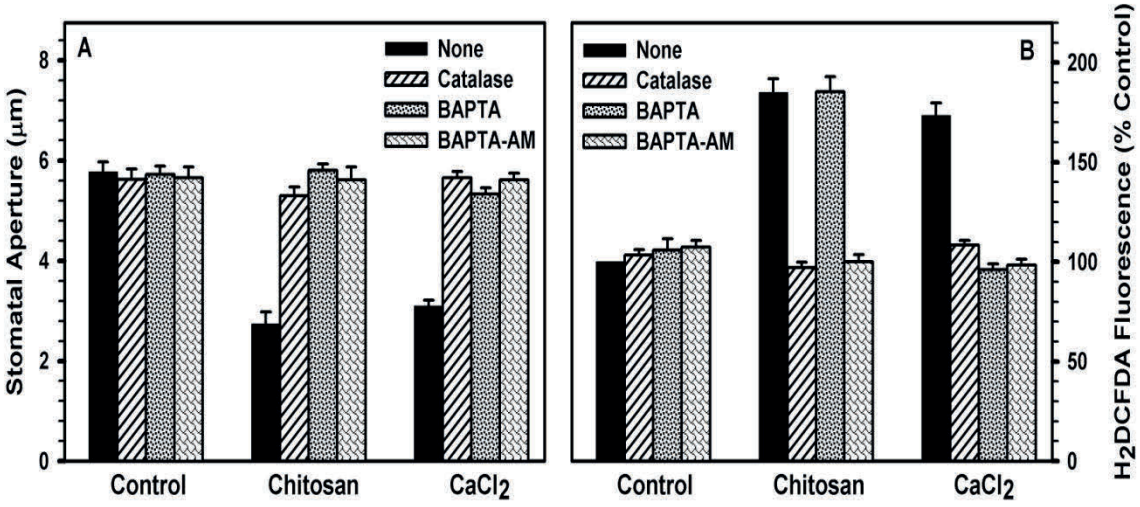

Figure 5.6. Ca^{2+} is required for chitosan dependent ROS production in the inhibition of stomatal opening. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min and then treated with 100 U mL^{-1} catalase, 20 μM BAPTA, 10 μM BAPTA-AM for 30 min. Strips were then treated for 30 min without control, 10 μg mL^{-1} chitosan or 0.1 mM $CaCl_2$. (A) Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. (B) ROS generation was determined by H_2DCFDA fluorescence either without (control) or with chitosan (10 μg mL^{-1}) or ABA (20 μM) in the presence of catalase (100 U mL^{-1}), BAPTA (20 μM), BAPTA-AM (10 μM). Fluorescence intensities were determined 30 minutes after treatment. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

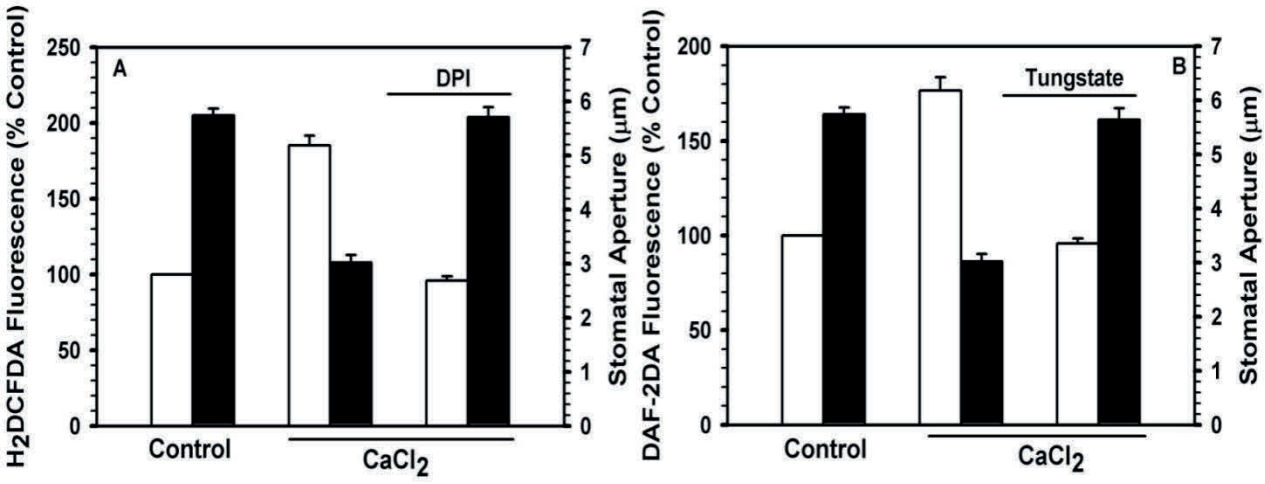

Figure 5.7. The NADPH oxidase inhibitor DPI and NR inhibitor tungstate effects on Ca^{2+}-mediated inhibition of stomatal opening and ROS or NO production. *Pisum sativum* epidermal strips preincubated in the dark for 2 h and 30 min and were treated with or without (A) 5 μM DPI or (B) 0.1 mM tungstate for 30 min and then treated for 30 min without (control) or with 0.1 mM $CaCl_2$ (Ca^{2+}). Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. Results are the averages ± SE from at least 3 independent experiments. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min were loaded with H_2DCFDA or DAF-2DA in opening buffer and were treated with (Ca^{2+}) or without (control) of 0.1 mM $CaCl_2$ in presence or absence of 5 μM DPI or 0.1 mM tungstate for 30 min in the dark and fluorescence expressed as % of control. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

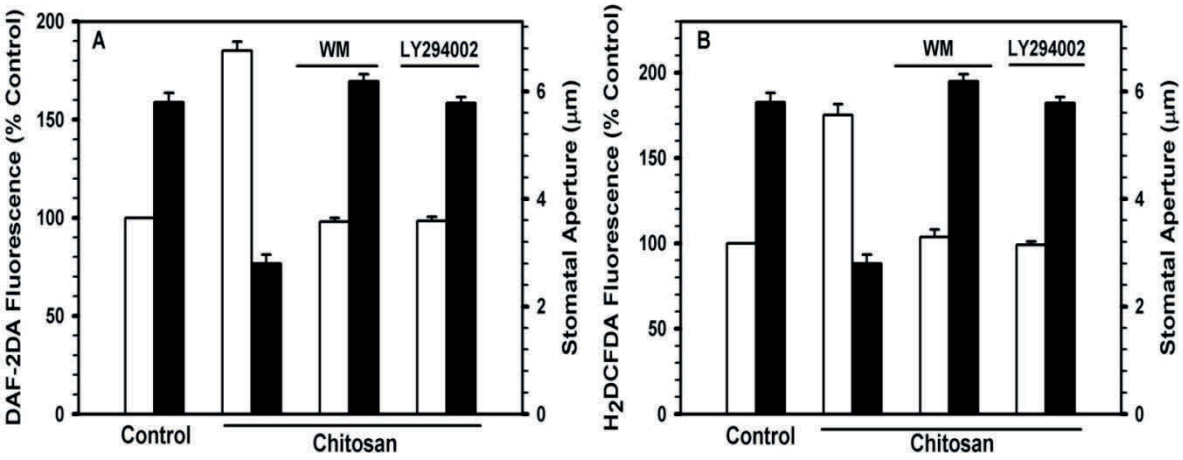

Figure 5.8. PI3K is required for chitosan dependent NO and ROS production in the inhibition of stomatal opening. *Pisum sativum* epidermal strips pre-incubated in the dark for 2 h and 30 min and then treated with 2 μM wortmannin, 50 μM LY294002 for 30 min. Strips were then treated for 30 min without control, 10 μg mL^{-1} chitosan. Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift. NO generation was determined by (A) DAF-2DA and (B) H_2DCFDA fluorescence either without (control) or with chitosan (10 μg mL^{-1}) in the presence of 2 μM wortmannin, 50 μM LY294002. Fluorescence intensities were determined 30 minutes after treatment. Results are the averages ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

Discussion

Rise in the levels of ROS and NO during inhibition of stomatal opening

In guard cells of several species (*Arabidopsis*, *Vicia*, tomato, *Commelina* and pea) production of ROS and NO is a common feature in response to ABA, MJ, bicarbonate or even chitosan/oligogalacturonic acid (Lee et al. 1999; Pei et al. 2000; Suhita et al. 2004; Kolla et al. 2007; Zhang et al. 2007; Srivastava et al. 2009). The involvement of ROS and NO during chitosan-mediated inhibition of stomatal opening is therefore not surprising and is demonstrated by multiple evidences: modulation of ROS and NO levels within cells by either scavenging these molecules or inhibition of source enzymes and finally real time monitoring of ROS/NO by using fluorescent dyes (Fig. 5.2).

In the present work, we noticed that cPTIO as well as catalase blocked the chitosan-mediated inhibition of stomatal opening (Fig. 5.3 and 5.4), suggesting that NO and ROS plays a role during chitosan-mediated inhibition of stomatal opening. The prevention of blue light-dependent H^+-pumping by ABA is restored by the addition of ascorbate, implying that ROS could regulate ABA inhibition of stomatal opening (Zhang et al. 2004). Yan et al (2007) reported that ROS and NO were involved during ABA-mediated inhibition of stomatal opening in *Vicia faba* guard cells.

Sources of ROS and NO and their importance

To determine the relationship between H_2O_2 and NO during the chitosan-mediated inhibition of stomatal opening, the epidermal strips were treated with H_2O_2 in the presence of either cPTIO or L-NAME. Both cPTIO and L-NAME reversed H_2O_2 inhibition of stomatal opening indicating that NO was required for H_2O_2-inhibition of

stomatal opening. Although NO is recognized as an important signaling molecule, the origin of NO synthesis in plants has remained controversial (Yamasaki et al. 1999; Wendehenne et al. 2001; Meyer et al.2005; Crawford et al. 2006; Tunet al. 2006). Two biosynthetic origins for NO in the guard cell ABA signalling cascade have been reported: NOS and nitrate reductase (NR). In the present studies, L-NAME inhibited both chitosan and ABA-mediated inhibition of stomatal opening (Fig. 5.3) suggesting that NOS-like activity was involved during the process. Similarly, tungstate prevented chitosan-mediated inhibition of light-induced stomatal opening much stronger than that by ABA (Fig. 5.3) suggesting that nitrate reductase (NR) could also be involved.

There are studies on the pattern of ABA-induced stomatal closure affected in mutants with impaired NR or NOS. For example, ABA-induced stomatal closure was affected in the NR double mutant *nia1/nia2*, while there were no differences between the mutant and the wild-type, regarding ABA-mediated inhibition of light-induced stomatal opening (Neill et al. 2002). Desikan et al. (2002) also demonstrated using pharmacological and genetical approaches that NR may not be a major source of NO during the ABA-mediated inhibition of stomatal opening. Similarly, in another report, ABA failed to inhibit light-induced stomatal opening in the *Arabidopsis* mutant *Atnos1* (Guo et al. 2003). Garcia-Mata and Lamattina (2007) showed that a NOS-dependent NO production is critical for ABA-mediated inhibition of stomatal opening process. DPI, a NADPH oxidase inhibitor, prevented the chitosan-mediated inhibition of stomatal opening (Fig. 5.4) which further suggest that NADPH oxidase is having a role during the process.

Both extracellular and intracellular Ca^{2+} are required during chitosan or $CaCl_2$-mediated inhibition of stomatal opening

Calcium (Ca^{2+}) is another ubiquitous intracellular second messenger, involved in many signal transduction pathways in both plants and animals. The increase of cytosolic Ca^{2+} not only is a common signal for most of the stimuli sensed by guard cells, but is also a common component in the signaling pathways leading to both inhibition of stomatal opening and stomatal closure induction (McAinsh et al. 1997; Garcia-Mata and Lamattina 2007). ABA have also been described to regulate cytoplasmic Ca^{2+} concentrations in different plant systems, including the regulation of stomatal movements (Sokolovski et al. 2005; Garcia-Mata and Lamattina 2007; Meimoun et al 2009).

The prevention of chitosan- and $CaCl_2$-mediated inhibition of stomatal opening (Fig. 5.5 and 5.6) by BAPTA **(**chelator of external calcium) and BAPTA-AM (chelator of internal calcium within the cell) has suggested that both extracellular and intracellular calcium could play an important role during chitosan-mediated inhibition of stomatal opening process.

Ca^{2+} inhibition of stomatal opening through a NR-dependent NO production and NADPH oxidase-dependent ROS production

In ABA-induced stomatal closing of *Arabidopsis*, ABA-induced H_2O_2 production and the H_2O_2-activated Ca^{2+} channels are important components of ABA signal transduction (Pei et al. 2000; Murata et al. 2001). In our present work, the Ca^{2+}-dependent production of

ROS was blocked by the NADPH oxidase inhibitor DPI (Fig. 5.7 A), suggesting that Ca^{2+} might be activating a NADPH oxidase- dependent ROS production.

Garcia-Mata and Lamattina (2007) studied the NO/Ca^{2+} interactions duringABA-mediated inhibition of stomatal opening in *Vicia faba* epidermal strips. Li et al. (2009)showed that NO generation required Ca^{2+} in *Arabidopsis* guard cells. Cytosolic Ca^{2+} was involved in auxin- and NO-induced adventitious root formation in cucumber explants (Lanteri et al. 2006). There are reports that Ca^{2+} might be activating a NOS-dependent NO production (Garcia-Mata and Lamattina 2007) in *Viciafaba* epidermal strips. We now show that, in *P. sativum* epidermal strips, there is a Ca^{2+}-dependent production of NO, which is blocked by the NR inhibitor tungstate (Fig. 5.7 B), suggesting that Ca^{2+} might be activating a NR-dependent NO production.

Role of PI3K during inhibition of stomatal opening

Two PI3K inhibitors: wortmannin and LY294002, blocked the chitosan-mediated inhibition of light-induced stomatal opening (Fig. 5.8 A and B) as well as decreases the increase in NO and ROS levels (Fig. 5.8 A and B) suggesting that PI3K might be acting upstream of ROS/NO.

A few differences were observed between the signaling pathways during inhibition of stomatal opening and induction of stomatal closure (Allen et al. 1999; Li et al. 2000; Wang et al.2001; Mishra et al.2006;Garcia-Mata and Lamattina 2007). For example, differences were noticed between stomatal opening and closure events, involving signaling components, such as Ca^{2+}, K^{+}, protein phosphatases and MAP kinases among others (Cousson et al. 1998; Allen et al. 1999; Gudesblat et al. 2007). We

can conclude that NOS-like enzyme is an important source of NO during chitosan mediated inhibition of stomatal opening. In contrast, during chitosan-induced stomatal closure, NR appeared to be the major source of NO. The present work supports the hypothesis that stomatal closure and inhibition of stomatal opening processes are commanded by the same signaling components in a broad sense, however, there are subtle differences.

Conclusions

1. Besides inducing closure, chitosan inhibits stomatal opening. ROS appears to act upstream of Ca^{2+} during such chitosan mediated inhibition of light induced stomatal opening.
2. The sources for chitosan-mediated Ca^{2+}-dependent increase in ROS and NO production are NADPH oxidase and NR respectively, in chitosan inhibition of light induced stomatal opening as indicated by the fluorescent probes DAF-2DA and H_2DCFDA.
3. Nitric oxide synthase-like enzyme is the source of NO during chitosan mediated inhibition of stomatal opening and there is a only partial involvement of nitrate reductase as well while the major source of NO during chitosan induced stomatal closure is nitrate reductase.
4. The effects of NO and ROS seem to converge at Ca^{2+} in the inhibition of stomatal opening as well as promotion of stomatal closure by chitosan or ABA.

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Chapter 6

Use of *Arabidopsis* mutants to assess the role of nitrate reductase, NAD(P)H oxidase and chitin elicitor receptor kinase during chitosan-induced stomatal closure

Chapter 6

Use of *Arabidopsis* mutants to assess the role of nitrate reductase, NAD(P)H oxidase and chitin elicitor receptor kinase during chitosan-induced stomatal closure

Plants are constantly challenged by microbial pathogens that are deposited on leaf surfaces and subsequently gain ingress to the leaf interior, often using stomata as the route of entry. Chitosan induces stomatal closure and limits the entry of pathogens. Chitosan is recognised by specific plant receptors and initiates signalling cascades that involve calcium fluxes, generation of ROS and NO, activation of signalling enzymes such as mitogen-activated protein kinasesand that culminate in defence responses such as stomatal closure, phytoalexin synthesis and expression of a large number of defence-related proteins (Lee et al. 1999; Hu et al. 2004; Zuppini et al. 2004, Lizama-Uc et al. 2007; Wang et al. 2008; Allègre et al. 2009; Srivastava et al. 2009; Khokon et al. 2010).

Stomatal closure in response to microbial challenge is important and such closure requires both H_2O_2 and NO (Gudesblast et al 2009; Zeng et al. 2010). Chitosan induces stomatal closure in tomato, *Commelina communis* and *Pisum sativum* (Lee et al. 1999; Srivastava et al. 2009) and this is accompanied by ROS and NO generation. NADPH oxidase participates in ROS production during hormonal responses in plants, and is named respiratory burst oxidase homologs (Rbohs) involved in oxidative burst in response to pathogens (Torres et al. 2002; Sagi and Fluhr 2006). In *Arabidopsis*, two genes *NIA1*and *NIA2*encoding nitrate reductase have been cloned. Evidence based on the double mutant of *nia1* and *nia2*, in which gene disruption impairs the activity of NR, indicated that both *NIA1* and *NIA2* mediate the ABA-induced NO generation and stomatal closure in *Arabidopsis* (Desikan et al. 2002). There are evidences that ABA

induces H_2O_2 generation via activation of the NADPH oxidase isoforms *RBOHD* and *RBOHF* and that H_2O_2 subsequently induces NO generation via activation of the nitrate reductase isoform NIA1 (Desikan et al. 2004; Bright et al. 2006). The binding of CERK1 to chitosan may be physiologically relevant as studied by Petutschnig et al. (2010). The present study was therefore extended to assess whether chitosan- or ABA-induced stomatal closure requires *CERK1*.

Extensive work have been done with pea, *Vicia* and *Commelina*, because of the ease of stripping epidermis and GCP isolation (She et al. 2004; Kolla et al. 2007; Srivastava et al. 2009; Ördög et al. 2011). However, *Arabidopsis* mutants evolved to be good models for validating the individual signaling components and their crosstalk (Suhita et al. 2004; Wang and Song 2008; Saito et al. 2009; Depuydt and Hardtke 2011; Hossain et al. 2011). Studies with *Arabidopsis* mutants have demonstrated the essential role of signaling components involved during ABA-induced stomatal closure or ABA-mediated inhibition of stomatal opening: for example, protein phosphatase type 2C or PP2C (*abi1* and *abi2* mutants); serine/threonine protein kinases (*ost1-1* and *ost1-2* mutants); (Pei et al. 1997; Murata et al. 2001; Mustili et al. 2002; Umezawa et al. 2009); a heterotrimeric GTP binding protein α-subunit (*GPA1* mutants) (Pei et al. 2000). NADPH oxidase, particularly RBOHD/RBOHF (*atrbohD/F* mutants) (Khokon et al. 2010); nitrate reductase (NR-deficient) (*nia1* and *nia2*mutants) (Desikan et al. 2002; Neill et al. 2008); chitin elicitor receptor kinase (*cerk1* mutants) (Miya et al. 2007) were studied during both ABA or chitosan-induced responses in guard cells.

In this study, a combined pharmacological and genetic approach was used with *Arabidopsis thaliana* to determine the requirements for *RBOH*, *NIA1* and *CERK1* during chitosan-induced rise in ROS/NO and subsequent stomatal closure.

Results

Concentration-dependent stomatal closure in Arabidopsis epidermal strips induced by chitosan and ABA

The stomatal closure in response to different concentrations of chitosan was studied in comparison with that of ABA (Fig. 6.1). Chitosan induced maximal closure at 20 µg mL^{-1} in wild type Ler while the *nia1* mutant was insensitive to chitosan in terms of stomatal closure (Fig. 6.1 A). ABA induced maximal closure at 20 µM (Fig. 6.1 B). The *nia2* mutant behaved very similar to wild type Col-0 and its stomata closed in response to chitosan or ABA, while the *rbohD/F* and *cerk1* mutants were also insensitive to chitosan or ABA in terms of stomatal closure (Fig. 6.1 C, D).

NO and ROS production and stomatal closure in wild types and mutants in response to chitosan

The levels of NO and ROS were monitored using suitable fluorescent probes and confocal microscopy. There was a substantial increase in NO levels of guard cells in the wild type Ler but not in the mutant *nia1*, following exposure to chitosan (Fig. 6.2 C & D, 6.3 A). While the wild type Col-0 showed an increase in NO production, the mutants *rbohD/F* and *cerk1* showed no such increase. However, *nia2* mutant showed an increase in NO in response to chitosan (Fig. 6.2 K-N, 6.3 B).

ROS production was monitored similarly using confocal microscopy (Fig. 6.4), and the fluorescence was quantified (Fig. 6.5). There was an increase in ROS production after chitosan elicitation in Ler wild type and the *nia1* mutant (Figs. 6.4 C & D, 6.5 A) and in the wild type Col-0 and *nia2* mutants while the mutants *rbohD/F* and *cerk1* showed no such increase (Fig.6.4 K-N, 6.5 B).

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

NO modulators had varying effects on both chitosan-induced NO production and stomatal closure (Fig. 6.6 A, B). Removal of NO via the NO scavenger cPTIO (2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide; NO scavenger) prevented both chitosan-induced NO production and stomatal closure as did addition of tungstate, an NR inhibitor. However, addition of L-NAME (N-nitro-L-Arg- methyl ester), a potential NOS inhibitor, was not able to prevent stomatal closure and NO production elicited by chitosan (Fig. 6.6 A, B). Removal of ROS (via addition of catalase, a ROS scavenger), or inhibition of ROS production (via addition of DPI, diphenyleneiodonium chloride, a NAD(P)H oxidase inhibitor), inhibited both chitosan-induced ROS generation and chitosan-induced stomatal closure (Fig. 6.6 C, D).

Stomata of mutants were sensitive to external SNP and H_2O_2

To confirm that the insensitivity of *nia1*, *rbohD/F* and *cerk1*mutants to chitosan are due to their reduced NR (NIA1) mediated NO generation or RBOH mediated ROS generation, the epidermal peels were exposed to NO (via incubation in SNP) or to H_2O_2.

NO (via SNP) induced stomatal closure in the *nia1*just in a way like the wild type Ler (Fig. 6.7 A) and also in *nia2, cerk1* and *rbohD/F* mutants similar to the wild types' responses Col-0 (Fig. 6.7 B). Exogenous H_2O_2 induced stomatal closure in Ler wild type but not in the *nia1* mutant (Fig. 6.7 A), while was able to induce stomatal closure in *rbohD/F*, *cerk1* and *nia2* mutants like wild type Col-0 (Fig. 6.7 B).

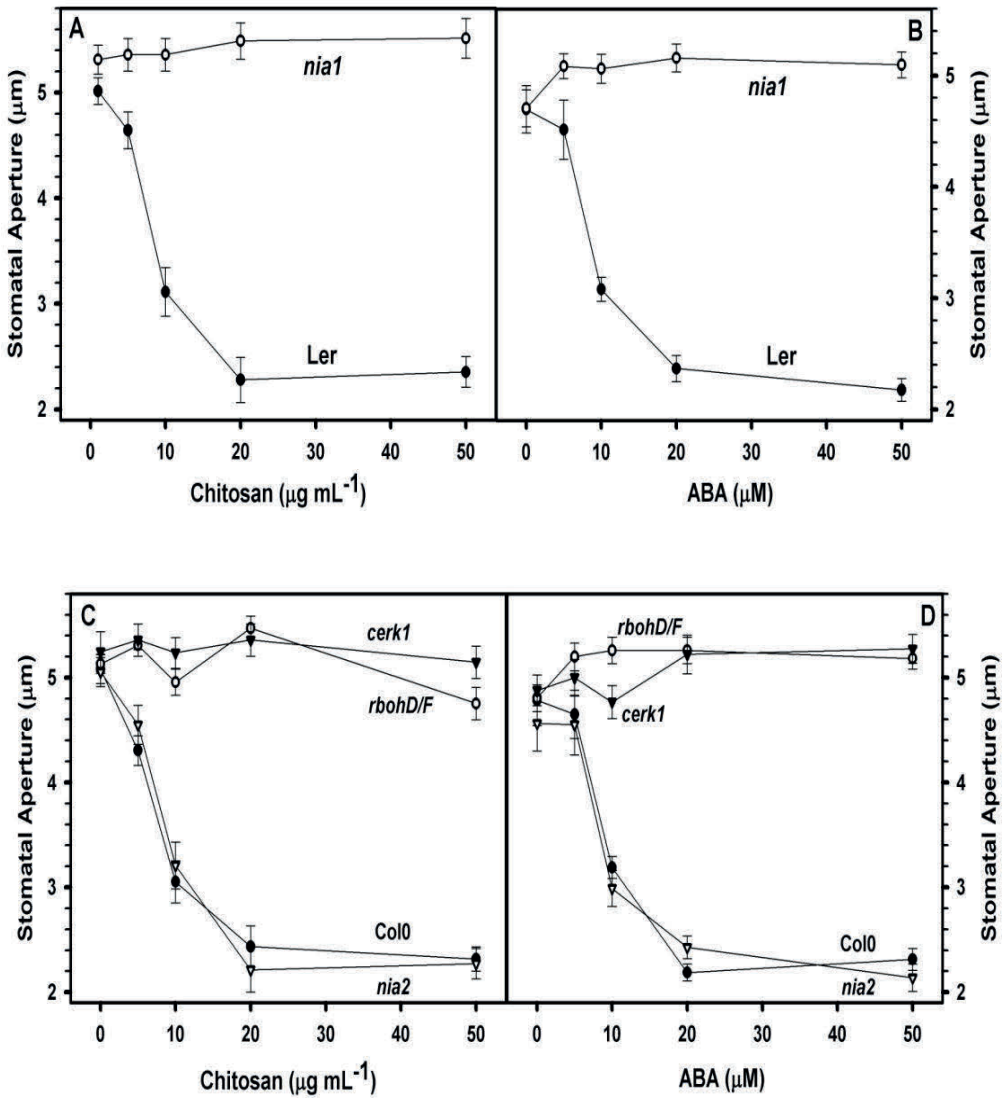

Figure 6.1. Concentration-dependent chitosan-or ABA- induced stomatal closure in abaxial epidermis prepared from *Arabidopsis* wild types andvarious mutants. Epidermal peels of *Arabidopsis* from wild type Ler (●), mutant *nia1* (o); wild type Col-0 (●), mutant *rbohD/F* (○), *cerk1* (▼) and *nia2* (∇) were incubated in light for 3 h and then treated with various concentrations of (A, C) chitosan or (B, D) ABA. The epidermal strips were again incubated in light for another 3 h and then stomatal apertures were measured. Data are the mean ± SE from at least 3 independent experiments.Further details are given in Materials and Methods.

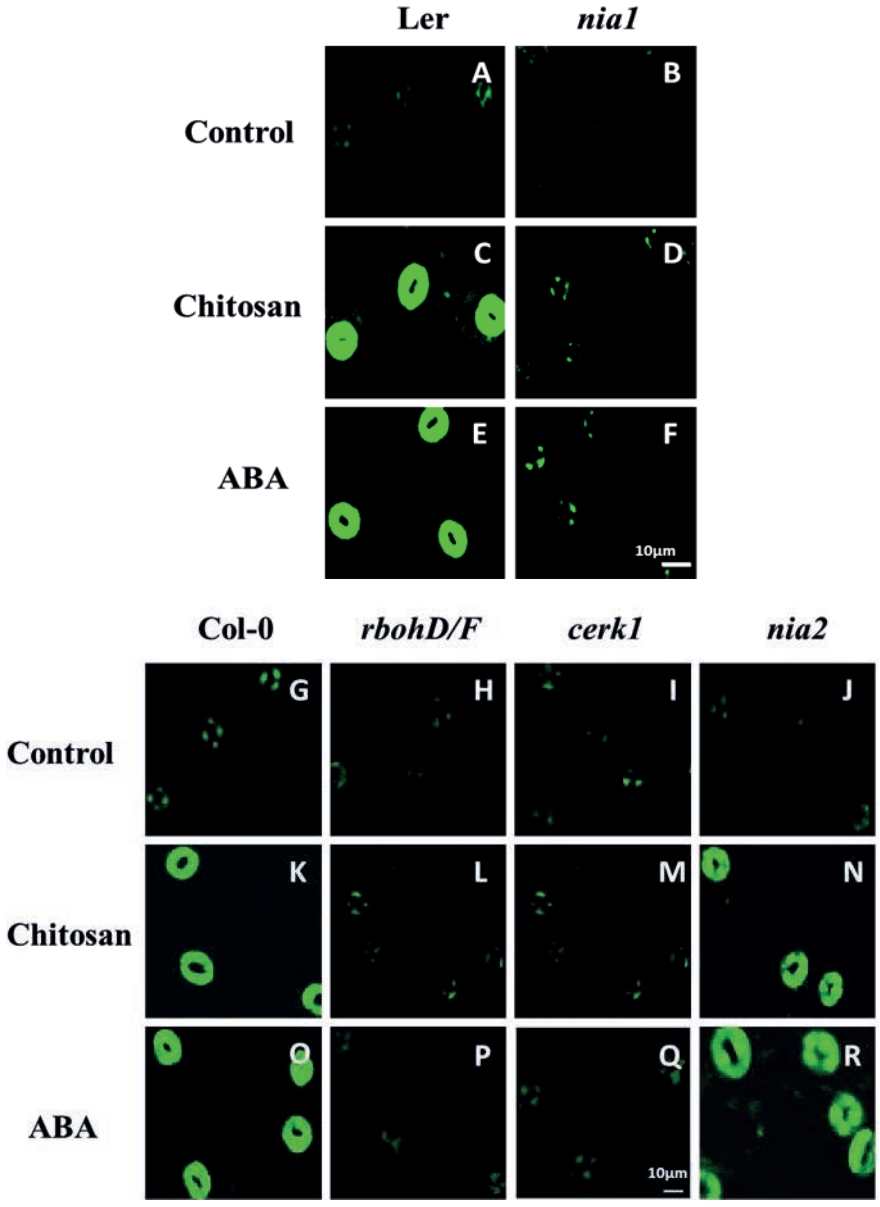

Figure 6.2. The extent of NO production in guard cells of (A-F) wild type Ler and *nia1* mutant and (G-R) wild type Col-0 and *rbohD/F*, *cerk1* and *nia2* mutants of*Arabidopsis thaliana*, as indicated by the fluorescent probe DAF-2DA. Panels A-B and G-J, represent control guard cells; C-D and K-N represent guard cells exposed to20 µgmL^{-1}chitosan; E-FandO-Rrepresent guard cells exposed to 20 µM ABA. A-E and B-F represent guard cells of Ler wild type and *nia1* mutant while G-O: representguard cells of Col-0 wild type while H-P: *rbohD/F* mutant**;** I-Q: *cerk1*mutant; J-R: *nia2* mutant. Images were taken 30 minutes after addition of chitosan and ABA. Further details are given in Materials and Methods. *Bar* 10 µm

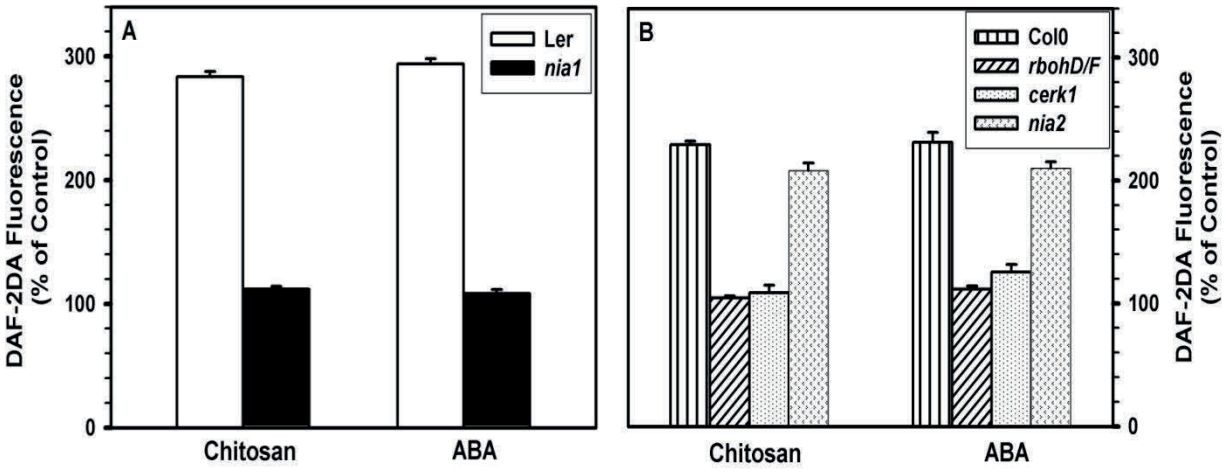

Figure 6.3. Quantitative evaluation of NO levels in guard cells of *Arabidopsis* wild type Ler and the *nia1* mutant and Col-0 wild types and *rbohD/F*, *cerk1*and *nia2* mutants. NO generation was determined by DAF-2DA fluorescence of (A) wild-type guard cells Ler (white bars) and mutant *nia1* (black bars) and (B) Col-0 wild type (linear striped bars), *rbohD/F* (diagonal striped bars), *cerk1* (spotted bars) and *nia2* (arrow bars) mutants with chitosan (20 μg mL^{-1}) or ABA (20 μM) and fluorescence expressed as a % of control. Fluorescence intensities were determined 30 minutes after treatment with chitosan or ABA. Data are the mean ± SE from at least 3independent experiments. Further details are given in Materials and Methods.

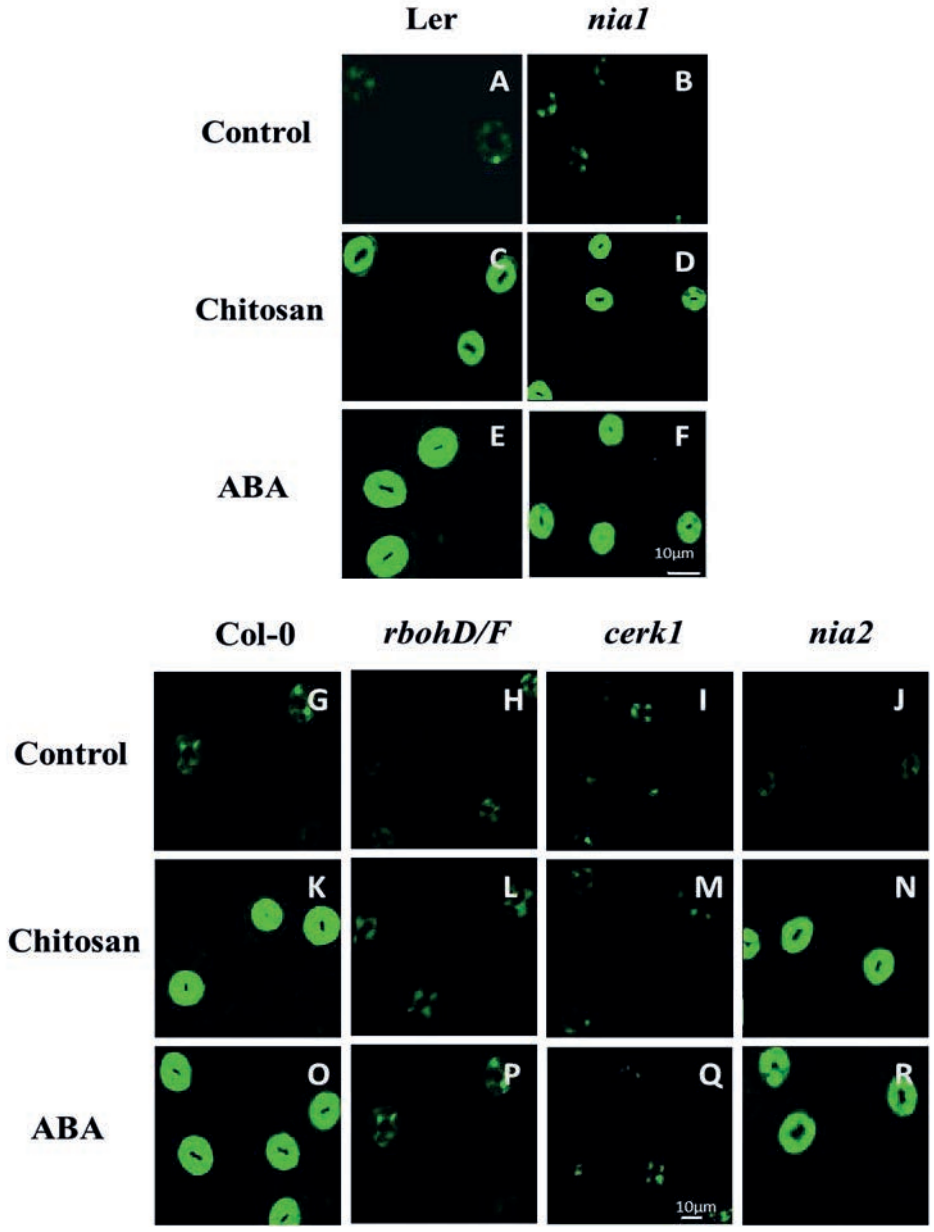

Figure 6.4. The extent of ROS production in guard cells of (A-F) wild type Ler and *nia1* mutant and (G-R) wild type Col-0 and *rbohD/F*, *cerk1* and *nia2* mutants of*Arabidopsis thaliana*, as indicated by the fluorescent probe H_2DCFDA. Panels A-B and G-J, represent control guard cells; C-D and K-N represent guard cells exposed to 20 µg mL^{-1}chitosan; E-F and O – R represent guard cells exposed to 20 µM ABA. A-E and B-F represent guard cells of Ler wild type and *nia1* mutant while G-O: represent guard cells of Col-0 wild type while H-P: *rbohD/F* mutant; I-Q: *cerk1* mutant; J-R: *nia2* mutant. Images were taken 30 minutes after addition of chitosan and ABA. Further details are given in Materials and Methods. *Bar* 10 µm

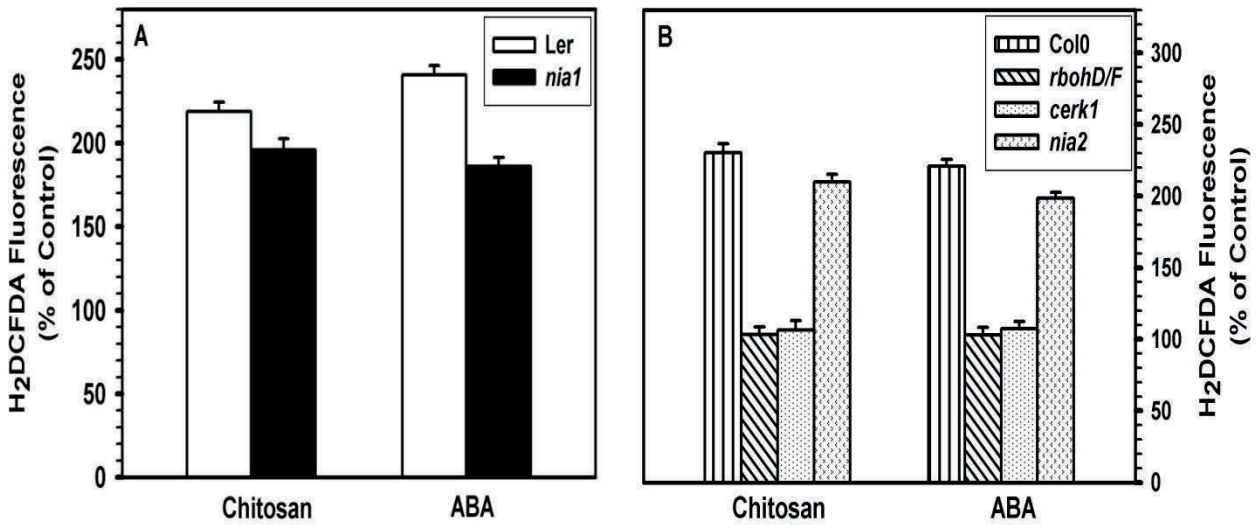

Figure 6.5. Quantitative evaluation of ROS levels in guard cells of *Arabidopsis* wild type Ler and the *nia1* mutant and Col-0 wild types and *rbohD/F*, *cerk1*and *nia2* mutants.ROS generation was determined by H_2DCFDA fluorescence of (A) wild-type guard cells Ler (white bars) and mutant *nia1* (black bars) and (B) Col-0 wild type (linear striped bars), *rbohD/F* (diagonal striped bars), *cerk*1 (spotted bars) and *nia2* (arrow bars) mutants with chitosan (20 μgmL^{-1}) or ABA (20 μM) and fluorescence expressed as a % of control. Fluorescence intensities were determined 30 minutes after treatment with chitosan or ABA. Data are the mean ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

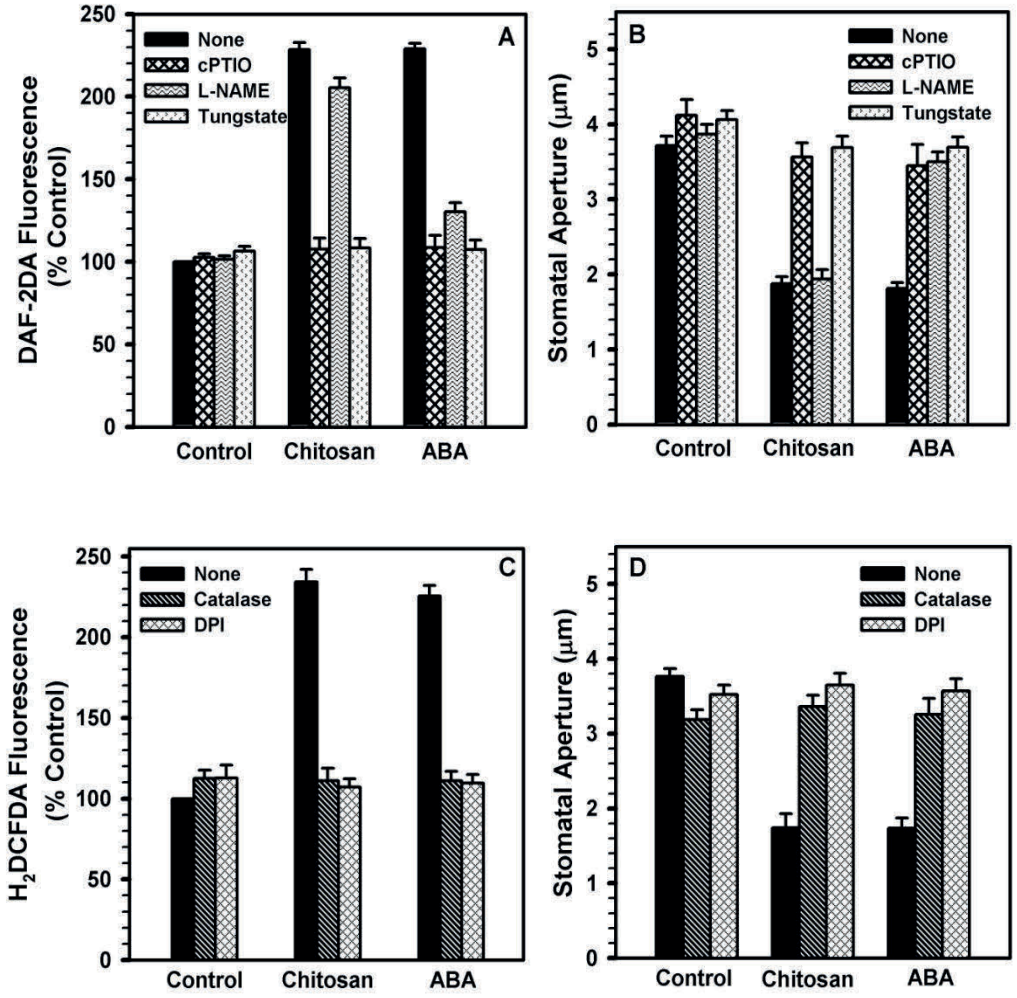

Figure 6.6. Chitosan or ABA- induced NO and ROS generation and its correlation with stomatal closure in abaxial epidermis of *Arabidopsis*. NO generation was determined by (A) DAF-2DA fluorescence of wild-type guard cells treated with chitosan (20 μg mL^{-1}) or ABA (20 μM) in the presence or absence of cPTIO (200 μM), L-NAME (25 μM) and tungstate (100 μM). (C) H_2DCFDA fluorescence of wild-type guard cells treated with chitosan (20 μg mL^{-1}) or ABA (20 μM) in the presence or absence of catalase (100 U mL^{-1}) and DPI (20 μM). Fluorescence intensities were determined 30 minutes after treatment. (B and D) are stomatal apertures measured for the same treatments in wild-type epidermal peels. Data are the mean ± SE from at least 3 independent experiments. Further details are given in Materials and Methods.

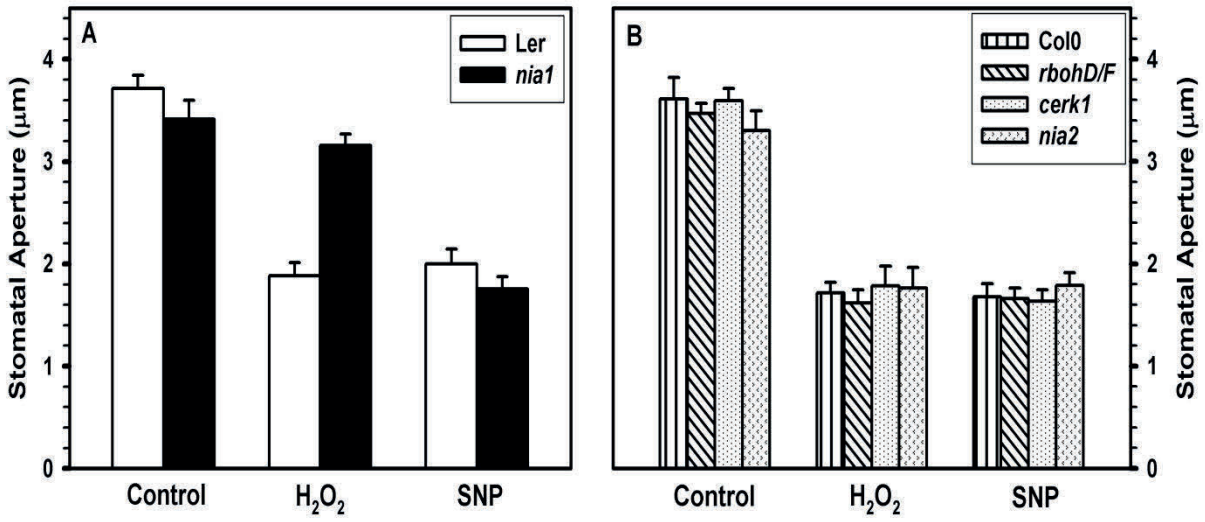

Figure 6.7. Stomatal sensitivity of *Arabidopsis* mutants *nia1, nia2, cerk1* and *rbohD/F* to external SNP and H_2O_2. Epidermal peels of either *Arabidopsis* (A) wild type Ler (white bars) and mutant *nia1* (black bars); Col-0 wild type (linear striped bars) and mutants rbohD/F(diagonal striped bars), *cerk1* (white spotted bars) and *nia2* (arrow bars) were incubated in light for 3 h in the absence (control) or presence of H_2O_2 (100μM) or SNP (50 μM), and stomatal apertures measured after 3 h. Results are the averages ± SE from at least 3 to 4 independent experiments. Further details are given in Materials and Methods.

Discussion

Previous studies have shown that chitosan induces stomatal closure in various species such as tomato, *Commelina communis, Pisum sativum*and *Hordeum vulgare*(Lee et al. 1999; Srivastava et al. 2009; Koers et al. 2011). The role and importance of ROS and NO as signaling components during chitosan-induced stomatal closure have been reported (Srivastava et al. 2009; Khokon et al. 2010). Our present work indicates that such ROS production is mediated by NAD(P)H oxidase during chitosan-induced stomatal closure in *Arabidopsis*. The present work also uses pharmacological and genetic approaches to demonstrate that *NIA1* and *RBOHD/F* are involved and that NO and ROS play an important role during chitosan-induced stomatal closure.

Requirement of NIA1, RBOHDF and CERK1, but not NIA2 during chitosan-induced stomatal closure

Being insensitive to chitosan or ABA effects, the stomata remained open for the mutants *nia1*, *cerk1* and *rbohD/F* while the *nia2* mutants stomata closed (Fig. 6.1). This observation suggests that *nia1*, *cerk1* and *rbohD/F* are playing an important role during chitosan-induced stomatal closure.

Two research groups found a receptor-like kinase designated *CERK1* or LysM RLK1 (lysine modified receptor-like kinase 1) which co-operate with CEBiP (chitin elicitor binding protein) for oligochitin elicitor signaling in *Arabidopsis* (Miya et al. 2007; Wan et al. 2008). The *cerk1* mutants also have impaired ability in responding normally to ABA apart from chitosan in terms of stomatal closure as evident from our

studies and as shown in (Fig. 6.1 C, D). From these data we can suggest that *rbohD/F*, *cerk1* and *nia1* are required but not *nia2* during chitosan-induced stomatal closure.

Chitosan-induced ROS and NO generation requires NIA1, RBOHD/F and CERK1 but not NIA2

Some workers have demonstrated that ABA-induced NO and H_2O_2 generation in *Arabidopsis* guard cells requires *NIA1*, but not *NIA2*, and both the D and H isoforms of the NADPH oxidase (Desikan et al. 2002; Bright et al. 2006). Our results show that chitosan-induced NO (Fig. 6.2 and 6.3) and H_2O_2 (Fig. 6.4 and 6.5) accumulation also required *NIA1* and *RBOHD/F*. We can also conclude from the results that *CERK1* plays an important role when induced by chitosan in stomatal guard cells. Also, being insensitive to ABA,*cerk1*can be linked directly into ABA signalling, at least in guard cells, and some way up the ABA signal transduction chain. The results hereby suggest that *NIA1* isoform of NR rather than *NIA2* plays an important role during the process. And *NIA1*, *RBOHD/F* and *CERK1* are required during chitosan-induced NO and ROS generation.

Chitosan-induced NO and ROS generation during chitosan-induced stomatal closure is mediated by NR and NADPH oxidase

NO scavenger, cPTIO prevented both the chitosan-induced stomatal closure as well as the rise in NO production suggesting that NO played a major role during chitosan-induced stomatal closure in *Arabidopsis* guard cells. The importance of nitrate reductase rather NOS-like enzyme during chitosan-induced stomatal closure was also seen (Fig. 6.6

A, B). The role of ROS and the importance of NADPH oxidase was also observed during the process of stomatal closure (Fig. 6.6 C, D).

The *nia1*, *rbohD/F* and *cerk1* mutants exhibited decreased sensitivity to chitosan, although they were still quite sensitive to external SNP or H_2O_2 indicating that only the events upstream of NO/H_2O_2 generation are affected. These data indicate that there are no inherent malfunctions in the mutant stomata and that their lack of response to chitosan is due to the lack of NIA1 or RBOHD/F. We can therefore conclude from our results that *NIA1*, *CERK1* and *RBOH* are required during chitosan-induced stomatal closure and also shares a common signaling pathway to ABA.

Conclusions

1. Chitosan enhanced NO levels of guard cells in a similar way, as that by ABA, during stomatal closure in *Arabidopsis* guard cells.
2. Pharmacological evidences indicated that NR plays an important role in production of NO, leading to chitosan or ABA-induced stomatal closure. The inability of L-NAME to affect chitosan-induced stomatal closure, suggested that NOS might not be essential for NO production during chitosan induced stomatal closure. However, NOS played an essential role during ABA induced stomatal closure.
3. Experiments with NR-deficient *nia1*, *nia2* and NADPH oxidase mutant *rbohD/F* indicated that *nia1* and *rbohd/F* were insensitive to chitosan or ABA, while those of *nia2* closed. These results suggest that *nia1* and *rbohD/F*, rather than *nia2* play a major role during chitosan or ABA induced stomatal closure in *Arabidopsis* stomatal guard cells.

4. The guard cells o*f cerk1* mutants do not show up any rise in either NO or ROS and their stomata remained open even on exposure to chitosan or ABA. These results suggested that *cerk1* couldalso play an important role in signaling pathway of chitosan-induced stomatal closure.

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Chapter 7

Chitosan-induced expression of nitrate reductase and NAD(P)H oxidase genes in leaves of *Arabidopsis* wild types and mutants

Chapter 7

Chitosan-induced expression of nitrate reductase and NAD(P)H oxidase genes in leaves of *Arabidopsis* wild types and mutants

Chitosan initiates signalling cascades, which include generation of reactive oxygen species (ROS) and reactive nitrogen species such as H_2O_2 and nitric oxide (NO) (Lee et al. 1999; Srivastava et al. 2009; Manjunatha et al. 2009), besides altered gene expression (Povero et al. 2011). The generation of ROS and NO is impaired in mutants such as *AtrbohD/F* (NADPH oxidase) and *nia1* and *nia2* (Desikan et al. 2002; Bright et al. 2006; Modolo et al. 2006) respectively. The plant membrane-bound respiratory burst oxidase homologue (*Rboh*, NADPH oxidase), which shows homology to mammalian gp91phox, has been identified as an important source of ROS. *Rboh* is involved in plant defence against pathogen and various abiotic stresses (Torres et al. 2006; Miller et al. 2008) and in hormonal (Kwak et al. 2003; Bright et al. 2006) and developmental responses (Torres and Dangl 2005). In *Arabidopsis*, NR was encoded by two genes *NIA1* and *NIA2*. *NIA2* accounts for 90% of the total NR activity, while *NIA1* is responsible for the remaining 10% of NR activity (Yu et al. 1998).

A number of *Arabidopsis* genes are induced upon chitin elicitation (Ramonell et al. 2005; Miya et al. 2007; Wan et al. 2008; Povero et al. 2011) and some genes are also induced following elicitation by chitosan/chitooloigosaccharides (Povero et al. 2011; Malerba et al. 2012). These include defense-related genes (such as, phenylalanine ammonia-lyase, chitinase, peroxidase and some camelexin biosynthetic genes) as well as other genes with functions not yet identified. ABA-induced biphasic expression of *RBOH A–D* in *Zea mays* (Lin et al. 2009), while the highest induction of *RBOHD* expression was by ABA in *Arabidopsis* guard cells (Kwak et al. 2003). In ABA-deficient mutants,

ABA-induced stomatal closure and expression of some of these genes are impaired leading to a wilty phenotype (Hoth et al. 2002). Since, chitosan is similar in action to that of ABA, it is possible that it may also affect the genes related to NO and H_2O_2 generation.

This chapter describes our efforts to determine the effect of chitosan on the expression of genes, involved in NO and H_2O_2 generation, namely *NIA1*, *NIA2*, *RBOHD* and *RBOHF*.

Results

The effects of chitosan on the expression of the genes required for H_2O_2 and NO generation, namely *RBOH* and *NIA1*, was determined, as also the reciprocal functional requirement of each of these genes on the chitosan-induced expression of each of the others. It was not possible to carry out these experiments with guard cells due to the technical limitation arising with the preparation of sufficiently large numbers of isolated guard cells. Thus, the effects of chitosan were determined on whole leaves.

The overall picture of *NIA1*, *NIA2*, *RBOHD* and *RBOHF* gene transcript accumulation are shown as gel images in Figures 7.1 and quantitative assessment of transcripts are presented in the next five Figures of 7.2 to 7.5.

The levels of NIA1 transcripts in the mutants nia2 and rbohd/f were induced quickly by chitosan

Accumulation of *NIA1* transcripts was elicited rapidly following chitosan treatment with a 5-fold increase occurring in leaves of both wild types Col-0 and Ler and approximately

10-fold increase in the *nia2* and *rbohD/F* mutants after 30 minutes (Fig. 7.1 A and Fig. 7.2 A). This accumulation peaked in 1 h and thereafter declined. Relative *NIA1* transcript accumulation was highest in leaves of the *rbohD/F* mutant, and was also higher in those of the *nia2* mutant when compared to the corresponding wild type (Fig. 7.2 B). In all the plants treated, with increasing time, the level of the *NIA1* transcript returned to levels prior to the treatment of the leaves with chitosan. The actual time taken for this to occur and the rate at which this happened depended on the plant genotype being investigated, being slow in the *nia2* and *rbohD/F* mutants, compared to the wild type.

The chitosan-induced increase in the levels of NIA2 transcripts was delayed in the mutants nia1 and rbohd/f

The *NIA2* transcript accumulation profile in response to chitosan followed a similar pattern to that of *NIA1*, but with different overall kinetics (Fig. 7.1 B and 7.3 A, B). Chitosan-induced increases in *NIA2* transcript accumulation occurred at slower rate and peaked later in 6 h. Again, the peak of *NIA2* transcripts was higher in the leaves of the two mutants, *nia1* and *rbohD/F*, than in those of the wild type Col-0. Similarly, after reaching a peak level of accumulation, the level of *NIA2* transcripts declined. However, in contrast with the rapid post-peak decline exhibited by *NIA1* transcripts, those encoded by *NIA2* declined at a slower rate such that in the leaves of the *nia1* and *rbohD*/*F* mutants they were still significantly elevated above the pre-treatment levels at the 48 h sampling time.

The levels of RBOHD transcripts in the mutants nia1 and nia2 when exposed to chitosan

The accumulation of transcripts encoded by the gene *RBOHD* was also followed in the leaves of wild type Col-0 and *nia1* and *nia2* mutant plants following their treatment with chitosan (Fig. 7.1 C and 7.4 A). The accumulation of *RBOHD* transcripts in the leaves of the mutants*nia1* and *nia2* started to increase by 1 h and reached its maximum in 2 h, remained higher till 6 h and then started to decline by 12 h and 24 h. However, the transcript accumulation in the leaves of *nia2* mutants were lower than those of Col-0 and *nia1*. Relative *RBOHD* expression was highest in the wild types Col-0 and the mutants*nia1* but in the *nia2* was lower when compared to its wild type (Fig. 7.4 B).

The chitosan-stimulated levels of RBOHF transcripts follow a similar pattern with that of RBOHD in the mutants nia1 and nia2

The accumulation of transcripts encoded by the gene *RBOHF* followed a similar pattern to those of *RBOHD* when induced by chitosan in the wild type Col-0 and the mutants *nia1* and *nia2* (Fig. 7.1 D and 7.5 A).A3-fold increase in *RBOHF* in the leaves of *nia1* mutants increased by 0.5 h and reached its maximum by 1 h and remained higher till 6 h and then declined in 24 h. While the *RBOHF* expression in the *nia2* mutants increased their expression by 0.5 h and reached their maximum by 1 h and remained higher till 2 h and then declined by 6 h till 24 h. Transcript accumulation of *RBOHF* in both *nia1* and *nia2* mutants levels are almost similar but two fold higher than those of wild type Col-0.

The relative gene expression of *RBOHF* in the wild types Col-0 and the mutants*nia1* and *nia2* was somewhat similar to the fold change increase (Fig. 7.5 B).

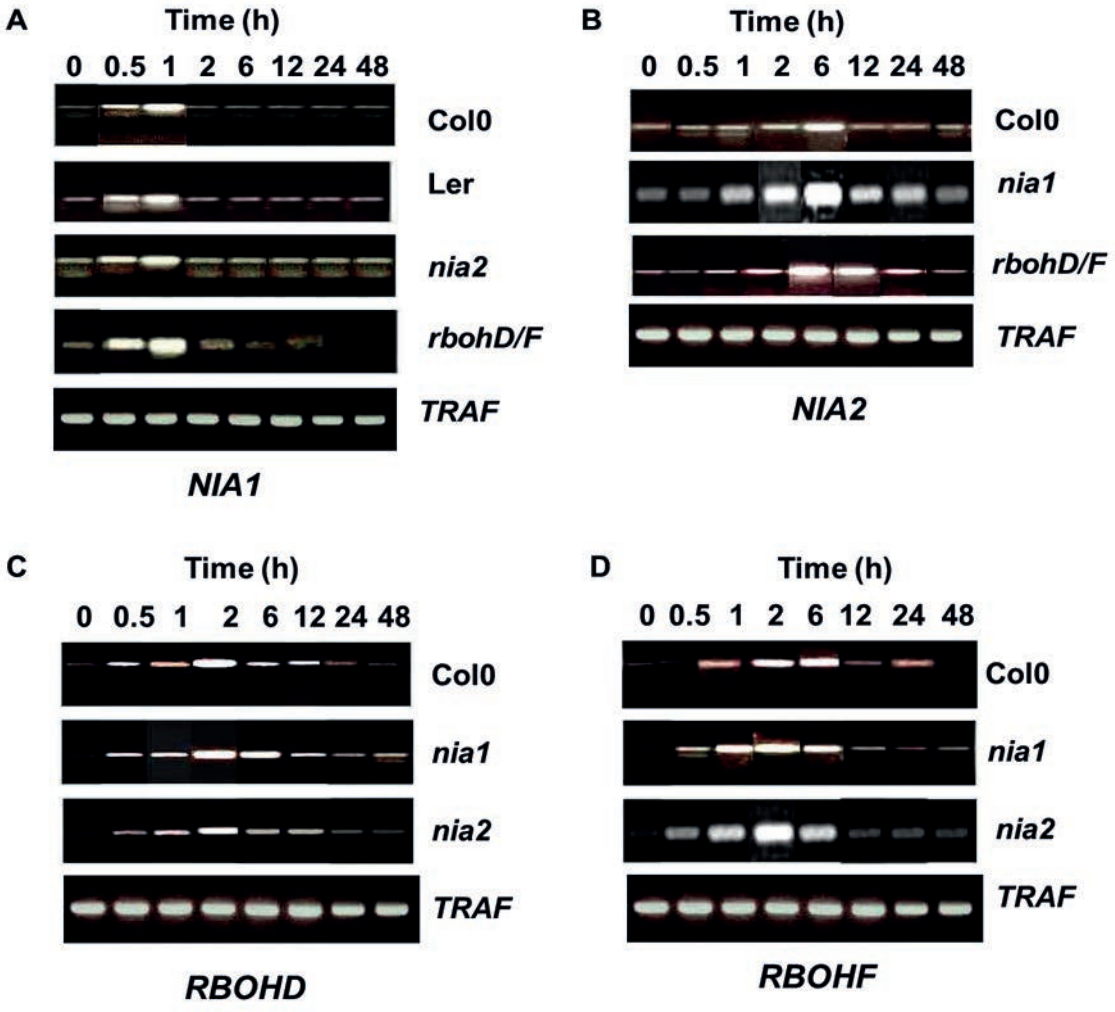

Figure 7.1. Accumulation of *NIA1*, *NIA2*, *RBOHD* and *RBOHF* gene transcript in chitosan treated *Arabidopsis* whole leaves, as observed with RT-PCR (A) *NIA1* transcript levels in the leaves of wild types Col-0 and *Ler* and the mutants *nia1* and *rbohD/F*. (B) *NIA2* transcript levels in the leaves of wild type Col-0 and the mutants *nia1* and *rbohD/F*. (C & D) *RBOHD* and RBOHF transcript levels in the leaves of the wild type Col-0 and the mutants *nia1* and *nia2*. Total RNA was extracted from treated and control leaves and used to perform RT-PCR analysis. Treatment was 20 µg mL^{-1} chitosan or solvent control.

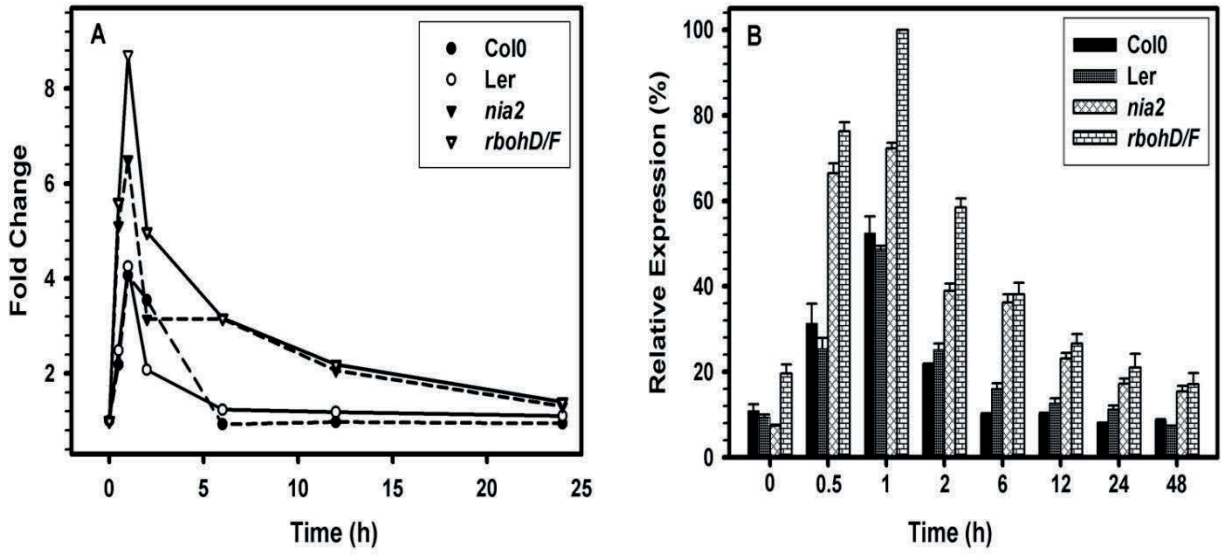

Figure 7.2. Transient increase in *NIA1* gene expression on exposure to chitosan in wild type and mutants of *Arabidopsis*. **A.** Effects of chitosan on *NIA1* gene expression (expressed as fold change) in leaves of Col-0 (●) and Ler (○) wild types and *nia2* (▼) and *rbohdf* (▽) mutants. Fold changes in expression of individual transcripts due to the treatments were calculated at each time point by dividing the normalised accumulation level of the transcript in the RNA samples derived from the treated plants by that from the untreated controls. **B.** Effects of chitosan on *NIA1* relative gene expression (expressed as %) in leaves of Col-0 (black bars) and Ler (white striped bars) wild types and *nia2* (criss cross bars) and *rbohdf* (bricked bars) mutants. Total RNA was extracted and used to perform quantitative RT-PCR for *NIA1*. Treatment was 20 µg mL^{-1} chitosan or solvent control. Each experiment included three replicates for each time point.

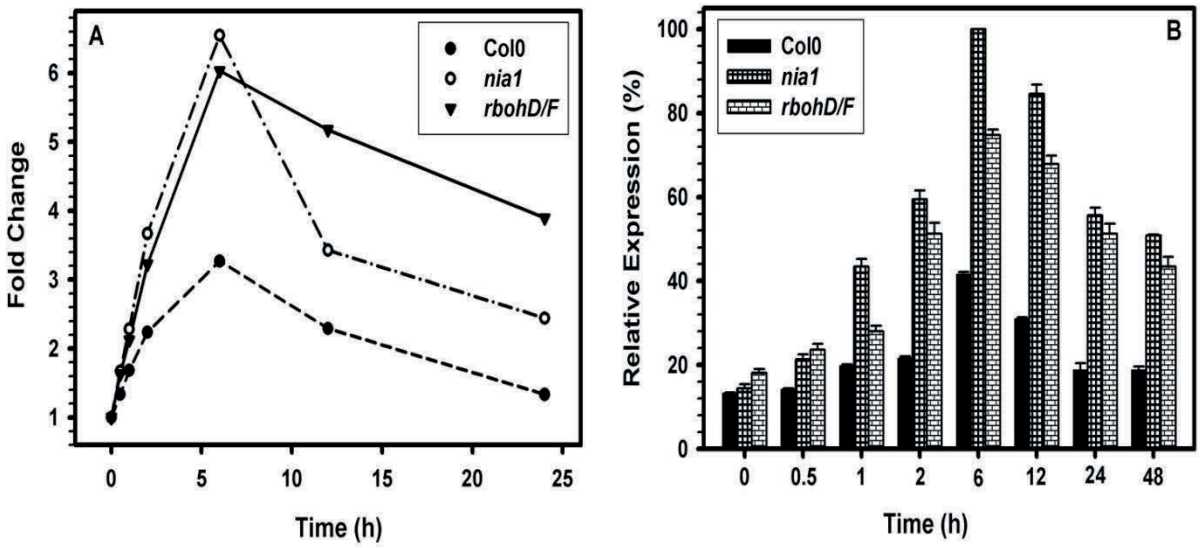

Figure 7.3. Transient increase in *NIA2* gene expression on exposure to chitosan in wild type and mutants of *Arabidopsis*. **A.** Effects of chitosan on*NIA2* gene expression (expressed as fold change) in leaves of Col-0 (●) and *nia1* (○) and *rbohdf* (▼) mutants. **B.** Effects of chitosan on*NIA2* relative gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and *nia1* (blocked bars) and *rbohdf* (bricked bars) mutants. Further details are given in Fig. 7.2 and Methods.

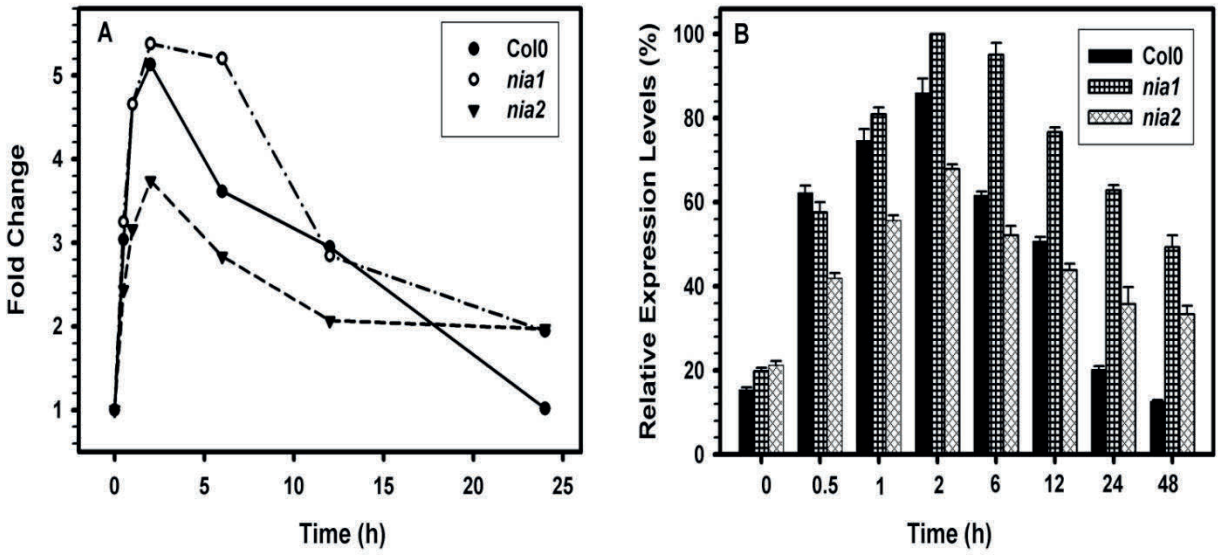

Figure 7.4. Transient increase in *RBOHD* gene expression on exposure to chitosan in wild type and mutants of *Arabidopsis*. **A.** Effects of chitosan on *RBOHD* gene expression (expressed as fold change) in leaves of Col-0 (●) and *nia1* (○)and *nia2* (▼) mutants. **B.** Effects of chitosan on *RBOHD* relative gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and *nia1* (blocked bars) and *nia2* (crossed bars) mutants. Further details are given in Fig. 7.2 and Methods.

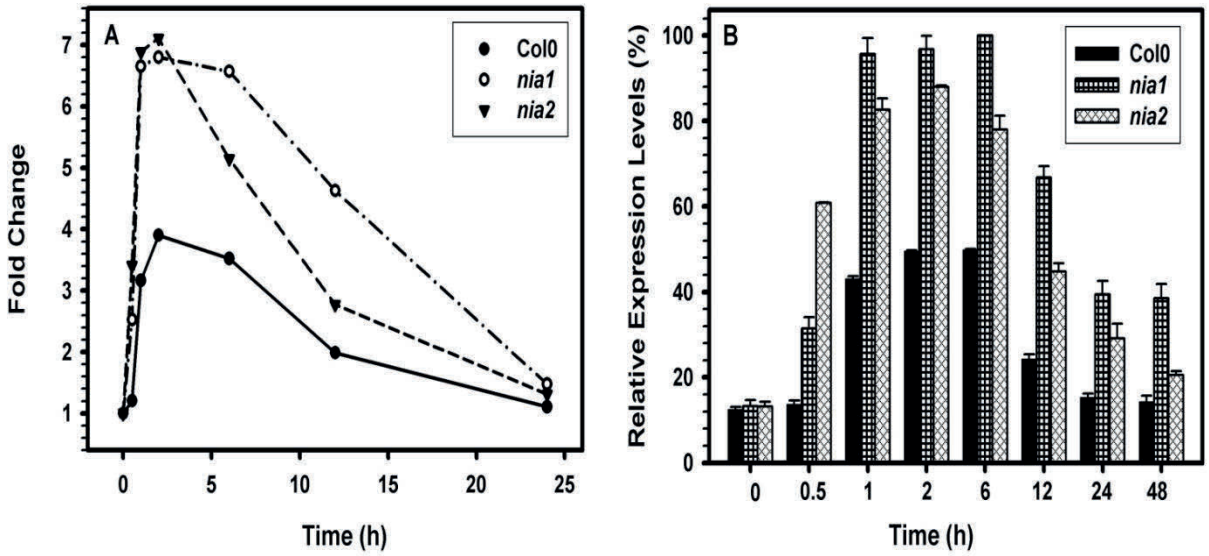

Figure 7.5. Transient increase in *RBOHF* gene expression on exposure to chitosan in wild type and mutants of *Arabidopsis*. **A.** Effects of chitosan on *RBOHF* gene expression (expressed as fold change) in leaves of Col-0 (●) and *nia1* (○) and *nia2* (▼) mutants *Arabidopsis* leaves. **B.** Effects of chitosan on *RBOHF* gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and *nia1* (blocked bars) and *nia2* (crossed bars) mutants. Further details are given in Fig. 7.2 and Methods.

Discussion

Chitosan-induced gene expression in Arabidopsis leaves may not be strictly dependent on H_2O_2 production

The significant increase in the levels of *NIA1* and *NIA2* transcripts by chitosan, in *rbohD/F* mutant (Fig. 7.2 and Fig. 7.3) indicates that the H_2O_2 generation associated with these isoforms of NADPH oxidase was not required for the downstream chitosan signaling that resulted in altered gene expression. However, whether or not this precluded an absolute requirement for H_2O_2 in such chitosan signaling is unclear, since multiple other isoforms of NADPH oxidase exist in *Arabidopsis* (Capper & Dolan 2006) and may have compensated for the lack of the D and F isoforms here. What is clear though, is that in the guard cells at least, chitosan failed to induce detectable levels of H_2O_2 in the *rbohD/F* mutant as determined by H_2DCFDA fluorescent imaging (Fig. 6.4 L). Thus, it may be possible to conclude that there is no requirement for H_2O_2 in order for chitosan to induce the expression of *NIA1* in *Arabidopsis* leaves.

The lack of NIA1 is compensated by enhancement of NIA2 during chitosan-induced gene expression in Arabidopsis leaves

The increase in the levels of either *NIA2* and *RBOH*, or *NIA1* and *RBOH* transcripts when induced by chitosan, in either the *nia1* or *nia2* mutants (Fig. 7.3, 7.4 and 7.5) respectively, indicates that neither *NIA1* nor *NIA2* expression and thus, the synthesis of NO was required. However, it is possible that other sources of NO exist.

It may be argued that *NIA1* is not the only source of NO in plants and plants may also possess a mammalian type nitric oxide synthase (NOS) which can convert arginine

to citrulline and NO (Corpas et al. 2009). However, the evidence for the existence of such a plant NOS has been on the basis of a non-specific inhibitor, L-nitro-arginine methyl ester (L-NAME). There is neither biochemical nor genetic evidence to support its existence (Neill et al. 2008). Whichever may be the case, chitosan did not induce NO accumulation in the guard cells of either the *nia1* or *rbohD/F* mutants (Fig. 6.2 D and L).

We therefore feel that the presence of NO may not by itself required for the expression of *NIA1* and *RBOHD/F* after exposure to chitosan. It is possible that chitosan treatment initially leads to an alteration in gene expression initially *via* an alternative signaling pathway that does not functionally depend on the presence of NO.

NIA1 and NIA2 genes are differentially expressed in Arabidopsis whole leaves

The transcripts of *NIA1* and *NIA2* were all higher in chitosan-treated leaves of the *rbohD/F* mutant than in those of similarly treated wild types (Fig. 7.1), indicating a possible role for *NIA1* in NO generation. The genetic evidence to date suggests that only *NIA1* is required for NO synthesis, at least in guard cells (Bright et al. 2006). However, here the increase in the accumulation of *NIA2* transcripts in leaves following chitosan treatment lagged behind that of those encoded by *NIA1* (Fig. 7.2). This suggest a differential expression of *NIA1* and *NIA2* genes when induced by chitosan in *Arabidopsis* leaves. Other reports have similarly demonstrated differential expression patterns for *NIA1* and *NIA2* in response to NO_3^-, light, circadian rhythm and cytokinin (Cheng et al. 1991; Yu, Sukumaran & Marton 1998).

The elevated chitosan-induced accumulation of *NIA2* transcripts in the leaves of the *nia1* mutant (Fig. 7.3 A, B) compared to those of wild types suggests that expression

of *NIA2* may compensate for a lack of *NIA1*. Further experiments are necessary to validate our suggestions. It would be interesting to determine the efficacy of NO mediated acclimation responses to stress in the *nia2* mutant. The present data suggests that the signaling molecules NO and H_2O_2 might not be required during chitosan-induced gene expression in leaves. The focus of future work may determine which signaling pathways are involved during this process.

Conclusions

1. Chitosan-induced gene transcript accumulation in *Arabidopsis* leaves is not dependent on RBOH (and possibly H_2O_2).
2. The increased levels of *NIA2* transcripts in the *nia1* mutants suggest that *NIA2* expression may compensate for a lack of *NIA1* and that NO production may not be essential for *NIA1* during chitosan-induced gene transcript accumulation in *Arabidopsis* leaves.
3. The expression of *NIA1* and *NIA2* genes are differentially modulated when exposed to chitosan in *Arabidopsis* leaves.
4. The ability of chitosan to induce stomatal closure and to activate expression of defense-related genes emphasizes its potential use for modulating the stomatal movement and defense responses.

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Chapter 8
General Discussion

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General Discussion

The present study elucidates the role and importance of two key secondary messengers, H_2O_2 and NO, involved during chitosan-induced stomatal closure in *Pisum sativum* and *Arabidopsis thaliana*. The fourth and fifth chapters of this work uses pharmacological inhibitors/modulators to ascertain the role of ROS, NO and Ca^{2+} during chitosan-induced stomatal closure as well as stomatal opening in *Pisum sativum* guard cells. In order to confirm the role and importance of H_2O_2 and NO, further stomatal bioaasays were carried out in different *Arabidopsis* mutants and wild type. In order to know the genes required /expressed for H_2O_2 and NO synthesis during chitosan elicitation, our work was further extended to gene expression analysis of genes, such as *RBOHD/F*, *NIA1*, *NIA2*.

Chitosan-induced stomatal closure requires H_2O_2 and NO in Pisum sativum guard cells

The ability of guard cells to generate H_2O_2 during ABA-induced stomatal closure have already been studied in *Vicia faba*, *Pisum sativum* and *Arabidopsis* (Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2004). Marked increases in H_2O_2 are induced in guard cells by exposure to methyl jasmonate, bicarbonate and elicitors, such as cryptogein or chitosan which is known to regulate stomatal aperture (Allan and Fluhr 1997; Kolla et al. 2007; Srivastava et al. 2009). The results from the present study demonstrate the importance of ROS during chitosan-induced stomatal closure, by using H_2DCFDA to monitor ROS production in guard cells of *Pisum sativum* (Fig. 4.2 F-H) The ability of catalase, a ROS scavenger, prevented chitosan-induced stomatal closure as well as ROS

production (Fig. 4.5 M, Table 4.1) which confirmed that H_2O_2 is indeed required to initiate stomatal closure.

ABA-induced increases in NO synthesis have been observed in guard cells of *Arabidopsis*, *Pisum sativum* and *Vicia faba* (Neill et al. 2002; Garcia-Mata and Lamattina 2003; Desikan et al. 2004). Guard cells generate NO in response to also elicitor challenge and methyl jasmonate (Lamotte et al. 2004; Saito et al. 2009).The increase in NO in guard cells, as monitored by DAF-2DA, in the present study (Fig. 4.2 A-C) demonstrates the importance of NO using cPTIO, a NO specific scavenger which led to prevention of stomatal closure as well as NO production (Fig. 4.4J, Table 4.1). These observations confirmed that NO is also an important factor during chitosan-induced stomatal closure.

Time course experiments in presence of H_2DCFDA (for ROS) and DAF-2DA (for NO) revealed that NO production occurred much after ROS production when induced by chitosan (Fig. 4.3). Thus, the present work elucidates that NO production occurs downstream of ROS and that ROS production was necessary for NO production during chitosan effects, as was the case with ABA (Bright et al. 2006; Neill et al. 2008; Srivastava et al. 2009). Besides H_2O_2 and NO, calcium also plays an important role in stomatal signal transduction pathway when induced by external stimuli (Islam et al. 2010). The importance of calcium during chitosan-induced stomatal closure is evident by use of both BAPTA (chelator of external Ca^{2+}) and BAPTA-AM (chelator of internal Ca^{2+} within the cell). These modulators prevented the chitosan-induced stomatal closure despite the high rise in NO or ROS levels (Fig. 4.4 O, P and Fig. 4.5 O, P).

Chitosan-mediated inhibition of stomatal opening requires ROS, NO and Ca^{2+} in Pisum sativum guard cells

It has been earlier reported that ABA inhibition of stomatal opening is a distinct process from ABA-induced stomatal closure (Mishra et al.2006; Yan et al. 2007; Garcia-Mata and Lamattina 2007). Although NO and ROS were found to be second messengers in the signaling cascade during chitosan-induced stomatal closure, little is known about the role of NO and ROS during chitosan inhibition of stomatal opening. In the present study, we investigated whether NO and ROS are also involved in chitosan inhibition of stomatal opening in *P. sativum*. We found that ROS and NO were important during chitosan-mediated inhibition of stomatal opening, as in case of closure (Fig. 5.3 and Fig. 5.4). However there was a difference in the source of NO production, as NOS-like enzyme played a major role during chitosan inhibition of stomatal opening, besides nitrate reductase (Fig. 5.3). Thus, chitosan-induced stomatal closure appears to be distinct in that nitrate reductase is the source of NO, and not nitric oxide synthase.

The roles of both intracellular and extracellular calcium during chitosan-mediated inhibition of stomatal opening were also studied. Since the rise in Ca^{2+} concentration is important in ABA-mediated inhibition of stomatal opening (Garcia-Mata and Lamattina 2007), it was interesting to check if Ca^{2+}plays an important role in the chitosan-mediated inhibition of light-induced stomatal opening as well. The ability of calcium chelators as BAPTA and BAPTA-AM to prevent the chitosan-mediated inhibition of stomatal opening, suggest that both extracellular and intracellular calcium play a major role during the inhibition of opening process (Fig. 5.5 and 5.6). Thus, stomatal closure and inhibition of stomatal opening processes are regulated by the same signaling components as ROS,

NO and Ca^{2+} but there may be a difference in the sources of ROS/NO and their relative dominance.

The major contributors for H_2O_2 and NO production are* RBOHD/F *and* NIA1 *during chitosan-induced stomatal closure in Arabidopsis guard cells

H_2O_2 can be generated during multiple routes in plant cells. Electron transport processes such as photosynthesis and respiration generate basal levels of H_2O_2, which increase in response to stress(Apel and Hirt 2004; Quan et al. 2008). Enzymatic sources of H_2O_2 include plasma membrane NADPH oxidase, cell wall peroxidases, amine oxidases, and other flavin containing enzymes (Mittler et al 2004; Gill and Tuteja 2010). The ability of DPI, an NADPH oxidase inhibitor, to restrict ABA- or chitosan-induced H_2O_2 synthesis (Fig. 6.6 D) and stomatal closure, suggests that NADPH oxidase is a potential source of H_2O_2 during stomatal closure.

Since, pharmacological data indicated a role for NADPH oxidase in guard cell functioning, reverse genetic approach was used to establish a functional role for NADPH oxidase. The inability of *AtrbohD/F* mutants of *Arabidopsis* to close stomata in response to chitosan (Fig. 6.4 L) confirms that NADPH oxidase-mediated H_2O_2 synthesis is required for chitosan effects on guard cells. This observation is consistent with the earlier studies on the role of gene silenced RBOH plants during ABA or elicitor-induced guard cell signaling in *Arabidopsis* or *Nicotiana benthamiana* plants (Kwak et al. 2003; Bright et al. 2006; Zhang et al. 2009).

Two enzymes capable of NO biosynthesis in plants are nitric oxide synthase (NOS) and nitrate reductase (NR). An apparent plant NOS (AtNOS1) in *Arabidopsis* was

first reported by Guo et al.(2003). Although animal nitric oxide synthase (NOS) activity has been detected in plants and inhibitors of mammalian NOS (as L-NAME) impair NO production in plants (Lamotte et al. 2004; Neill et al. 2008), the gene encoding NOS in plants is still not clear. The nature as well as function of *AtNOS1* is in debate (Crawford et al. 2006; Guo 2006; Zemojtel et al. 2006).Several studies have confirmed that *Atnos1* mutant had low NO accumulation and impaired ability to generate NO in response to lipopolysaccharides, ABA, oxidative stress as well as salicylic acid (Zeidler et al. 2004; Bright et al. 2006; Zhao et al. 2007; Zottini et al. 2007).Based on the sensitivity to L-NAME, the present study provides an indication that NOS-like enzyme activity may have a role during chitosan or ABA-induced stomatal closure as well as inhibition of stomatal opening.

The other enzyme for NO synthesis is NR. In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2*. Of the two isoforms, NIA2 accounts for 90% of the total nitrate assimilatory activity of NR in seedlings, while NIA1 accounts for the remaining 10% (Yu et al. 1998). The prevention of NO production by tungstate, an inhibitor of nitrate reductase, is frequently cited as the evidence for the role of NR in NO biosynthesis (Bright et al. 2006; Srivastava et al. 2009; Hao et al. 2010; Wang et al. 2010). The NR-deficient *nia1nia2*double mutant, which has only 0.5% of wild-type NR activity, exhibits neither guard cell NO synthesis nor stomatal closure in response to either ABA or nitrite (Desikan et al. 2002). The use of single mutants,*nia1* and *nia2*,suggested that *NIA1* played more important role than *NIA2* in NO production in response to ABA or cold stress (Bright et al. 2006; Zhao et al. 2009).In our studies, chitosan-induced NO production as well as stomatal closure was inhibited by tungstate (Fig. 6.6 A, B),

indicating the importance of NR. Further, the pattern of NO production in *Arabidopsis* mutants, confirmed that *nia1*plays more important role than *nia2*(Fig. 6.2 D, N) during chitosan-induced stomatal closure.

In *Arabidopsis* plants, the lysin motif (LysM)-containing chitin elicitor receptor kinase 1 (CERK1) is essential for perception of the fungal cell wall component chitin and for resistance to fungal pathogens (Miya et al. 2007; Wan et al. 2008). The binding of CERK1 to chitosan may be physiologically relevant as *cerk1-2* knockout mutants showed neither a reactive oxygen burst nor MAP kinase activation in response to chitosan (Petutschnig et al. 2010). Our preliminary data provide sensitivity of *cerk1* mutants to chitosan. As per Figs. 6.1, 6.2 and 6.4, *cerk1* mutants were insensitive to stomatal closure or NO/ROS generation in response to chitosan or ABA. Thus, the present study demonstrates that chitosan-or ABA-induced stomatal closure requires*CERK1*. However, due to technical reasons and time limitations, we were unable to take up further the study of *CERK1* gene expression in *Arabidopsis* plants when induced by chitosan. These are necessary to confirm the role of CERK1 in chitosan signaling.

Possible limitations in the present study

Our experiments still raise and leave some questions. For e.g. the specificity of DAF-2DA and H_2DCFDA to detect NO or ROS are questionable (Planchet and Kaiser 2006). However, in view of relevant controls and evidence from scavengers of NO or ROS during these experiments (Fig. 4.4, Fig. 4.5, Fig. 5.3; Fig. 5.4; Fig. 6.6), we are confident that the monitored fluorescence is related to NO/ROS, as intended. Similarly, one may argue that catalase may not enter the guard cells, but the efficacy of catalase to decrease

ROS and sustain stomatal opening was consistent and significant. External catalase was used earlier to demonstrate the importance of ROS in plant tissues (Zhang et al. 2007) and even guard cells (Lee et al. 1999; Zhang et al. 2001). Thus, the possible criticisms would not affect the conclusions drawn in the present work, and indicate that the increase in NO-levels occurred after that of ROS.

Chitosan-induced intracellular signal transduction chains differ between Arabidopsis leaf (mesophyll) cell and stomatal guard cells

There are a few studies on ABA-induced expression of genes required for H_2O_2 synthesis (Kwak et al. 2003). In order to know, whether chitosan-induced gene expression similarly requires H_2O_2 and NO, we used *Arabidopsis* mutants to determine the effects of chitosan, and any reciprocal requirement therein for NR1 and NADPH oxidase, on the expression of the genes essential for NO and H_2O_2 synthesis, namely *NIA1*, *NIA2*, *RBOHD* and *RBOHF*.

During chitosan-induced gene expression in *Arabidopsis* leaves, the lack of NO production of *NIA1* was compensated by *NIA2* (Fig. 7.4 and Fig. 7.5). H_2O_2 generation might also not be required for the chitosan-induced gene expression signaling in leaves. Other isoforms of NADPH oxidase exist in *Arabidopsis* (Capper & Dolan 2006) which might have compensated for the lack of the D and F isoforms. This can also be supported by our data that the levels of *NIA1* and *NIA2* transcripts increased in the *rbohD/F* mutant when induced by chitosan. However, chitosan failed to induce detectable levels of H_2O_2 in the *rbohD/F* mutant as determined by H_2DCFDA fluorescent imaging (Fig. 6.4 L). Unlike stomatal guard cells, H_2O_2 might not be required for NIA1 expression during

chitosan-induced gene expression in *Arabidopsis* leaves as *NIA1* and *NIA2* transcript levels increased in *rbohD/F* mutants (Fig. 7.2 and Fig. 7.3). The data presented here also suggest that the two NIA isoforms are differentially expressed when induced by chitosan, a conclusion reached by other workers (Yu et al. 1998; Lu et al. 2011)with similar conclusions, but in different systems and treatments, like, cyotkinin (hormone) and PB90, a proteinaceous elicitor. Thus, the nuclear modifications induced by chitosan in leaves differ from the effects on membranes induced in isolated guard cells, in that the former do not require RBOHD/F and NIA1 whereas the latter do.

The summary and conclusions are presented in the next chapter, along with a schematic presentation of possible signaling pathway during chitosan-induced stomatal closure.

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Chapter 9

Summary and Conclusions

Chapter 9

Summary and Conclusions

Signal transduction mechanisms in guard cells integrate a multitude of different abiotic and biotic signals as light signals, water satus, CO_2, temperature, hormones, elicitors, and other environmental conditions to modulate stomatal apertures (Sirichandra et al. 2009; Kim et al. 2010). A detailed analysis of guard cell signal transduction are quite useful for studies of other plant signaling systems. Guard cells respond to plant hormone ABA through several secondary messengers as ROS, NO, cytosolic pH, calcium, G-proteins, protein kinases, phospholipases. The signaling components and sources of ROS and NO during ABA-induced stomatal closure have been examined extensively (Desikan et al. 2004; Bright et al. 2006; Neill et al. 2008; Gonugunta et al. 2008, 2009; Raghavendra et al. 2010). However, very limited work has been done to identify the signaling components during stomatal closure induced by microbial elicitors, such as chitosan (Lee et al. 1999; Srivastava et al. 2009).

The present work is an attempt to elucidate the role and importance of secondary messengers as ROS and NO during chitosan-induced stomatal closure in guard cells of *Pisum sativum* and *Arabidopsis thaliana*. In most of the experiments, ABA was used for comparison. The initial attempt was to study the patterns of changes in the levels of NO and ROS (Fig. 4.2, Fig. 4.4 and Fig. 4.5). Reverse genetic approaches were followed using *Arabidopsis* mutants as *rbohD/F*, *nia1*, *nia2*so as to validate in *P. sativum* abaxial epidermis, the sources of NO and ROS production (Fig. 6.2, Fig. 6.4 and Fig. 6.6). The work was further extended to determine the effect of chitosan on the expression of genes,

in *Arabidopsis* leaves, involved in NO and H_2O_2 generation, namely *NIA1*, *NIA2*, *RBOHD* and *RBOHF*.

Chitosan induced stomatal closure in *P. sativum* guard cells in a concentration dependent manner, an effect similar to those of ABA. The use of fluorescent probes, demonstrated that the levels of ROS and NO rise and thus play an important during chitosan-induced stomatal closure. Several studies have shown that production of H_2O_2 occurs in response to many physiological stimuli in plant cells, including pathogen attack, hormone signaling and gravitropism (Kwak et al. 2006; Rachel and Dolan 2006; Vellosillo et al. 2010). The present results suggest the involvement of H_2O_2 during chitosan-induced stomatal closure which is demonstrated by two evidences: Firstly, production of H_2O_2 in guard cells (Fig. 4.2 G-H, Fig. 4.3 B) and secondly, the prevention of chitosan-induced stomatal closure and H_2O_2 by scavenging agents: catalase or DPI (Table 1.1). The production of H_2O_2 have also been shown to be a common signaling element during stomatal closure caused by ABA or MJ (Zhang et al. 2001; Suhita et al. 2004).

The next step was to examine the role of NO, another signaling molecule. Previous studies have indicated that NO regulates stomatal movement in response to ABA via nitric oxide synthase (NOS) like and nitrate reductase activity (Desikan et al. 2002; Neill et al. 2002, 2008). Suppression of NO levels, by either scavenging in the presence of cPTIO or inhibiting NOS (by L-NAME) or NR (by sodium tungstate) reversed the stomatal closure, indicating the essentiality of NO during chitosan-induced stomatal closure (Fig. 4.4 L-N, Table 1.1). Real time monitoring with the help of fluorescent probes indicated that NO production in guard cells of *P. sativum* peaked at 20

min (Fig. 4.3 A), compared to 5 min in case of ROS (Fig. 4.3 B). Thus, ROS precedes NO production in pea guard cells with chitosan-induced stomatal closure. The reversal of chitosan-induced stomatal closure by calcium chelators, BAPTA and BAPTA-AM, provides evidence for the role of calcium downstream of ROS/NO (Table 1.1).

There are studies which indicate that ABA-induced stomatal closure not only similar in some aspects, but also different from ABA-mediated inhibition of stomatal opening (Yan et al. 2007; Garcia-Mata and Lamattina 2007). The present studies also emphasizes the roles of ROS and NO during chitosan-mediated inhibition of stomatal opening. Using pharmacological compounds to modulate ROS and NO, such as DPI, L-NAME and tungstate, respectively, our studies provide evidence that during the inhibition of stomatal opening, the sources of ROS and NO production are NADPH oxidase and nitric oxide synthase-like (NOS) enzyme, respectively with only a partial involvement of nitrate reductase (NR) (Fig. 5.3 and Fig. 5.4). However, during chitosan-induced stomatal closure, the major source of NO production appears to be NR (Table 1.1).

In a reverse genetic approach, mutants of *Arabidopsis* have been used. The mutants used are deficient in plasma membrane NADPH oxidases (*AtrbohD/F*), nitrate reductase (*nia1* and *nia2*) and chitin elicitor receptor kinase (*cerk1*). Such studies were earlier made to analyse and validate the importance of ROS and NO in chitosan-induced stomatal closure, in comparison with ABA (Desikan et al. 2002; Kwak et al. 2003; Bright et al. 2006; Neill et al. 2008). During chitosan-induced stomatal closure, the *rbohD/F* mutants were insensitive to chitosan, as the stomata of these mutants remained open even

when exposed to chitosan (Fig. 6.1 C). Similarly, the stomata of *nia1* mutant remained open when exposed to chitosan, while those of *nia2* closed (Fig. 6.1 A& C).

The confocal studies using fluorescent probes H_2DCFDA (for ROS) and DAF-2DA (for NO), provided further evidence that *RBOHD/F* and *NIA1*, rather than *NIA2* were playing a major role during chitosan-induced stomatal closure. The mutants*nia1*and *rbohD/F* did not show any increase in NO levels when exposed to chitosan, while *nia2* did (Fig. 6.2 D & L, Fig. 6.2 N). However, the *nia1* and *nia2* mutants showed up a rise in ROS levels, while the *rbohD/F* did not show any increase in ROS levels (Fig. 6.2 D & N, Fig. 6.2 L). This observation also implies that NO is required for ROS during chitosan-induced stomatal closure. The importance of chitin elicitor receptor kinase 1 (*cerk1*) during chitin signaling has been indicated by Miya et al. (2007) and Wan et al. (2008). Our results with stomatal bioassays and confocal fluorescent experiments, provide preliminary evidence that *cerk1* may be important during chitosan-induced stomatal closure (Fig. 6.1 C, Fig. 6.2 M, Fig. 6.4 M). However, this hypothesis needs further detailed examination.

The expression of genes required for H_2O_2synthesis during ABA-induced signaling have earlier been studied in *Arabidopsis* (Kwak et al. 2003). The present studies attempted to characterize the expression of genes, mainly *NIA1*, *NIA2*, *RBOHD* and *RBOHF*. (required for H_2O_2 and NO synthesis), in response to chitosan, in *Arabidopsis* mutants as *nia1*, *nia2* and *rbohD/F*. From the present studies, we imply that, chitosan-induced downstream gene expression differs in these mutants, from the wild types (Fig. 7.1 to Fig. 7.5). Chitosan-induced gene expression in leaves might not require H_2O_2generation associated with NADPH oxidase isoforms and also, the *NIA1* and *NIA2*

genes are differentially expressed when exposed to chitosan (Fig. 7.2 and Fig. 7.3). The observation of differential expression of the genes *NIA1* and *NIA2*is consistent with studies by Yu et al. (1998) and Lu et al. (2011). Thus, both chitosan-induced stomatal closure and chitosan-induced gene expression follow a slightly different pattern, in their requirements for H_2O_2 and NO.

A possible model that integrates the present results and the sequential role of signaling elements that are involved during chitosan-induced stomatal closure is presented in Fig. 9.1. This scheme is broadly similar to the events during ABA-induced stomatal closure (Kim et al. 2010; Raghavendra et al. 2010; Lee and Luan 2012).

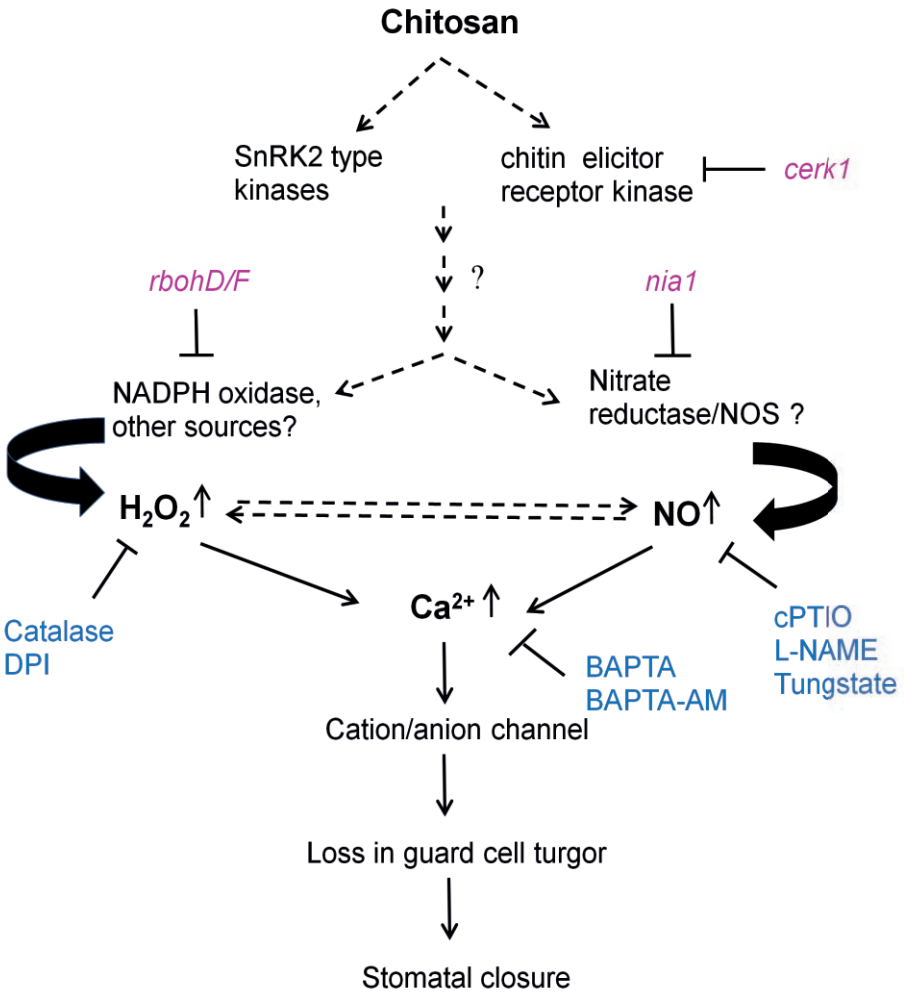

Figure 9.1. Schematic representation the sequence of events in the chitosan signaling cascade leading to stomatal closure. This linear model integrates our resultsfrom the different mutants and the use of inhibitors. There is a rise in ROS or NO in guard cells when exposed to chitosan. Both ROS and NO lead to rise in cytosolic Ca^{2+} and subsequent stomatal closure. These interactions between ROS, NO and Ca^{2+}need further detailed examination. The sequence of changes for which the evidences are either ambiguous or lacking, are indicated by dotted arrows, while the solid arrows represent signaling pathways for which experimental evidence is provided in this work.

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

1. The levels of H_2O_2 and NO rise during stomatal closure induced by chitosan. Therefore, H_2O_2, NO and Ca^{2+} appear to play an important role during the action of chitosan, as in case of ABA. The main sources for H_2O_2 and NO production during chitosan-induced stomatal closure appear to be NADPH oxidase and NR respectively.
2. Besides inducing closure, chitosan inhibits stomatal opening in abaxial epidermis of *P. sativum*. Although the patterns of H_2O_2 and NO during inhibition of opening appear to be similar to that during closure, there was a difference. The source of NO during chitosan mediated inhibition of stomatal opening appeared to be nitric oxide synthase-like enzyme and there is a only partial involvement of nitrate reductase.
3. Among the two mutants of *Arabidopsis*, the NR-deficient *nia1* and NADPH oxidase-deficient *rbohD/F* were insensitive to chitosan or ABA, while stomata of *nia2* closed in response to chitosan/ABA. These results suggest that *nia1* and *rbohD/F*, rather than *nia2*, play a major role during chitosan or ABA induced stomatal closure in *Arabidopsis* stomatal guard cells. The insensitivity of stomata to chitosan in the mutant *cerk1* indicates that *cerk1* could also play an important role in signaling pathway of chitosan-induced stomatal closure.
4. The patterns of chitosan-induced gene transcript accumulation in *Arabidopsis* leaves deficient in NADPH oxidase suggests that *NIA1* expression might not be completely dependent on RBOH (and possibly H_2O_2).The increased levels of *NIA2* transcripts in the *nia1* mutants suggest that *NIA2* expression may compensate for a lack of *NIA1*and

that NO production may not be essential for *NIA1* during chitosan-induced gene transcript accumulation in *Arabidopsis* leaves.

Further work is required to establish the mechanism of action as well as biosynthesis of H_2O_2 or NO in guard cells, after exposure to chitosan. Similarly, the patterns of changes in gene expression induced by chitosan need to be examined in detail.

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Chapter 10
Literature Cited

Chapter 10

Literature Cited

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Appendix

Research Articles Published

By

Nupur

Plant, Cell and Environment (2008) **31**, 1717–1724 doi: 10.1111/j.1365-3040.2008.01872.x

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.

Planta (2009) 229:757–765
DOI 10.1007/s00425-008-0855-5

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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Received: 11 June 2008 / Accepted: 28 October 2008 / Published online: 16 December 2008

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

[Plant Signaling & Behavior 4:6, 561-564; June 2009]; ©2009 Landes Bioscience

Article Addendum

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

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

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

Introduction

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

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

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

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

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Submitted: 04/19/09; Accepted: 04/22/09

Previously published online as a *Plant Signaling & Behavior* E-publication: http://www.landesbioscience.com/journals/psb/article/8847

Addendum to: Gonugunta VK, Srivastava N, Puli MR, Raghavendra AS. Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid. Plant Cell Environ 2008; 31:1717–24; PMID: 18721267; DOI: 10.1111/j.1365-3040.2008.01872.x.

Plant Physiology and Biochemistry 48 (2010) 931–935

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation

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ARTICLE INFO

Article history:
Received 17 June 2010
Accepted 22 September 2010
Available online 1 October 2010

Keywords:
GYY4137
Hydrogen sulfide
NaSH
Nitric oxide
Stomata

ABSTRACT

Effects of hydrogen sulfide (H_2S) on plant physiology have been previously studied, but such studies have relied on the use of NaSH as a method for supplying H_2S to tissues. Now new compounds which give a less severe H_2S shock and a more prolonged exposure to H_2S have been developed. Here the effects of one such compound, GYY4137, has been investigated to determine its effects on stomatal closure in *Arabidopsis thaliana*. It was found that both NaSH and GYY4137 caused stomatal opening in the light and prevented stomatal closure in the dark. Nitric oxide (NO) has been well established as a mediator of stomatal movements and here it was found that both NaSH and GYY4137 reduced the accumulation of NO in guard cells, perhaps suggesting a mode of action for H_2S in this system. GYY4137, and future related compounds, will be important tools to unravel the effects of plant exposure to H_2S and to determine how H_2S may fit into plant cell signalling pathways.

1. Introduction

It is now well established that a variety of reactive chemicals are involved in the control of cellular events in plants. These chemicals include reactive oxygen species [1] such as hydrogen peroxide and reactive nitrogen species such as nitric oxide [2]. Such chemicals may be produced endogenously by the cell itself or may in fact arrive at a plant cell from the outside, perhaps from another cell or tissue. Many of the downstream events modulated by ROS and NO are now well established and include the alteration of the activity of proteins such as kinases, phosphatases and transcription factors [1]. However, other compounds such as carbon monoxide and hydrogen sulfide have also been suggested to have effects on cell signalling pathways [3].

Hydrogen sulfide is often thought to be a phytotoxin, being harmful to the growth and development of plants. It was found to inhibit oxygen release from young seedlings of six rice cultivars [4], but it was also noted that although in some cultivars nutrient uptake was reduced in other cultivars it was increased. Phosphorus uptake was inhibited in this plant species. Thompson and Kats [5] treated a variety of plants with continuous fumigation of H_2S. In Medicago, grapes, lettuce, sugar beets, pine and fir 3000 parts per billion (ppb) H_2S caused lesions on leaves, defoliation and reduced growth of the plants supporting the role of H_2S as a phytotoxin. However lower levels of fumigation, 100ppb, caused a significant increase in the growth of Medicago, lettuce and sugar beets [5]. Quite recently, Zhang et al. [6], showed that the H_2S donor NaSH would alleviate the osmotic-induced decrease in chlorophyll concentration in sweetpotato. Furthermore, spraying NaSH increased the activity of the antioxidant enzymes superoxide dismutase, catalase, ascorbate peroxidase while decreasing the concentration of hydrogen peroxide and lipoxygenase, and it was suggested that H_2S has a role in protection against oxidative stress in plants. Supporting this hypothesis are the findings that fumigation of spinach increased glutathione levels [7], and it was estimated that approximately 40% of the H_2S was converted to glutathione in the leaves. On cessation of fumigation glutathione levels once again fell, with the levels being comparable to control levels after 48 h of no H_2S treatment. Therefore, clearly H_2S can have intracellular effects which impinge on cell signalling events in the cells.

As well as effects on plants, many species of plant have been found to generate H_2S, suggesting that it may be an endogenous chemical and suitable to be acting as signalling molecule. Using

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0981-9428/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2010.09.016