Plant guard cell anion channel SLAC1 regulates stomatal closure

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Abbreviations

ABA	abscisic acid
Ca^{2+}	calcium
C_a	ambient CO_2 concentration
C_i	intracellular CO2 concentartion
CDPK	calcium dependent protein kinase
CPK	calcium dependent protein kinase
Cl^-	chloride
CO_2	carbon dioxide
EMS	ethylmethylsulfonate
ExtCaM	extracellular calmodulin
FTase	farnesyltransferase
H_2O_2	hydrogen peroxide
JA	jasmonic acid
K^+	potassium
K_{in} channel	K^+ inward rectifying channel
K_{out} channel	K^+ outward rectifying channel
MAPK	mitogen-activated protein kinase
MJ	methyl jasmonate
NADPH	nicotinamide adenine dinucleotide phopsphate
NO	nitric oxide
O_2^-	superoxide radical
O_3	ozone
OH^-	hydroxyl radical
PA	phosphatidic acid
PLD	phospholipase D
ROS	reactive oxygen species
RTD	rapid transient decrease in stomatal conductance
R-type	rapid type
S-type	slow type
S1P	sphingosine-1-phosphate
SA	salicylic acid
UV	ultraviolet
V-ATPase	vacuolar-type H^+-ATPase
WT	wild type

Original publications

This thesis is based on three original publications. The publications are referred to in the text by Roman numerals.

I Kollist, T*.; Moldau, H.; Rasulov, B.; Oja, V.; Rämma, H.; Hüve, K.; Jaspers, P.; Kangasjärvi, J.; Kollist, H. (2007). A novel device detects a rapid ozone-induced transient stomatal closure in intact Arabidopsis and its absence in abi2 mutant. Physiologia Plantarum, 129(4), 796–803.
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II Vahisalu, T.; Kollist, H.; Wang, Y-F.; Nishimura, N.; Chan, W-Y; Valerio, G. Lamminmäki, A.; Brosché, M.; Moldau, H.; Desikan, R.; Schroeder, J.I.; Kangasjärvi, J. (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature, 452(7186), 487–491.

III Vahisalu, T.; Puzõrjova, I.; Brosché, M.; Valk, E.; Lepiku, M.; Lindgren, O.; Moldau, H.; Pechter, P.; Wang, Y-S.; Salojärvi, J.; Loog, M.; Kangasjärvi, J.; Kollist, H. (2010). Ozone-triggered rapid stomatal response involves production of reactive oxygen species and is controlled by SLAC1 and OST1. Plant Journal, 62(3), 442–453.

Abstract

Plants are rooted to their growth place; therefore it is important that they react adequately to changes in environmental conditions. Stomatal pores, which are formed of a pair of guard cells in leaf epidermis, regulate plant gas-exchange. Importantly, guard cells protect the plant from desiccation in drought conditions by reducing the aperture of the stomatal pore. They serve also as the first barrier against the major air pollutant – ozone, but the behaviour of guard cells during ozone exposure has not been sufficiently addressed. Aperture of the stomatal pore is regulated by the influx and efflux of osmotically active ions via ion channels and transporters across the guard cell membrane, however the molecular identity of guard cell plasma membrane anion channel has remained unknown.

In the frame of this study, guard cell behaviour during ozone exposure was studied using the newly constructed *Arabidopsis* whole-rosette gas-exchange system. Ozone induced a Rapid Transient Decrease (RTD) in stomatal conductance within 10 min from the start of exposure, which was followed by a recovery in the conductance within the next 40 min. The decrease in stomatal conductance was dependent on the applied ozone concentration. Three minutes of ozone exposure was sufficient to induce RTD and further ozone application during the closure-recovery process had no effect on RTD, demonstrating that the whole process is programmed within the first three minutes. To address the molecular components responsible for RTD, the ozone response was measured in 59 different *Arabidopsis* mutants involved in guard cell signalling. Four of the tested mutants – *slac1* (originally *rcd3*), *ost1*, *abi1-1* and *abi2-1* lacked RTD completely.

As the ozone sensitive mutant *slac1* lacked RTD, the next aim of this study was to identify and characterize SLAC1. SLAC1 was shown to be a central regulator in response to all major factors regulating guard cell aperture: CO_2, light/darkness transitions, ozone, relative air humidity, ABA, NO, H_2O_2, and extracellular Ca^{2+}. It encodes the first guard cell plasma membrane slow type anion channel to be identified at the molecular level. Interestingly, the rapid type anion conductance was intact in *slac1* mutant plants.

For activation, SLAC1 needs to be phosphorylated. Protein kinase OST1 was shown to phosphorylate several amino acids in the N-terminal tail of SLAC1, Ser120 was one of its main targets, which led to SLAC1 activation. The lack of RTD in type 2C protein phosphatase mutants *abi1-1* and *abi2-1*, suggests that these proteins have a regulatory role in ozone-induced activation of the slow type anion channel.

1. Introduction

Stomatal pores on the leaf epidermis regulate the flow of gases in and out of plants and they are formed of a pair of guard cells surrounding the pore (Figure 1). Most importantly stomata control the exchange of water vapour and carbon dioxide (CO_2) between the leaf interior and the atmosphere, thus controlling the water and carbon cycles in the world. They also restrict the entry of air pollutants, for example ozone (O_3), which has an increasingly negative impact on crop yields and natural vegetation. Importantly, guard cells play a crucial role in regulating the plant water status – in drought conditions stomatal aperture is reduced, thereby protecting the plant from transpirational water loss. As they play an important role in global carbon fixation and atmospheric water cycles, they assist also global climate change. Different environmental factors like light intensity and quality, water content in the soil and air, CO_2 and pollutants content in the air regulate stomatal opening and closure. In response to those factors plants produce different hormones and second messengers, which influence different aspects of plant physiology, including stomatal aperture. The aperture of stomatal pore is regulated by the turgor pressure of guard cells. As mature guard cells lack plasmodesmata, the uptake and efflux of osmotically active ions and metabolites takes place through ion channels and transporters in guard cell membranes. During stomatal opening, the increase in osmotically active solutes leads to water influx and increase in turgor pressure. On the contrary, during stomatal closure osmotically active ions flow out, this leads to water efflux and reduction in turgor. Potassium (K^+), chloride (Cl^-) and malate are the main osmotically active solutes. Guard cells have an asymmetric structure – their cell walls in parallel with each other are thicker, therefore when guard cell turgor increases, they bend and between the two cells the stomatal pore is formed (Figure 1). Guard cell chloroplasts are considerably smaller and play a different role compared to the mesophyll cells – they are considered to be important in signalling.

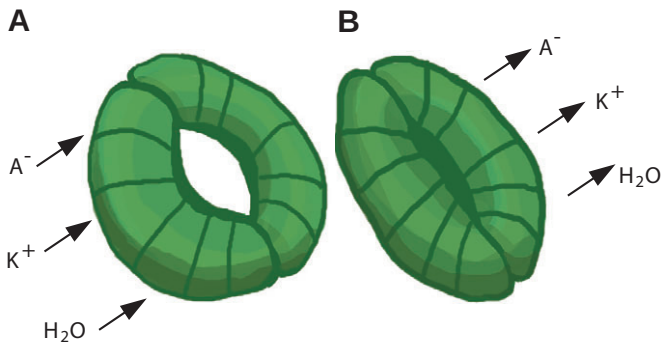

Figure 1 Stomatal opening and closure.

The aperture of the stomatal pore is regulated by the turgor pressure of guard cells. During stomatal opening (left) the osmotically active solutes and water flow in and during stomatal closure they flow out (right) across the guard cell plasma membrane.

The chapters of the introductory part of this thesis aim to give a short overview of guard cell regulation in response to different environmental factors, the role of different hormones and second messengers in guard cell signalling and different ion channels and transporters regulating the aperture of stomatal pore. The role of different *Arabidopsis thaliana* (*Arabidopsis*) genes and proteins in guard cell regulation will be addressed. This thesis work focused on studying guard cell regulation in response to O_3 and the involvement of different guard cell specific proteins in response to O_3 was addressed in more detail. The model plant *Arabidopsis* was used throughout the study. The role of guard cell anion channels in controlling the stomatal aperture was studied. Finally, a model about anion channel regulation in response to O_3 was proposed.

1. 1 Factors regulating stomatal opening and closure

1.1.1 Light

Light quality and quantity affects plants physiology and development at all levels. Plants depend on light for photosynthesis and use it as an informational cue in several processes including stomatal regulation and seed germination, stem elongation, leaf expansion, dormancy, blooming, phototropism and senescence. Guard cells sense light and induce stomata to open in order to allow CO_2 influx, which is required for photosynthesis. Vice versa, darkness induces stomata to close. Stomatal opening is dependent on wavelength and blue light is most efficient in driving stomatal opening (Vavasseur and Raghavendra, 2005). Light is sensed by different photoreceptors. Phytochromes recognize light in the red portion of the spectrum, while cryptochromes and phototropins perceive blue and UV-A light. Zeaxanthin, which is a carotenoid in chloroplasts and implicated in dissipation of excess light, has a role in blue light sensing.

Plants have a two-phase mechanism, which contributes to guard cell swelling during the day. In the morning phase, guard cell opening is mostly correlated with K^+ uptake, while during the afternoon phase K^+ content declines and sucrose becomes the dominant osmoticum (Talbott and Zeiger, 1996). Red light activates guard cell photosynthesis and stimulates sucrose accumulation in the absence of K^+ uptake, whereas blue light stimulates K^+ and Cl^- uptake, malate synthesis and starch hydrolysis (Zeiger et al., 2002). Red light induced stomatal opening is mediated through a decrease of in the intercellular CO_2 concentration, which inhibits guard cell anion channels (Roelfsema et al., 2002). Green light reverses the processes established by red and/or blue light, having a negative effect on stomatal opening; it may be functioning in a manner similar to far-red light, informing the plant of photosynthetically unfavourable conditions (Folta et al., 2007).

Blue light in guard cells is perceived by phototropins, cryptochrome and zeaxanthin (Kinoshita et al., 2001; Talbott et al., 2003a; Mao et al., 2005). Its perception through phototropins PHOT1 and PHOT2 activates plasma membrane H^+-ATPase, which creates an inside-negative electrical potential across the plasma membrane (Assmann et al., 1985;

Shimazaki et al., 1986) and drives the uptake of K^+ through inward-rectifying voltage-gated K^+ channels (Schroeder et al., 1987; Assmann and Shimazaki, 1999; Schroeder et al., 2001a; Roelfsema et al., 2001).

PHOT1 and PHOT2 are plant specific serine/threonine protein kinases with two LOV (Light, Oxygen, or Voltage) domains. The light-stimulated phototropins undergo autophosphorylation and regulate the downstream signalling leading to H^+-ATPase activation (Kinoshita et al., 2001, Kinoshita et al., 2003). Type 1 protein phosphatase is activated upon blue light illumination, positively regulating the blue light signal between phototropins and the H^+-ATPase. However, the exact mechanism from phototropins to H^+-ATPase activation is largely unknown (Takemiya et al., 2006; Hayashi et al., 2010). It is known that the C terminus of the H^+-ATPase is phosphorylated, with a subsequent binding of 14-3-3 protein. This leads to activation of the H^+-ATPase and stimulation of downstream events in stomatal guard cells (Kinoshita et al., 1999: Kinoshita et al., 2002). H^+-ATPase activation results in membrane hyperpolarisation, which cannot be achieved without inactivation of anion channels. Blue light has been shown to inhibit the slowly activating (S-type) anion channels further contributing to membrane hyperpolarisation (Marten et al., 2007). Light-induced stomatal opening is inhibited by abscisic acid (ABA) via inhibition of H^+ ATPase activation by phosphorylation (Zhang et al., 2004). In addition to phototropins, the cryptochromes CRY1 and CRY2 function additively with PHOT1 and PHOT2 and regulate the blue light induced stomatal opening (Lin et al., 1998; Mao et al., 2005).

Zeaxanthin is a chloroplastic carotenoid, which contributes to blue light response. Its levels in guard cells are positively correlated with photosynthetically active radiation and stomatal opening (Srivastava and Zeiger, 1995). Furthermore, NON-PHOTOCHEMICAL QUENCHING1 (NPQ1), which converts violaxanthin to zeaxanthin, has a role in blue light sensing (Talbott et al., 2003).

Generally, cytosolic Ca^{2+} elevations are known to contribute to stomatal closure via inhibition of the guard cell H^+ ATPase (Kinoshita et al., 1995). Interestingly, a positive role for Ca^{2+} in blue light signalling has been reported. Patch-clamp assay of Ca^{2+} permeable channels has shown that blue light triggers Ca^{2+} fluxes via the phototropin-activated Ca^{2+}-permeable channel (Stoelze et al., 2003; Harada et al., 2003). Ca^{2+} increase is dependent on membrane hyperpolarization and takes place after H^+ ATPase activation (Harada et al., 2009).

1.1.2 Carbon dioxide

Plants use CO_2 and energy from sunlight during photosynthesis for making organic matter, the basis of all animal life. Guard cells allow CO_2 influx in exchange for transpirational water loss from plants to the atmosphere. The CO_2 concentration in the air has an influence on stomatal aperture - elevated CO_2 stimulates stomatal closure, while reduced CO_2 concentrations have the opposite effect (Assmann, 1993).

The continuing rise in atmospheric CO_2 causes reduction in stomatal apertures across diverse

plant species influencing water use efficiency and leaf heat stress (Hu et al., 2010). On the other hand, elevated CO_2 enhances plant growth rate, maturation and biomass production. Changes in stomatal aperture have an effect also on global climate in addition to elevated leaf temperatures. Transpiration through stomata acts as a cooling system both for plant leaves and also as a global air conditioner. Decreasing such a system brings about both leaf temperature and global temperature elevation. Thus, guard cells play an important part in global warming. CO_2 elevation could lead to changes in biodiversity, for example there are differences in growth rates and reproductive success giving advantage to some species over the others. In some species CO_2 elevation causes increase in reproductive biomass and seed number, in others decrease in reproductive biomass or no response at all (LaDeau and Clark, 2001).

The understanding of guard cell reactions to changes in CO_2 concentration at the molecular level is still incomplete. It has been shown that guard cells sense the intercellular CO_2 concentration (C_i), not the external concentration or the concentration within the pore (Mott, 1988; Mott, 1990). There are also day/night changes in leaf tissue CO_2 concentrations, which are caused by photosynthesis and respiration. Since C_i is influenced both by mesophyll photosynthesis and by ambient CO_2 concentrations (C_a), CO_2 is both an endogenous and an exogenous signal. C_i, regulated by photosynthesis, can also contribute to red light induced stomatal opening via a decrease in C_i (Roelfsema et al., 2002). In addition to photosynthesis dependent CO_2 response, guard cells are proposed to sense CO_2 also independent of photosynthesis (Messinger et al., 2006). A photosynthesis independent pathway has been supported experimentally as chlorophyll deficient albino *Vicia faba* leaves can sense CO_2 changes and respond to blue light but lack the photosynthesis dependent red light-induced stomatal opening (Roelfsema et al., 2006).

The involvement of second messengers and ion channels in guard cell CO_2 response has been studied. CO_2 elevation induces cytosolic Ca^{2+} rise (Webb et al., 1996; Young et al., 2006), which activates the S-type anion channels, such as SLAC1 (publication II) and the quickly activating (rapid type/ R-type) anion conductance (Raschke et al., 2003). Anion channel activation leads to K^+ outward rectifying channel activation (Brearley et al., 1997). Additionally, the second messenger H_2O_2 is produced during elevated CO_2-induced stomatal closure in guard cells (Kolla et al., 2007).

So far only a few *Arabidopsis* genes that are responsible for guard cell CO_2 signalling response have been identified. The first recessive CO_2-insensitive mutant to be identified was *gca2* (*growth controlled by abscisic acid 2*). *gca2* is also insensitive to ABA and impaired in the regulation of CO_2-induced cytosolic Ca^{2+} pattern (Young et al., 2006). Different protein kinases are involved in guard cell CO_2 signalling. The kinase HT1 (HIGH LEAF TEMPERATURE1) and *Nicotiana tabacum* MAP kinase NtMPK4 are responsible for guard cell CO_2 responses, but are not involved in ABA signalling. Thus, they are proposed to function upstream of the convergence point of ABA and CO_2 signalling. HT1 was suggested

to function as a negative regulator of CO_2-induced stomatal closure. NtMPK4 is a positive regulator in guard cell CO_2 signalling as it is responsible for elevated CO_2 induced stomatal closure and anion channel activation (Hashimoto et al., 2006; Marten et al., 2008).

Ion channels are essential in regulating the stomatal aperture. The SLAC1 anion channel and the ABC transporter AtABCB14 are engaged in guard cell CO_2 responses; SLAC1 is a positive regulator in elevated CO_2-induced stomatal closure as mutation in the gene abolishes elevated CO_2-induced stomatal closure. AtABCB14 modulates stomatal opening by transporting malate from the apoplast into the guard cells, thereby increasing their osmotic pressure. *AtABCB14* mutant plants have a faster high CO_2-induced stomatal closure response when compared to the WT (Negi et al., 2008; publication II; Lee et al., 2008).

Carbonic anhydrase proteins catalyse the reversible reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Carbonic dioxide binding carbonic anhydrases are proposed to function early in guard cell CO_2 signalling and are implicated in CO_2-induced stomatal closure but not in light or ABA reactions. As carbonic anhydrases change water use efficiency, their modulation may provide an approach for engineering gas-exchange and transpirational water loss or protection against heat-induced damage of plants (Hu et al., 2010).

1.1.3 Water in the soil and air humidity

Water has a major influence on plant physiology, its content both in the soil and in the air influences plant physiology, influencing guard cell regulation and stomatal opening. Lack of water in the soil induces stomata to close in order to prevent plants from wilting.

Fresh water scarcity is one of the principal problems of the 21st century and plants account for around 65% of global fresh water use. Desiccation of crops and horticultural plants during drought periods causes severe damage and yield losses (Schroeder et al., 2001). Plant water deficit occurs when the rate of transpiration exceeds water uptake from soil, which is a component of several different stresses – drought, salinity and low temperature. After the perception of water stress, signalling mechanisms are activated to induce specific genes. One of the major signals during drought stress is the plant hormone ABA. ABA is involved also in other abiotic stresses like salinity, freezing and chilling (Bray, 1997). Gene expression studies have revealed different clusters of genes, which are induced during drought stress, thus plants have different mechanisms to avoid water deficit (Bray, 2004). Under water stress, guard cells display a short-term response based on osmoregulation and a long-term response involving modification of major metabolic enzymes due to alterations in guard cell gene expression (Vavasseur and Raghavendra, 2005). ABA accumulation in guard cells induces rapid stomatal closure and prevents water loss by transpiration via triggering a signalling cascade that causes the efflux of K^+ and anions, sucrose removal and the conversion of malate to osmotically active starch, resulting in turgor reduction (MacRobbie 1998). The ABA-signalling network is complex and the abundance of ABA-signalling components in guard cells may reflect the importance of stomatal closure in plant survival (Assmann and Shimazaki, 1999). Guard cell

ABA signalling is discussed in more detail in the following chapters.

The relative humidity of air influences stomatal opening. Reduction in ambient humidity causes stomatal closure and an increase drives stomata to open. Of all the stomatal responses, the humidity response is probably the least understood. The challenge has been to define which humidity parameter guard cells actually sense. Are guard cells sensing relative humidity, which is related to the chemical activity of water in the atmosphere, vapour pressure difference, which is the gradient in vapour pressure from the leaf to the atmosphere or transpirational water loss, which is the actual rate of water loss from the leaf (Assmann, 1993)? Current evidence supports the hypothesis that stomata do not detect alterations in relative humidity as such, but rather perceive the change in transpiration rate (Xie et al., 2006). Stomatal closure in response to reduction in relative humidity occurs very rapidly. Despite its role in stomatal movements, molecular components of the humidity-signalling cascade are largely unknown. ABA dependent and independent signalling pathways have been proposed. Using the ABA biosynthesis mutant *aba1* and ABA signalling mutants *abi1-1* and *abi2-1*, has shown that ABA is not involved in the guard cell humidity response (Assmann et al., 2000). In contrast, Xie et al. (2006) have suggested that ABA plays a role in the signal transduction pathway connecting decreases in relative humidity to reductions in stomatal aperture. They isolated two genes as positive modulators in relative humidity signalling pathway – protein kinase OST1, which is involved in ABA-mediated stomatal closure and ABA2, which encodes an enzyme involved in ABA biosynthesis. Additionally, the SLAC1 anion channel, which is regulated by OST1, mediates guard cell rapid responses to decreased relative air humidity (publication II; publication III).

Relative humidity is also a key environmental factor mediating the changes in stomatal sensitivity to CO_2, elevation of relative humidity results in enhanced CO_2 response (Talbott et al., 2003b). Nevertheless, the early humidity sensing mechanisms remain largely unknown.

1.1.4 Ozone

O_3 is the trimolecular allotrope of oxygen. Its function in the atmosphere is contradictory – stratospheric O_3 shields biologically harmful UV-B radiation from reaching the earth's surface, whereas tropospheric O_3 is toxic to living organisms. The phytotoxicity of O_3 was discovered in the 1950-s (Richards et al., 1958), which prompted widespread studies on the effect of O_3 on plants (Kley et al., 1999; Rao and Davis 2001). Nowadays O_3 is the most widespread air pollutant in the industrial areas causing severe damage both on crop plants (Krupa et al., 1994) and natural communities (Hogsett et al., 1997) having a negative effect on global carbon fixation (Hopkin 2007). The damage depends on the concentration and duration of O_3 exposure.

Background O_3 concentrations have doubled during the last 100 years (Campbell, 1996). Increased O_3 concentration in troposphere is caused mainly by traffic and industry. Nitrous oxides (NO_x), produced by factories and cars react with oxygen in the presence of heat and

sunlight and as a result O_3 is formed. Increasing problems caused by elevated tropospheric O_3 concentrations are expected in the future, particularly in developing countries (Sitch et al., 2007).

When O_3 concentrations, even for a short time, exceed the tolerance threshold of a given plant, the formation of visible damage and cell death may occur. The plant's response to O_3 resembles the biotic defence response and includes a biphasic oxidative burst (Schraudner et al., 1998) and induction of the hypersensitive response and systemic acquired resistance (Conklin and Last, 1995; Sharma et al., 1996; Sandermann et al., 1998; Rao et al., 2000; Sandermann, 2000). Because the O_3 response is similar to other biotic and abiotic elicitors, O_3 has become established as a model system for studying the role of ROS in cell death regulation (Allan and Fluhr, 1997; Rao and Davis 2001). From an experimental point of view, the gaseous nature of O_3 allows precise control of its concentration and duration of application.

O_3 enters the plant through open stomata, where it rapidly reacts with the cell wall and plasma membrane and degrades into ROS, such as hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-) and hydroxyl radicals (OH^-) (Mehlhorn et al., 1990, Moldau et al., 1998). Laisk, Moldau and Kull (1989) established that O_3 concentration in intercellular spaces is extremely low irrespective of the concentration outside the leaf, suggesting that O_3 degradation into ROS, and scavenging by antioxidants is very rapid.

The question of what determines O_3-sensitivity or tolerance in various plant species or genotypes has been one of the central topics in O_3 research. First of all, the rate of O_3 influx into the plant leaf may determine subsequent O_3 responses. Thus, the number and size of stomata and the stage of their opening, i.e. stomatal conductance, affect O_3 uptake (Rich et al., 1970). The O_3-sensitivity of different *Arabidopsis* ecotypes is correlated with their steady state stomatal opening (Brosché et al., 2010).

O_3 induces stomatal closure and also reduction in photosynthesis (Hill and Littlefield, 1969); hence the ability to close stomata in response to changes in environmental conditions is correlated with O_3 sensitivity (Grulke et al., 2006). High air humidity may affect O_3 sensitivity through increased stomatal opening and higher O_3 influx into the plant. Accordingly, it has been shown that drought stress may protect from O_3-induced foliar damage, possibly due to decrease in stomatal conductance and reduced influx of O_3 into plant cells (Robinson et al., 1998). There is much interest how stomatal responses might influence plant responses to the combined effects of O_3 pollution and atmospheric CO_2 enrichment. It has been hypothesized that as elevated CO_2 concentrations can cause partial stomatal closure, it is possible that increasing CO_2 would provide protection against O_3 damage by reducing the dose of O_3 entering the leaf (Robinson et al., 1998). Recently it has been demonstrated that elevated CO_2 can only temporarily compensate for elevated O_3 (Kontunen-Soppela et al., 2010).

The molecular identities of signalling components involved in guard cell O_3 signalling have been described. For example mitogen-activated protein kinase (MAPK) NtMPK4-silenced plants, *ost1*, *abi1-1*, *abi2-1* and *slac1* mutant plants have enhanced sensitivity to O_3, which

is caused by higher stomatal conductance and an aberrant regulation of stomatal closure in response to O_3 (Gomi et al., 2005; publication II; publication III). This indicates the involvement of protein phosphorylation/dephosphorylation events and anion channels in guard cell O_3 response. O_3 exposure induces a rapid reactive oxygen species (ROS) production in guard cells, which arises primarily from chloroplasts (Joo et al., 2005; publication III). Heterotrimeric Gα and β proteins are involved in the activation of choroplastic ROS in guard cells, while the spread of ROS production to adjacent cells is governed by membrane-bound AtRBOHD and AtRBOHF NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (Joo et al., 2005). O_3 perception and initiation of downstream signalling is still an unsolved question.

1.1.5 Hormones

Different plant hormones affect various aspects of plant physiology from germination to senescence including responses to different environmental stressors. The involvement of hormones, primarily ABA, in guard cell regulation is well established.

1.1.5.1 Abscisic acid

ABA is a plant hormone that controls important developmental and abiotic stress responses, including seed dormancy, seed development, growth regulation and stomatal closure. It was identified in plants in 1960s (Ohkuma et al., 1963). The role of ABA in guard cell signalling is among the foremost characterized plant signalling pathways. Recent major breakthroughs include the identification of ABA receptors and their molecular mechanism and structure. ABA has a central role in protecting plant against water deficiency by regulating the stomatal aperture and minimizing water loss through stomata.

In response to water deficiency, enhanced biosynthesis of ABA occurs, foliar ABA moves to apoplast and accumulates preferentially in guard cells where its concentration is increased up to 30-fold (Outlaw, 2003). ABA accumulation induces stomatal closure and differential gene expression. Furthermore, it inhibits light-induced stomatal opening to prevent water loss by transpiration (Garcia-Mata and Lamattina, 2007; Schroeder et al., 2001b). Stomatal closure is detectable within about 5 min after ABA application (Pei et al., 2005) and such rapid events are linked to early signalling, rather than to changes in gene expression.

Considerable amount of information has been acquired about downstream signalling network of ABA (Yamaguchi-Shinozaki and Shinozaki, 2006) and a number of signalling components have been isolated. Briefly, ABA triggers a signalling cascade that ultimately causes the efflux of K^+ and Cl^- and the removal of organic osmolytes, thereby reducing cellular turgor (MacRobbie, 1998; Bray, 1997; Schroeder et al., 2001a).

The second messengers ROS, nitric oxide (NO) and Ca^{2+} are known components regulating the stomatal aperture in the ABA signalling network. ABA induces a rapid burst of H_2O_2 production, which promotes stomatal closure (Pei et al., 2000; Murata et al. 2001; Zhang et al., 2001). Different studies have reported changes in cytosolic pH to take place both before

and after ROS production (Xiao et al., 2001a; Suhita et al., 2004). NO is produced and acts downstream of ROS in ABA-induced stomatal closure (Neill et al., 2002; Garcia-Mata and Lamattina, 2002; Desikan et al., 2002; Bright et al., 2006). ABA-induced increase in cytosolic Ca^{2+} in guard cells activates S-type anion channels in the plasma membrane. This leads to activation of outward rectifying K^+ channels and inhibits inward K^+ channels and plasma membrane H^+-ATPases, thereby regulating the stomatal aperture (McAinsh et al., 1990; Schroeder and Hagiwara, 1989; Pei et al., 1997; Li et al., 2000; Schroeder, 2001a; Kinoshita et al., 1995). SLAC1 anion channel and protein kinase OST2 (OPEN STOMATA 2), have been identified to act as central components in ABA-induced stomatal closure (publication II, Negi et al., 2008; Merlot et al., 2007). ABA-induced Ca^{2+} elevation is achieved both by Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular space (Schroeder and Hagiwara, 1990; MacRobbie, 2000; Pei et al., 2000). ABA induces H_2O_2 production in guard cells, which activates plasma membrane Ca^{2+}-permeable channels. This process is regulated by AtRBOHD and AtRBOHF NADPH oxidases (Pei et al., 2000; Kwak et al., 2003). Interestingly, both Ca^{2+} dependent and independent pathways mediate ABA responses (reviewed in Israelsson et al. 2006).

In addition to inducing stomatal closure, ABA also inhibits stomatal opening. The effect of ABA on stomatal closure and opening are genetically separable (Wang, et al., 2001). ABA-induced raise in cytosolic Ca^{2+} inhibits H^+-ATPase pumping and K^+ inward rectifying channels (K_{in} channels) (Schroeder and Hagiwara, 1989; Wang et al., 1998; Kinoshita et al., 1995), thereby preventing an increase in guard cell turgor and stomatal opening. Later it has been demonstrated that both ROS and NO contribute to ABA-induced inhibition of stomatal opening (Garcia-Mata and Lammatina 2007; Yan et al., 2007). The role of Ca^{2+}, ROS and NO in guard cell signalling will be discussed in more detail below.

Moreover, sphingosine-1-phosphate (S1P), phospholipase D (PLD), phosphatidic acid (PA) and farnesyltransferases function as second messengers in the guard cell ABA signal transduction pathway (Pei et al., 1998; Ng et al, 2001; Wang, 2005). S1P is a Ca^{2+}-mobilizing molecule in plants; its levels increase in response to drought and it is involved in ABA promotion of stomatal closure and inhibition of stomatal opening (Ng et al, 2001). It is proposed to act upstream of G proteins and downstream of ABA (Coursol et al., 2003). Phospholipase D (PLD) hydrolyzes membrane lipids to generate phosphatidic acid (PA), a lipid-signalling mediator (Wang, 2005).

PA binds to ABI1 thereby decreasing its activity as a negative regulator in ABA signalling (Zhang et al. 2004). PLDa1 and PA interact with the heterotrimeric G protein and mediate ABA inhibition of stomatal opening (Mishra et al., 2006). Farnesyltransferases position upstream of cytosolic Ca^{2+} in the guard cell ABA signalling cascade, regulating S-type anion current activation and stomatal closure (Cutler et al., 1996; Pei et al., 1998; Allen et al., 2002).

Heterotrimeric GTP binding (G) proteins consist of three subunits: α, β and γ, which are

important communicators in guard cell ABA signalling. G protein α mediates ABA inhibition of guard cell K_{in} channels and activation of anion channels (Wang et al., 2001). G protein–coupled receptors (GPCRs) interact with G protein α (GPA1) subunit and regulate GPA1-mediated responses negatively (Pandey et al., 2004).

Protein phosphorylation/dephosphorylation events play an important role in ABA-induced stomatal movements (Schroeder et al., 2001). Several different classes of protein kinases and phosphatases have been identified to regulate guard cell ABA signalling. The SnRK2 type protein kinase OST1/SRK2e was identified as a key positive regulator in ABA signal transduction in guard cells (Mustilli et al., 2002; Yoshida et al., 2002). ABA-activated protein kinase (AAPK), calcium dependent protein kinases CPK3, CPK4, CPK6 and CPK11, mitogen-activated protein kinases MPK3, MPK9 and MPK12 all mediate ABA signalling in guard cells (Li et al., 2000; Mori et al., 2006; Zhu et al., 2007; Jammes et al., 2009; Gudesblat et al., 2006). Protein phosphatase type 2C enzymes ABI1, ABI2 and HAB1 are all negative regulators of ABA signalling (Leung et al., 1994; Merlot et al., 2001; Rodriguez et al., 1998; Saez et al., 2004). In contrast, the protein phosphatase type 2A (PP2A) RCN1 functions as a general positive regulator of ABA signalling (Kwak et al., 2002).

It has been proposed that ABA can be perceived both inside and outside the cell, thus multiple ABA receptors have been proposed to exist (Bray et al., 1997).

The existence of cytosolic ABA receptors was recently confirmed by the discovery of the PYR/PYL/RCAR proteins (Ma et al., 2009, Park et al., 2009). This protein family consists of 14 START domain proteins in *Arabidopsis* showing dual cytosolic and nuclear localisation. There have been multiple reports over the past years that have claimed identification of the receptor for the plant hormone ABA. Although it remains possible that there are multiple types of ABA receptors, the recent work presents a major breakthrough in our understanding of hormonal regulation of plant growth and development. After the identification ABA PYR/PYL/RCAR receptors, the crystal structure was reported by several groups (Melcher et al., 2009; Miyazono et al, 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009). ABA binds to the receptor cavity and the loops surrounding the cavity fold over the ABA molecule bringing about conformational changes of the receptor. These changes are required for direct interactions with PP2C protein phosphatases (Santiago et al., 2009, Nishimura et al., 2009; Yin et al., 2009). ABA-bound PYR/PYL/RCAR receptor tightly interacts with PP2C, forming a heterodimer and inhibiting phophatase activity (Miyazono et al., 2009; Yin et al., 2009; Melcher et al. 2009; Ma et al., 2009; Park et al., 2009; Nishimura et al., 2009). A model has been drawn where in the absence of ABA PP2Cs are active and inactivate SnRKs by dephosphorylation. In the presence of ABA a tertiary complex between the hormone, PYR/PYL/RCAR receptors and PP2Cs forms, which in turn releases SnRK2s. Activation of SnRK2s leads to the stimulation of downstream signalling, for example transcription activation or SLAC1 anion channel activation (Figure 2) (Umezawa et al., 2009; Fujii et al. 2009; Klinger et al., 2010).

OST1 has been demonstrated to activate SLAC1 by phosphorylation, while ABI1 and ABI2 inhibit OST1 activity (Geiger et al., 2009; Lee et al., 2009; publication III). Furthermore it has been demonstrated that PYR1 is able to control SLAC1 phosphorylation status in an ABA dependent manner. In the presence of ABA PYR1 interacts with PP2C, which disrupts the interaction between PP2C and OST1, leading to OST1 activation and SLAC1 phosphorylation (Figure 2) (Klinger et al., 2010). This demonstrates that PYR/PYL/RCAR proteins control the most immediate responses to stress signals mediated by ABA.

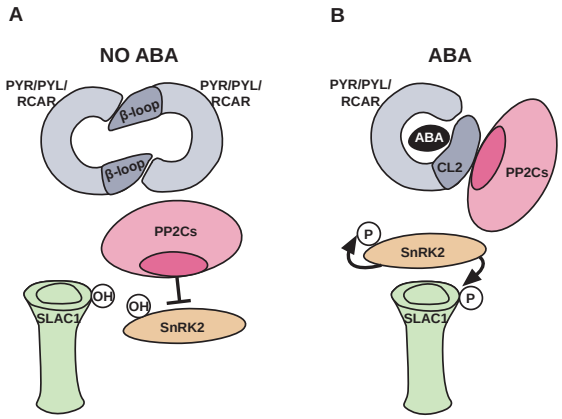

Figure 2 Schematic view of ABA signalling.

In the absence of ABA (A), the PYR/PYL/RCAR receptors form a homodimer and conformation of the CL2 loop (β-loop) in the receptors is incompatible to bind PP2Cs. The PP2Cs inhibit both the autophosphorylation of SnRK2 and phosphorylation of SLAC1 (A). In the presence of ABA (B), the β-loop folds over ABA and the resulting conformational change of the receptor allows the active site of PP2C (indicated in dark purple) to bind the receptor thereby inhibiting PP2C activity. This permits autophosphorylation of SnRK2 and phosphorylation of SLAC1 (B).

In addition to the PYR/PYL/RCAR proteins, two homologous G proteins, GTG1 and GTG2 have been characterised as plasma membrane-localised ABA receptors (Pandey, 2009) giving further evidence for the diversity of ABA receptors.

Conclusively, numerous molecular players of guard cell and ABA signalling have been identified, which suggest a complex ABA signalling network in guard cells. It seems possible that some mechanisms are central mediators of ABA signalling and other mechanisms are peripheral modulators. Recent findings have simplified the process of ABA signalling to just four steps from ABA perception to regulation of gene expression or SLAC1 anion channel activation.

1.1.5.2 Ethylene

The plant hormone ethylene influences many aspects of plant growth and development, including fruit ripening, senescence, seed germination, seedling growth and abscission, as well as mediating stress and pathogen defence responses (Guo and Ecker, 2004; Schaller and Kieber, 2002). Despite the well-known association between ethylene and stress signalling, its effects on stomatal movements have been studied only relatively recently.

Ethylene is perceived by the ethylene receptor (ETR1), which is localized in guard cell endoplasmic reticulum. Its signalling in guard cells has been linked with the widespread signalling molecule H_2O_2. Thus ETR1 mediates both ethylene- and H_2O_2-induced stomatal closure via NADPH oxidase AtRBOHF dependent H_2O_2 production (Desikan et al., 2005; Desikan et al., 2006). Though, it should be noted that ETR1 localisation in the endoplasmic reticulum makes its connection with NADPH oxidases complicated. ETR1 has been shown to interact with EIN2 (ethylene insensitive 2) thereby mediating ethylene and H_2O_2 signalling in guard cells (Desikan et al., 2006; Bisson et al., 2009). In contrast to its role in H_2O_2 signalling, several *etr1* alleles behaved as WT in response to O_3 (publication III). Interestingly, ethylene is also able to counteract stomatal closure by inhibiting the ABA signalling pathway (Tanaka et al., 2005). The dual functions of ethylene receptors proposed in those studies — transduction of stomatal closure and inhibition of ABA-induced stomatal closure in *Arabidopsis* — will require further analyses (Kwak et al., 2006).

1.1.5.3 Methyl jasmonate

Methyl jasmonate (MJ) is a linolenic acid derivate, known to regulate plant development, fruit ripening and various plant defence responses including response to drought stress (Creelman and Mullet, 1997). MJ also regulates guard cell aperture by inhibiting stomatal opening and inducing stomatal closure (Raghavendra and Reddy, 1987). MJ- and ABA-induced stomatal closure comprise of similar events – cytosolic alkalization, NADPH oxidase dependent H_2O_2 production, NO production, activation of S-type ion channels, Ca^{2+} channels and K^+ outward rectifying channels (K_{out}). Still, the two hormones are sensed separately as MJ insensitive mutants *jar1-1* and *coi1* (*coronatine-insensitive 1*) react to ABA while ABA insensitive mutant *ost1-2* reacts to MJ (Evans et al., 2003; Suhita et al., 2004; Munemasa et al., 2007).

1.1.5.4 Salicylic acid

Salicylic acid (SA) is a plant hormone known to mediate the induction of systemic acquired resistance (Gaffney et al., 1993), but can induce also stomatal closure and inhibit stomatal opening (Manthe et al., 1992). During SA signalling H_2O_2 accumulation occurs in guard cells, possibly through SA inhibition of catalase activity, leading to stomatal closure (Chen et al., 1993; Joon-Sang 1998; Mori et al., 2001).

1.1.6 Second messengers

Different second messengers, like Ca^{2+}, ROS and NO are crucial mediators in guard cell regulation in response to different factors regulating the stomatal aperture.

1.1.6.1 Calcium

Ca^{2+} is a ubiquitous signal in plants and no other single messenger has been demonstrated to respond to more stimuli than cytosolic free Ca^{2+}. Changes in cytosolic Ca^{2+} concentration take place in response to a wide variety of abiotic and biotic signals - light, heat shock, cold, touching, nodulation factors, salinity, drought, ABA, gibberellins, oxidative stress and fungal elicitors (Sanders et al., 1999; Sanders et al., 2002). Ca^{2+} is also a central regulator in guard cell signalling in response to versatile stimuli like ABA, ROS, NO, CO_2, darkness, cold and extracellular Ca^{2+} (McAinsh et al., 1995; McAnish et al., 1996; Allen et al., 2000; Young et al., 2006; Marten et al., 2008).

Ca^{2+} is stored both in extra- and intracellular stores. Inside the cell, the vacuole is the most prominent Ca^{2+} storage place, but its concentration is higher than in the cytosol also in the endoplasmic reticulum, mitochondria and chloroplasts. It should be noted that most of the Ca^{2+} in the cytosol is rapidly bound by different proteins, primarily by calmodulin. Therefore the Ca^{2+} content in the cytosol is considerably higher than the cytosolic free Ca^{2} (Sanders et al., 1999). For Ca^{2+} homeostasis, Ca^{2+} is transported out of the cytosol, which is mediated by ATP powered pumps. The entry of Ca^{2+} into the cytosol is mediated by ion channels (Schroeder and Keller, 1992; Sanders et al., 1999). Thus the unequal Ca^{2+} distribution in the cell makes rapid Ca^{2+} concentration changes and fluxes possible (Kudla et al., 2010).

Different effectors can induce an elevation in cytosolic Ca^{2+} in guard cells. This activates S-type anion channel rapidly and at the same time blocks K_{in} channels, which leads to stomatal closure (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; McAinsh et al., 1990; Schroeder and Hagiwara, 1990; McAnish et al., 1992; publication II; Stange et al., 2010). Cytosolic Ca^{2+} elevation can be achieved by Ca^{2+} influx from the apoplast into the cytosol via hyperpolarisation activated Ca^{2+} channels in the plasma membrane of guard cells and by its release from intracellular stores, primarily from the vacuole. The TPC1 (TWO-PORE CHANNEL 1) slow vacuolar channel in the vacuole membrane is responsible for intracellular Ca^{2+} release (Schroeder and Hagiwara 1990; McAnish et al., 1992; Sanders et al., 1999; Hamilton et al., 2000; Pei et al., 2000¸ Peiter et al., 2005). H_2O_2 and ABA can activate Ca^{2+} channels in the plasma membrane, while InsP6 (*myo*-inositol hexakisphosphate), which is involved in ABA-induced stomatal closure, induces Ca^{2+} release from the endomembrane compartments including the vacuole by activating the Ca^{2+} channels in the tonoplast (Hamilton et al., 2000; Pei et al., 2000; Lemtiri-Chlieh et al., 2003).

In response to ABA, CO_2 and darkness, a Ca^{2+}-independent activation of anion channels exists. Still, the anion channel activity is enhanced by increases in the cytosolic Ca^{2+} concentration (Levchenko et al., 2005; Marten et al., 2007; Marten et al., 2008; Siegel et al., 2009).

Interestingly, cytosolic Ca^{2+} elevation takes place also during stomatal opening. Low CO_2, which opens stomatal pores, induces repetitive cytosolic Ca^{2+} elevations (Irving et al., 1992; Young et al., 2006). It needs clarification how these opposite responses could be directed via elevations in the same second messenger, Ca^{2+}.

As Ca^{2+} is involved in so many different responses, it has been frequently speculated on how specificity of the response could be achieved. Different effectors induce oscillations in cytosolic Ca^{2+} and it is proposed that those oscillations increase the amount of information generated by different stimuli by creating a stimulus-specific Ca^{2+} signature (McAainsh et al., 1997). Different stimuli induce Ca^{2+} oscillations of different amplitudes and frequencies correlating with the degree of stomatal closure, thus Ca^{2+} oscillations are stimulus specific (Schroeder and Hagiwara, 1990; McAinsh et al., 1995; Staxen et al., 1999; Allen et al., 2001; Allen et al., 2000).

In guard cells, Ca^{2+} sensing is mediated by Ca^{2+}-sensing receptor (CAS), localised to the chloroplast thylakoid membrane. It should be noted that CAS was initially localised to the plasma membrane and only later its localisation in the chloroplasts was revealed. CAS binds Ca^{2+}, and it is essential for extracellular Ca^{2+}-induced cytosolic Ca^{2+} increases and stomatal closure. This demonstrates the importance of chloroplasts in generating Ca^{2+} signals in guard cells (Han et al., 2003; Nomura et al., 2008; Vainonen et al., 2008; Weinl et al., 2008). Additionally, several families of Ca^{2+} sensors have been identified in higher plants. The best known are calmodulins (CaM), which contain multiple Ca^{2+} binding domains and CaM-related proteins. In addition, calcineurin B–like (CBL) proteins and CDPKs/CPKs are involved in Ca^{2+} sensing and regulating downstream processes (Luan et al., 2002). CPK3 and CPK6 regulate S-type anion channel activation in response to cytosolic Ca^{2+} (Mori et al., 2006). Furthermore, NtMPK4 is a proposed regulator of Ca^{2+}-independent as well as Ca^{2+}-dependent activation of guard cell anion channels (Marten et al., 2007b). Extracellular calmodulin (ExtCaM) stimulates a cascade of intracellular signalling events which regulate stomatal movement. ExtCaM-induced stomatal closure involves GPA1-dependent activation of H_2O_2 production, changes in cytosolic Ca^{2+} and subsequent AtNOA1-dependent NO accumulation (Chen et al., 2004; Li et al., 2009).

As concluded by Kudla et al., (2010) in their recent review, the major task still remains to identify molecular identities of true Ca^{2+} channels. Another major challenge remains to understand mechanistically how Ca^{2+} sensor proteins sense and transduce Ca^{2+} oscillations and signatures (Kudla et al., 2010).

1.1.6.2 Nitric oxide

NO was first identified as an important messenger in animal cells (Mayer and Hemmens, 1997). The highly reactive NO is a versatile signalling molecule regulating basic physiological processes throughout the plant life cycle like seed germination, growth, development, flowering, fruit maturation, senescence, programmed cell death and stress tolerance (Delledonne et al., 1998; Pei and Kuchitsu, 2005). Depending on the concentration and the tissue where it is acting, NO can be considered either toxic or protective in animals as well as in plants (Garcia-Mata and Lamattina, 2001). It induces stomatal closure in different plant species and is suggested to increase plant drought tolerance in stress conditions by reducing transpirational water loss (Garcia-Mata and Lamattina, 2001). NO has been found

to act in guard cell ABA signalling and its generation is dependent on H_2O_2 production by NADPH oxidases (Neill et al., 2002; Garcia-Mata and Lamattina, 2002; Desikan et al., 2002; Bright et al., 2006). Similarly to ABA, NO is also involved in the inhibition of stomatal opening (Yan et al., 2007). ABA-induced ROS production is followed by nitrate reductase (NIA1, NIA2) dependent NO synthesis (Desikan et al., 2002; Bright et al., 2006; Crawford et al., 2006; Gonugunta et al., 2008). NO is involved also in darkness-induced stomatal closure, which is dependent on NIA1 (Neill et al., 2008). NO promotes intracellular Ca^{2+} release from intracellular stores and through that regulates guard cell Ca^{2+} sensitive K^+ and Cl^- channels (Garcia-Mata et al., 2003; Sokolovski et al., 2005). ExtCaM is involved in guard cell regulation by activating G protein α to induce H_2O_2 generation by NADPH oxidases, which increases AtNOA1 dependent NO production; NO subsequently induces stomatal closure (Li et al., 2009).

However, it should be noted that studies with NO have been complicated due to the lack of proper NO synthesis mutants, and some of the commonly used NO releasing chemicals have unwanted side effects. The exact route of NO synthesis in plant cells remains a matter of debate. First, nitrate reductase (NR) was reported to synthesize NO in plants (Yamasaki et al., 1999), but its contribution is still controversial. The situation was further complicated after the identification of the NO synthase NOS1, which was later renamed AtNOA1 (NO Associated1) after it was found not to have NO synthase activity. Defective NO production in the mutant is most likely an indirect effect according to recent data (Guo et al., 2003b; Crawford et al., 2006; Gas et al., 2009). Still, it is possible that despite the pleiotropic phenotype of NOA1, it could be used as a mutant with lowered NO production in response to some stimuli. Additionally, sodium nitroprusside, a widely used NO donor, produces in addition to NO also side products (cyanide) that might have an effect on the experimental data.

1.2 Reactive Oxygen Species in guard cell signalling

Reactive oxygen species (ROS) are important signals mediating stomatal movements and other physiological processes in plants (Desikan et al., 2004a; Foyer and Noctor, 2005). ROS comprise of super oxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Plants accumulate ROS continuously as a side product from metabolism, which is strictly controlled. There are different sources of ROS like mitochondria, chloroplasts, plasma membrane bound NADPH oxidases, cell wall associated peroxidases, peroxisomes and glyoxysomes (Foyer and Harbinson, 1994; Asada, 2006; del Rio et al., 2006; Allan and Fluhr, 1997; Pei et al., 2000; Zhang et al., 2001).

During stress situations the formation of ROS increases and regulates important downstream signalling events in plant defence reactions. When the production of ROS exceeds the degrading capacity, it can lead to oxidative stress and possible injury (Zhang et al., 2001).

To date no ROS receptor has been identified in plants, and thus a key question is: how is increased ROS production sensed? Many targets may exist and ROS can possibly modify protein structure and activity by causing the formation of disulfide bonds or sulfenic acid groups (Reviewed by Mori and Schroeder, 2004).

In guard cells ROS are produced in response to numerous stimuli such as ABA, MJ, SA, CO_2, ExtCaM, ethylene, darkness, drought, extreme temperatures, UV radiation, O_3 and pathogen elicitors (Pei et al., 2000; Zhang et al., 2001; Suhita et al., 2004; Munemasa et al., 2007; Mori et al., 2001; Kolla et al., 2007; Chen et al., 2004; Li et al., 2009; Desikan et al., 2004; Desikan et al., 2004; Joo et al., 2005; publication III; Lee et al., 1999; Desikan et al., 2005; Desikan et al., 2006). H_2O_2 causes an increase in guard cell cytosolic free Ca^{2+}, anion channel activation and stomatal closure, at the same time inhibiting K_{in} channels (McAinsh et al., 1996; Zhang et al., 2001; publication II). H_2O_2 modulates protein phosphorylation and gene expression (Neill et al., 2002; Gudesblat et al., 2006; Jammes et al., 2009). Similarly to ABA and NO, in addition to inducing stomatal closure, ROS also inhibits stomatal opening (Yan et al., 2007). NADPH oxidases AtRBOHD and AtRBOHF have been identified as a source of ROS in guard cells (Kwak et al., 2003). Furthermore, guard cell chloroplasts are involved in ROS production (Joo et al., 2005). Since the amount of Rubisco is very low in guard cells as compared to mesophyll cells (Reckmann et al., 1990), ROS production in response to different stimuli suggest a possible role for their function in signalling (Zhang et al., 2001). Consistent with this, O_3-induced ROS production in guard cells is initiated from chloroplasts and later ROS diffuses to other cell compartments (publication III; Joo et al., 2005).

Ascorbic acid is the major antioxidant that scavenges ROS. Ascorbic acid levels are regulated diurnally in guard cells and whole leaves so that ROS increases during the afternoon while stomata close, whereas ascorbic acid decreases. Consequently ascorbic acid concentration correlates with stomatal opening (Chen and Gallie, 2004). In addition to ascorbic acid, Gutathione peroxidases serve as H_2O_2 detoxifiers, controlling H_2O_2 homeostasis and regulating ABA-induced ROS production and stomatal closure (Miao et al., 2006).

Different molecular components have been identified to act in guard cell ROS signalling. For example the ABI1, ABI2 and OST1 are central in this signalling and G protein subunits Gα and Gβ are required for ROS generation in guard cells (Murata et al., 2001; Mustilli et al., 2002; Chen et al., 2004; Joo et al., 2005; publication III)

1.3 Guard cell ion transporters and channels

Stomatal opening and closure are regulated by changes in the turgor pressure of guard cells. During stomatal opening the content of osmotically active solutes in the guard cell increases, which leads to water uptake and increase in guard cell turgor. When stomata open, K^+, Cl^- (Humble and Raschke, 1971; Raschke and Fellows 1971) and malate (Pearson 1975; Van Kirk and Raschke 1978) migrate into the guard cells. During stomatal closure, on the contrary, solutes flow out, which leads to water efflux and decrease in guard cell turgor (Figure 1). The volume of guard cells can change by more than 40% during stomatal movement.

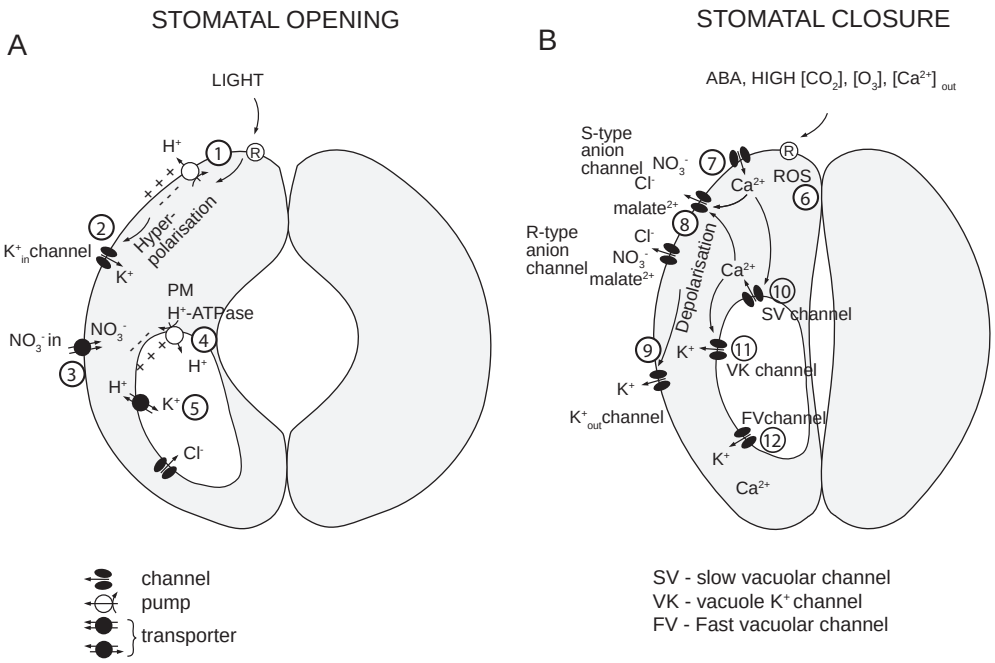

Figure 3 Model of ion channels and transporters functioning in stomatal movements.

Light induces stomatal opening (A). First, plasma membrane H^+-ATPases are activated (1), which leads to membrane hyperpolarisation and K^+ inward rectifying channel activation (2). NO_3^- uptake is driven by the nitrate transporter (3). At the vacuolar membrane vacuolar-type H^+-ATPases are active (4), which acidify the vacuole lumen and drive K^+/H^+ antiporters (5). During stomatal opening Cl^- and K^+ accumulate in the vacuole (A).

ABA, high [CO2], [O3] and high extracellular [Ca^{2+}] induce the stomatal closing (B). Stomatal closing requires cellular efflux of solutes, in particular K^+, Cl^- and malate. ABA and O_3 activate reactive oxygen species (ROS) production (6), which activates Ca^{2+} permeable Ca^{2+} channels (7). Elevation in cytosolic Ca^{2+} concentration activates S-type anion channels, slow vacuolar (SV) channels and vacuole K^+ channels (VK). Cl^-, NO_3^- and malate flow out through S-type and R-type anion channels (8). This causes membrane depolarisation and K^+ efflux via K^+ outward rectifying channels (9). At the vacuolar membrane SV channels are activated by cytosolic Ca^{2+} and are permeable to Ca^{2+} (10). VK channels are activated by cytoplasmic Ca^{2+} and allow K^+ release from the vacuole into the cytoplasm during stomatal closing (11). Fast vacuolar (FV) channels are inhibited by elevated cytosolic Ca^{2+} (12).

Unlike other cell types, mature guard cells lack plasmodesmata. Therefore all solute uptake and efflux must occur via ion channels and transporters in the plasma membrane. Ion transporters are pumps, carriers, symporters and antiporters, which use energy, usually from ATP, to drive transport against a free energy gradient. Ion channels are proteins that mediate energetically downhill ion fluxes via ion movement through a regulated pore. Solute flux through channels is orders of magnitude faster than through transporters (Pandey et al., 2007). See figure 3 for the model of ion channels and transporters functioning in stomatal movements. Different guard cell ion transporters and channels discussed in this chapter are listed in table 1.

Table 1. List of guard cell ion transporters and channels discussed in this thesis

Name of transporter or channel	Genes encoding the channel or transporter in guard cells
Plasma membrane ion transporters and channels	
H^+-ATPase	AHA1-AHA11
NO_3^- transporter	AtNRT1.1 (CHL1)
K^+_{in} channel	KAT1, KAT2, AKT1, AKT2/3, AtKC1
K^+_{out} channel	GORK
Slow type anion channel	SLAC1
Rapid type anion channel	NA (no information available)
Ca^{2+} channel	NA (20 CNGC genes, 20 GLR genes)
Vacuolar ion transporters and channels	
V-ATPase	DET3, 26 VHA genes in Arabidopsis
Fast vacuolar channel	NA
Vacuole K^+ channel	TPK1
Slow vacuolar channel	TPC1
Vacuolar anion channel	NA

1.3.1 Guard cell plasma membrane ion channels and transporters

During stomatal opening H^+-ATPase activation causes H^+ efflux from the guard cells leading to membrane hyperpolarisation (Assmann et al., 1985; Shimazaki et al., 1986) and K^+ uptake via K_{in} channels (Schroeder et al., 1984; Schroeder et al., 1987). Conversely, during stomatal closure anion channels are activated, leading to membrane depolarization and K_{out} channel activation. At the same time K_{in} channels and H^+-ATPases are inactivated (Schroeder and Hagiwara, 1989; Keller et al., 1989).

1.3.1.1 H^+-ATPases

Blue light activates the H^+-ATPase through phosphorylation and subsequent binding of 14-3-3 protein to the C-terminus, leading to stomatal opening (Kinoshita and Shimazaki, 1999; Kinoshita and Shimazaki, 2002; Kinoshita et al., 2003). Ca^{2+} and ABA can inhibit H^+-ATPase phosphorylation and binding of 14-3-3 protein (Kinoshita et al., 1995; Zhang et al., 2004).

Arabidopsis H^+-ATPases (AHA1-11) belong to a gene family with 11 members; all of them are expressed in guard cell plasma membrane (Ueno et al., 2005). Dominant mutation in the major *Arabidopsis* guard cell plasma membrane H^+-ATPase AHA1 (OST2) causes constitutive activity of the pump. OST2 deactivation is essential step in membrane depolarization prior to stomatal closure (Merlot et al., 2007).

1.3.1.2 Anion transporters

During stomatal opening guard cells take up Cl^- ions, but the molecular basis of this mechanism is still unknown (Pandey et al., 2007). NO_3^- uptake contributes to stomatal opening, especially when Cl^- is not available. In *Arabidopsis* guard cells the dual-affinity nitrate transporter AtNRT1.1 (CHL1) mediates the NO_3^- uptake. The *chl1* mutant plants have reduced stomatal opening and decreased water loss rates when compared to the WT (Guo et al., 2003).

1.3.1.3 K^+ channels

K^+_{in} and K^+_{out} channels regulate K^+ transport across the guard cell plasma membrane. K^+ is the main osmoticum in guard cells and K^+_{in} channels are activated by membrane hyperpolarisation during stomatal opening (Schroeder et al., 1988). At dawn blue light activates H^+-ATPase leading to membrane hyperpolarisation, K^+_{in} channel activation and stomatal opening. K^+_{in} channel activity underlies the circadian rhythm of stomatal opening, thus stomatal opening anticipates changes in light conditions (Lebaudy et al., 2007).

K^+_{in} channels are multimeric proteins. Three α-subunit families which form K^+ channels have been identified in plants: Shaker, TPK and Kir-like. A functional channel is built of 2 (TPK) or 4 (Shaker and Kir-like) α -subunits (Véry and Sentenac, 2003; Pilot et al., 2003; Lebaudy et al., 2007). *KAT1*, *KAT2*, *AKT1*, *AKT2/3*, and *AtKC1* K^+ channels are expressed in guard cells and all belong to the Shaker family (Schachtman, et al., 1992; Szyroki A, et al. 2001). Disrupting the multimeric Shaker family *KAT1* or *KAT2* gene alone does not have an effect on stomatal opening (Szyroki A, et al. 2001; Pilot et al., 2001). Studies with *Arabidopsis* plants containing a dominant negative point mutation in the pore region of KAT1 have shown that the K^+_{in} channel subunit KAT1 is responsible for K^+_{in} current activity and light-induced stomatal opening (Kwak et al., 2001). A transgenic approach where a dominant negative *KAT2* mutation was introduced in the *KAT2* knockout background resulted in a plant named *kincless*. Knock out of *KAT2* alone or introducing the dominant negative *KAT2* mutation in WT background did not cause any changes in the phenotype. In *kincless* the K^+_{in} channel activity in guard cells is absent, stomatal movement in response to light and low CO_2 are defective and the mutant has decreased transpirational water loss (Lebaudy et al., 2008).

In *Arabidopsis* the K^+_{out} rectifying channel in the guard cell membrane is encoded by a single gene - *GORK*. It belongs to the Shaker K^+ channel superfamily and is responsible for guard cell closure in response to darkness, ABA and O_3. Guard cell depolarization and anion efflux activates GORK, resulting in K^+ efflux, water outflow and stomatal closure (Ache et al., 2000; Hosy et al., 2003; publication III).

It has been suggested that fast K^+ uptake upon membrane hyperpolarization during stomatal opening is more essential and complex for stomatal physiology than K^+ secretion upon membrane depolarization during stomatal closure. The K^+_{in} channels are encoded by several redundant genes, whereas only a single gene *GORK*, is responsible for the K^+_{out} conductance. Protein trafficking is involved in regulating K^+ channel activity during stomatal opening

(Sutter et al., 2006). It was suggested that vesicles containing both K^+_{in} and K^+_{out} rectifying channels are transported to the plasma membrane during guard cell swelling, causing an increase in membrane surface area and K^+ currents (Kwak et al., 2008). ABA can trigger K^+ channel endocytosis and reduction in KAT1 expression thereby enhancing stomatal closure (Sutter et al., 2007). Arabidopsis SYP121, a protein from SNARE superfamily is responsible for delivery of KAT1 K^+ channel to the plasma membrane (Sutter et al., 2006).

1.3.1.4 Anion channels

Guard cell anion channels localized in the plasma membrane are central regulators of stomatal closure. Voltage dependent anion channel activation and the resulting anion efflux from guard cells leads to membrane depolarization, K^+ channel activation and K^+ efflux required for stomatal closing (Keller et al., 1989; Schroeder, 1995). Anion channels are classified as S-type and R-type. The R-type channels are activated rapidly by depolarization over a narrow voltage range and deactivated rapidly at hyperpolarized potentials. The S-type channels are activated over a broader voltage range and have slow activation and deactivation kinetics (Schroeder and Hagiwara, 1989; Schroeder et al., 2001, Schroeder and Keller, 1992). Both R- and S-type anion channels are permeable to different anions like $malate^{2-}$, NO_3^- and Cl^- and can be activated by ABA and cytosolic Ca^{2+} elevation leading to guard cell depolarization (Figure 3) (Hedrich et al., 1990; Schmidt and Schroeder, 1994; Schmidt et al., 1995; Pei et al., 1997; Roelfsema et al., 2004; Marten et al., 2007). CO_2 elevation, darkness and O_3 activate S-type anion channels via phosphorylation (Brearley et al., Planta 1997; Marten et al., 2008; Allen et al., 1999; Schmidt et al., 1995; publication II; publication III). ABA has been shown to enhance the ability of cytosolic Ca^{2+} to activate S-type anion channels, although a Ca^{2+} independent activation also exists (Siegel et al., 2009). Hyperpolarized voltage pulses induce elevation of cytosolic free Ca^{2+} concentration, which evokes rapid activation of S-type anion channels (Stange et al., 2010).

The molecular identity of the long sought anion channel SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) in the plasma membrane of guard cells was revealed relatively recently. SLAC1 is a general regulator in guard cells, being responsible for stomatal closure in response to elevated CO_2, O_3, ABA, H_2O_2, extracellular Ca^{2+}, darkness and low humidity (Negi et al., 2008; publication II; Saji et al., 2008). SLAC1 encodes an S-type anion channel, in contrast the R-type anion channel and Ca^{2+} channels are intact in the *slac1* mutant. (publication II;). SLAC1 is regulated via phosphorylation at the N-terminal tail of SLAC1 by OST1 leading to the channel activation. In addition, the protein phosphatase ABI1 prevents SLAC1 activation, demonstrating that SLAC1 is controlled by OST1/ABI1-dependent phosphorylation/ dephosphorylation. SLAC1 has weak voltage-dependence and is permeable to NO_3^- and Cl^- (Geiger et al., 2009). The Ca^{2+}-dependent protein kinases CPK21 and CPK23 stimulate SLAC1 activation. However, only CPK21 is involved in Ca^{2+}-dependent SLAC1 activation as CPK23 is Ca^{2+}-independent. Similarly to activation by OST1, CPK activation of the guard cell anion channel is suppressed by ABI1 (Geiger et al., 2010). Additionally, G proteins

(Wang et al., 2001), sphingosine-1-phosphate (Coursol et al., 2003) and Ca^{2+} dependent protein kinases (Mori et al., 2006) have been shown to be involved in the regulation of S-type anion channels suggesting that the regulation might be very complex.

1.3.1.5 Ca^{2+} channels

Ca^{2+} channel activities in the plasma membrane are involved in many physiological processes. During ABA-induced stomatal closure Ca^{2+} entry activates many physiological processes. However, no genes encoding plasma membrane Ca^{2+} channels have been identified (Pandey et al., 2007; Ward et al., 2009). The possible candidate for plasma membrane Ca^{2+} channels belong to the cyclic nucleotide gated channel family (CNGC) with 20 members (Kaplan et al., 2007) and to the glutamate receptor family (GLR) with 20 members (Lacombe et al., 2001). Ca^{2+} channels are activated by hyperpolarisation and ABA enhances Ca^{2+} channel activity (Pei et al., 2000; Hamilton et al., 2000). ABA-induced Ca^{2+} channel activation can occur via ROS production, which induces increases in cytosolic Ca^{2+} concentration (Pei et al., 2000; Kwak et al., 2003). The involvement of ROS has been further confirmed as the NADPH oxidase *atrbohD, atrbohF* mutants lack ABA-induced ROS production and Ca^{2+} channel activation (Kwak et al., 2003). Furthermore, ABA activated Ca^{2+} channels are regulated by phosphorylation and Ca^{2+}-dependent protein kinases CPK3 and CPK6 (Köhler and Blatt, 2002; Mori et al., 2006).

1.3.2 Guard cell vacuolar ion transporters and channels

Solute trafficking during stomatal movements does not take place only across the plasma membrane, but also between the vacuole and cytoplasm. Vacuoles take up to 90% of the guard cell's volume. Thus stomatal closing requires also the efflux of K^+ from the guard cell vacuole; more than 90% of the released K^+ during stomatal closure originates from the vacuole (Ward and Schroeder, 1994; MacRobbie, 2006).

1.3.2.1 Vacuolar ATPases

Vacuoles accumulate H^+, which depends on the activity of vacuolar-type H^+-ATPases (V-ATPases). H^+ uptake mediates also the movement of other solutes between vacuoles and cytoplasm (Pandey et al., 2007). V-ATPases comprise of multiple subunits and in *Arabidopsis* genome at least 26 V-ATPase subunits have been identified (Padmanaban et al 2007). The guard cell V-ATPase mutant *de-etiolated 3* (*det3*) lacks the external Ca^{2+} and oxidative stress induced stomatal closure (Allen et al., 2000).

1.3.2.2 Vacuolar ion channels

Vacuolar channels consist of three different types: the fast vacuolar, K^+-selective vacuolar and slow vacuolar channels (Pandey et al., 2007). K^+-selective vacuolar channels are activated upon increases in the cytoplasmic Ca^{2+} concentration, whereas fast vacuolar channels activate quickly in response to voltage and cytosolic alkalinisation and are inhibited by elevated cytosolic Ca^{2+} (Ward and Schroeder, 1994; Allen and Sanders, 1996). Genes encoding fast vacuolar channels have not been identified so far, they are suggested to function as a

pathway for K^+ flux from the vacuole into the cytoplasm during Ca^{2+} independent stomatal closure (Ward et al., 2009). Slow vacuolar channel is activated by cytosolic free Ca^{2+} and by cytosol-positive potentials, playing an important role in Ca^{2+}-induced Ca^{2+} release from the vacuole during stomatal closure. Cytosolic Mg^{2+} sensitizes slow vacuolar channels to physiological Ca^{2+} elevations (Ward and Schroeder, 1994; Allen and Sanders, 1995; Pei et al., 1999). The only slow vacuolar channel identified at the molecular level is TPC1 (TWO-PORE CHANNEL1), lacking the stomatal response to extracellular Ca^{2+} (Peiter et al., 2005). The K^+-selective vacuolar channel TPK1 encodes the TWO PORE K^+ (TPK) channel. It is expressed in the tonoplast mediating K^+ selective currents between the cytoplasm and the vacuole. TPK1 is activated by cytoplasmic Ca^{2+} and cytoplasmic pH, but not by tonoplast membrane potential (Gobert et al., 2007). Additionally, CDPKs can activate Cl^- and malate conductances at the guard cell tonoplast (Pei et al., 1996).

1.3.3 Protein phosphorylation and dephosphorylation in ion channel regulation

Protein phosphorylation and dephosphorylation plays a significant role in regulating a wide range of cellular processes. A number of protein kinases and phosphatases have been identified, which are involved in controlling different aspects of guard cell physiology from development to rapid stomatal closure. Their role in guard cell regulation in response to different factors has been discussed above. Protein phosphorylation and dephosphorylation events are involved also in guard cell ion channel regulation.

Using the patch-clamp technique and protein kinase and phosphatase modulators, it has been shown that ABA activates S-type anion channels via phosphorylation, which induces stomatal closure. When stomata open, S-type anion channels are dephosphorylated (Schmidt et al., 1995). Additionally, ABA activates Ca^{2+} permeable channels (Pei et al., 2000) by phosphorylation leading to stomatal closure (Köhler and Blatt, 2002) and K^+ channel activity is modulated via (de)phosphorylation (Armstrong et al., 1995).

Protein phosphatases ABI1 and ABI2 play an important role in this regulation. They mediate the ABA activation of Ca^{2+} (Murata et al., 2001), K^+ (Armstrong et al., 1995) and S-type anion channels (Pei et al., 1997). Different protein kinases have been identified to activate S-type anion channels. For example two MAP kinases, MPK9 and MPK12, have been shown to be involved in ABA and Ca^{2+} activation of S-type anion channels in guard cells (Jammes et al., 2009). Ca^{2+}-dependent protein kinases, CPK3 and CPK6 are required for ABA activation of both, S-type and Ca^{2+} permeable channels (Mori et al., 2006). The regulation of SLAC1 S-type anion channel by OST1, CPK21, CPK23 and ABI1 is quite well understood and was discussed in more detail above. All of these components play an important role in ABA and Ca^{2+} activation of anion channels and stomatal closure.

Some of the components responsible for K^+ channel phosphorylation have been identified – the K_{in} channel KAT1 is phosphorylated by CDPK in a Ca^{2+} dependent manner (Li et al., 1998) and OST1 phosphorylation regulates KAT1 negatively by reducing its activity (Sato et al., 2009).

2. Aims of the Study

The aim of the current work was to obtain novel information about the role of guard cells in determining plant O_3 sensitivity in the model plant *Arabidopsis*. This included performing *Arabidopsis* gas-exchange measurements during O_3 treatment, identification and characterization of an O_3-sensitive mutant *rcd3* (*slac1*). Finally, the regulation of SLAC1 was addressed and the role of different mutants in O_3-induced rapid transient decrease in stomatal conductance was studied.

The specific aims were following:

- To monitor *Arabidopsis* gas-exchange in a non-invasive manner during O_3 exposure with the *Arabidopsis*-specific gas-exchange device.

- To clone and characterize the O_3-sensitive mutant *rcd3* (*slac1*).

- To characterize the regulation of SLAC1.

- To characterize the O_3-triggered rapid transient decrease in stomatal conductance and the role of different genes/proteins and ROS in its regulation.

3. Materials and methods

The materials and methods used in this study are described in detail in publications I, II and III as indicated in table 2.

Table 2. Methods used in publications I, II and II. Parentheses indicate that the method was conducted only by the co-authors of the respective publication

Method	Publication
Construction of a device for *Arabidopsis* gas-exchange measurements: set-up and fine-tuning	I
Plant material	I, II, III
Growth conditions	I, II, III
Whole-plant stomatal conductance measurements	I, II, III
rcd3 (*slac1*) mapping	II
Array analysis	(II)
Leaf stomatal conductance and water loss measurements	II
Stomatal responses to H_2O_2 and NO	(II)
Stomatal responses to Ca^{2+} pulses	(II)
ABA-induced stomatal closure analyses	(II)
Electrophysiological analyses	(II)
Stomatal density measurements	(II)
Complementation analysis	II
GUS activity analysis	II
Transient gene expression analyses	II
Confocal microscopy experiments	II, III
ROS measurements in guard cells	(III)
Split-ubiquitin yeast two-hybrid assay	(III)
Bimolecular fluorescence complementation assay	III
In vitro kinase assays	(III)
Mass spectrometry	(III)

Table 3. List of *Arabidopsis* ecotypes and mutants used in this study

Genotype	Annotation	Function
Group1 ecotypes		
C24		
Col-0		
Col-*er*		Has *erecta* mutation
Cvi-0		
Est-1		
Kas-1		
Kin-0		
L*er*		Has *erecta* mutation
Te-0		
Tsu-1		
Ws-0		
Ws-2		
Group 2. Hormone biosynthesis or signalling		
aba1-3	*ABA deficient 1*	Zeaxanthin epoxidase
coi1-16	*coronatine-insensitive1-16*	F-BOX protein
ein2	*ethylene insensitive 2*	In EIN2 the amino-terminal integral membrane domain shows similarity to the disease-related Nramp family of divalent cation transporters
etr1-1	*ethylene response 1*	ETR1 encodes a histidine kinase forming a hybrid ethylene receptor
etr1-3	*ethylene response 1*	ETR1 encodes a histidine kinase forming a hybrid ethylene receptor
etr1-9	*ethylene response 1*	ETR1 encodes a histidine kinase forming a hybrid ethylene receptor
jar	*jasmonic acid resistant 1*	Jasmonate-amido synthetase
npr1	*nonexpresser of PR genes 1*	NPR1 is transcription coactivator and a master regulator of plant immunity
oji1	*ozone-sensitive and jasmonate-insensitive 1*	Mutation has not been identified
sid2	*salicylic acid induction deficient 2*	Isochorismate synthase
Group 3. Ion channels		
atmrp5	*ATP binding casette transporter 5*	ATP binding cassette transporter
gork1	*gated outwardly-rectifying K+ channel*	Guard cell voltage-gated outwardly rectifying K^+ channel
kco1	*two pore K^+ channel 1*	2-pore K-channel
kincless	*K^+ inward current-less*	Knockout of the KAT2 and expression of a dominant negative *kat2* construct
slac1-1	*slow anion associated 1*	Protein required for guard cell plasma membrane S-type anion channel functioning
Group 4. Regulators of guard cell opening or closure to different stimuli		
abi1	*ABA insensitive 1*	Protein Phosphatase 2C
abi2	*ABA insensitive 2*	Protein Phosphatase 2C
agb1	*Heterotrimeric G-protein beta subunit*	Heterotrimeric G-protein beta subunit
Atmyb61	*MYB domain protein 61*	R2R3-MYB family transcription factor

atrbohD	*A.thaliana respiratory burst oxodase protein D*	NADPH oxidase catalytic subunit
atrbohD atrbohE	*A.thaliana respiratory burst oxodase protein D×E*	NADPH oxidase catalytic subunit
atrbohD atrbohF	*A.thaliana respiratory burst oxodase protein D×F*	NADPH oxidase catalytic subunit
atrbohE	*A.thaliana respiratory burst oxodase protein E*	NADPH oxidase catalytic subunit
atrbohE atrbohF	*A.thaliana respiratory burst oxodase protein E×F*	NADPH oxidase catalytic subunit
atrbohF	*A.thaliana respiratory burst oxodase protein F*	NADPH oxidase catalytic subunit
cpk3-1×6-1	*Ca-dependent protein kinase 3×6*	Ca-dependent protein kinase 3/6 double mutant
cpk32-4×7-1×8-1	*Ca-dependent protein kinase 32×7×8*	Ca-dependent protein kinase 32/7/8 triple mutant
cpk4-2×11-1	*Ca-dependent protein kinase 4×11*	Ca-dependent protein kinase 4/11 double mutant
gca2	*growth controlled by abscisic acid*	Mutation has not been identified
gpa1	*Heterotrimeric G-protein alpha subunit*	Heterotrimeric G-protein alpha subunit
gpa×agb	*gpa1×agb1 double mutant*	Lacks alpha and beta subunit of the heterotrimeric G-protein
hab1-1×abi1-2	*hypersensitive to ABA1 x abi1-2*	Double mutant of knockout alleles for HAB1 and ABI1
ht1-1	*high leaf temperature 1*	Serine threonin tyrosine kinase
mpk3	*mitogen-activated protein kinase 3*	Mitogen-activated protein kinase 3
mpk6	*mitogen-activated protein kinase 6*	Mitogen-activated protein kinase 6
nia1×nia2	*nitrate reductase 1×2 double mutant*	Nitrate reductase
oxi1	*OXidative signal -Inducible1*	Serine/threonin kinase
rcd1	*radical induced cell death 1*	Has WWE domain and a [Poly(ADP-Ribose) Polymerase]-like ADP-ribose transferase catalytic domain
rcn1	*roots curl in NPA 1*	Phosphoprotein phosphatase 2A regulatory subunit A
rpk1-1	*receptor-like protein kinase 1*	Receptor-like protein kinase 1
ost1-1	*SnRK2-type protein kinase / open stomata 1*	Encodes a calcium-independent ABA-activated protein kinase
srk2e/ost1-3	*SnRK2-type protein kinase / open stomata 1*	Encodes a calcium-independent ABA-activated protein kinase

Group 5. Miscellaneous

Atnoa1	*NO associated*	Encodes a protein with similarity to the bacterial YqeH GTPase required for proper ribosome assembly.
gri	*grim reaper*	Small apoplastic protein
serk3-1	*somatic embryogenesis receptor kinase*	Somatic embryogenesis receptor kinase/BRI1 associated receptor kinase
vtc1	*vitamin C defective 1*	GTP-mannose pyrophosphorylase
re-8	*reticulata 8*	Encodes a protein with unknown function

4. Results and Discussion

4.1 A novel gas–exchange system for studying rapid O_3-induced stomatal responses

Several different methods can be used to study guard cell behaviour. Following plant stomatal conductance is one option and thus monitoring stomatal behaviour in intact plants. *Arabidopsis* is a complicated species for gas-exchange measurements as its leaves are small and the stem is very short. Thus the use of traditional, commercially available gas-exchange devices was complicated, in particular in 2004, at the time this work was started. Furthermore no whole-rosette *Arabidopsis* gas-exchange measurement device was available commercially. Today, single-cell *Arabidopsis* gas-exchange measurement devices are commercially produced by LI-COR Biosciences (www.licor.com) and WALZ Mess- und Regeltechnik (www.walz.com). In these devices CO_2, humidity, temperature and light can be adjusted within physiologically relevant ranges. Chlorophyll fluorescence measurements, important for photosynthesis studies, can be conducted with WALZ whole-plant cuvettes.

The lack of commercial devices led to the construction of custom-made *Arabidopsis* gas-exchange devices. A multi-cuvette *Arabidopsis* gas-exchange device was constructed, where stomatal conductance and CO_2 assimilation could be simultaneously monitored in 6 cuvettes (Dodd et al, 2004). This device has been used for studying circadian rhythms of gas-exchange (Dodd et al., 2005). Similarly, a device with three different *Arabidopsis* cuvette sizes was designed where CO_2 assimilation and stomatal conductance could be followed (Tocquin et al., 2004). However, a system with the possibility to add O_3 and follow rapid changes in stomatal conductance had not been reported before.

In order to measure O_3-induced stomatal responses of different *Arabidopsis* O_3-sensitive accessions, a through-flow gas-exchange system, which enables monitoring 8 plants in a non-invasive manner (publication I), was constructed. Among several different parameters, the system measures *Arabidopsis* whole-shoot stomatal conductance, general photosynthesis and O_3 uptake rate and dose absorbed by the plant. Another basic difference of the system design is that in order to avoid any mechanical stress, plants are grown through a hole in the glass plate covering the pot with growth substrate. This plate forms the bottom rim of the measuring cuvette and separates gas-exchange of the shoots from the roots and soil (publication I). When plants are ready for measurements, they are inserted to stainless steel cuvettes, which is covered with a glass plate on top (publication I), forming a tight chamber. The cuvettes have similar basal conditions to the growth chambers in respect to light intensity (110 µmol m^{-2} s^{-1} photosynthetic photon flux density at rosette level), relative air humidity (60%) and temperature (23ºC) (publication I). Additionally, it is possible to set O_3 and CO_2 content in each chamber individually. Humidity changes are applicable to all chambers simultaneously (it should be noted that options for rapid changes in cuvette air CO_2 and humidity concentrations were developed after the publication I). Light responses can be studied by switching the light on and off in each chamber at a desired time. CO_2, O_3

and humidity content is sampled from the inflow and outflow air of each chamber. Based on changes in water vapour, stomatal conductance (g_s) is calculated according to von Caemmerer and Farquhar (1981). The actual response time to H_2O is 60sec and 90 sec to O_3. Duration of each sampling is 56 seconds (reference and chamber are sampled sequentially). It is possible to work in the single-cell mode when recordings from the chamber are taken every 112 seconds or 8-cell mode, while each plant is measured after 15 minutes.

A similar machine which would allow simultaneous stomatal conductance measurement of 8 *Arabidopsis* plants in a non-invasive manner online and after the application of O_3 had not been constructed before. Traditionally, most of the measurements studying guard cell responses of *Arabidopsis* plants have been performed using isolated epidermal peels (Allen et al., 2001). In these studies guard cell responses to different stimuli are usually recorded over longer time points (2h after treatment being very common).

Studies with epidermal peels have been instrumental for guard cell signalling research, however, the biggest problem with them is the mechanical stress. Mechanical stimulus has been shown to induce an oxidative burst, Ca^{2+} channel activation, ethylene production and changes in gene expression, all of which could obscure the accurate measurement of guard cell responses (Yahraus et al., 1995; Legue et al., 1997; Braam et al., 2005). This demonstrates the importance of non-invasive measurement devices.

On basis of the current research rapid O_3 response was detected already within minutes from the start of exposure (publication I). This demonstrated the existence of early guard cell signalling events in response to O_3 and the need for a measuring device with high time resolution and without applying any mechanical stimulus. The type 2C protein phosphatase mutant *abi2-1* lacked the O_3-induced RTD in stomatal conductance completely (publication I). Previously, ABI2 was shown to function downstream of ROS in guard cell signalling (Murata et al., 2001). The lack of RTD in *abi2-1* demonstrates that O_3-induced ROS has a biological function in the guard cell signalling cascade and indicates the useful role of O_3 in studying guard cell signalling. It also suggestes that the system is helpful in identifying novel molecular components in rapid guard cell O_3 responses (publication III). Interestingly, RTD was absent in one previously isolated O_3-sensitive mutant, *rcd3*, which suggested that map-based-cloning of *rcd3* might reveal a new regulator for stomatal function. Later the device was instrumental for the detailed characterization of the SLAC1 anion channel (publication II). Furthermore, the device was useful in identifying components responsible for RTD and the study of SLAC1 regulation. The lack of RTD in *ost1* and SLAC1 mutants with mutations in its N-terminus serine 120 and 146 suggested that OST1 might regulate SLAC1 and that these amino acids might be of functional importance (publication III). Recently the device was used to study the role of stomatal conductance in regulating O_3 entry to the plant and the resulting damage (Brosché et al., 2010).

4.2 SLAC1 anion channel is a central component in guard cell regulation

Guard cells serve as the first barrier against air pollutants. O_3 is one of the major pollutants in the air, causing serious crop losses each year. The entry of O_3 into the plants is regulated by guard cells and as a consequence stomatal regulation has a central role in determining plant O_3-sensitivity. One of the O_3-sensitive mutants, *rcd3 (radical-induced cell death 3*), which was originally isolated from a screen of EMS mutants for O_3-sensitivity (Overmyer et al., 2000; Overmyer et al., 2008), completely lacked RTD (publication II) and had constitutively higher stomatal conductance (publication II). Later *rcd3* was renamed *slow anion channel-associated 1-1* (*slac1-1*) according to its function. Constitutively higher stomtal conductance and lack of RTD might give an explanation to the *slac1* high O_3-sensitivity (publication II). It also illustrates the importance of guard cells in O_3 sensing and protecting the plant against O_3 damage.

The lack of fast O_3-induced processes in *slac1* gave an insight that SLAC1 was responsible for rapid stomatal closure. The mutant also had significantly higher water loss (publication II), which was used as the trait for identification of true recombinants from a L*er* x *rcd3* cross used in map based cloning. The mutation in *slac1-1* was mapped to the loci At1g12480. SLAC1 encodes a predicted membrane protein with 10 transmembrane domains, a long N-terminal tail and shorter C-terminal tail (publication II). SLAC1 was localised to the plasma membrane and it was preferentially expressed in guard cells (publication II). Several *slac1* mutant alleles showed strong insensitivity to CO_2-, ABA-, and O_3-induced stomatal closure (publication II). The aperture of stomatal pore is regulated by influx and efflux of osmotically active ions across guard cell plasma membrane. Anion channels have been shown to play a central role in guard cell regulation already more than 20 years ago, although the molecular identity of guard cell anion channels had not been revealed (Schroeder et al., 1984; Keller et al., 1989). Patch clamp analysis, which measure the activation of ion conductance across the plasma membrane, have led to the discovery of two distinct types of anion currents, the R-type and the S-type (Keller et al., 1989, Schroeder and Hagiwara, 1989, Hedrich et al. 1990; Schroeder and Keller 1992; Raschke et al., 2003). Measuring the activation of ion conductance of two *slac1* mutant alleles showed that S-type anion channel currents were greatly impaired, whereas the rapid (R-type) anion channels and the plasma membrane Ca^{2+} permeable channels were intact in *slac1* mutants (publication II). This data provides strong evidence that S-type anion channels play a central role in stomatal closure induced by different signals as had been proposed previously (Schroeder and Hagiwara, 1989). Linder and Raschke (1992) used the acronyms QUAC for the quickly activating anion channel and SLAC for the slowly activating anion channel, therefore RCD3 was renamed as “SLAC1”.

As *slac1* mutants had a slowed or partial stomatal closure in response to light-dark transitions and transitions to low humidity (publication II), it suggests that those responses might be mediated by R-type anion conductance. Furthermore, this data suggests that R-type and S-type anion channels do not share identical membrane proteins.

SLAC1 was discovered in parallel from a screen of CO_2-insensitive mutants by Negi et al. (2008), who showed similar results concerning its expression and localisation and that *slac1* mutants had impaired responses to CO_2, ABA and darkness. Additionally, they demonstrated that mutation in SLAC1 causes elevated accumulation of osmoregulatory ions like malate, Cl^- and K^+ in the guard cell protoplasts.

To address whether SLAC1 forms an anion channel itself or contributes to the formation of an anion transporting pore as a functional subunit and also to elucidate which anions it transports, SLAC1 was expressed hererologously in *Xenopus oocytes*. Unfortunately expression of SLAC1 in *Xenopus oocytes* failed to generate anion currents (publication II). Recently, Geiger et al. (2009) and Lee et al. (2009) showed that in order to produce S-type anion currents in *Xenopus oocytes* SLAC1 has to be co-expressed with the protein kinase OST1. This gives an explanation why experiments with *Xenopus oocytes* failed in publication II and demonstrates that SLAC1 activation is regulated by phosphorylation. Additionally, it was shown that SLAC1 is an anion selective channel with preferred permeability to Cl^- and NO_3^- (Geiger et al., 2009; Lee et al., 2009).

Guard cells decrease the stomatal aperture in response to drought, thereby decreasing transpirational water loss via stomata and protecting plants from desiccation. Drought leads to increased ABA production, resulting in stomatal closure. SLAC1 has been shown to be essential in guard cell ABA responses. As water scarcity is an increasing problem in the world, it is of great importance to understand how plants cope with drought at the molecular level. Further studies on the regulation of SLAC1 might be useful in the future for engineering or breeding of more drought resistant crops.

The continuing rise in atmospheric CO_2 causes reduction in stomatal apertures influencing water use efficiency and leaf heat stress. Consequently, changes in stomatal aperture also have an effect on the global climate. *slac1* was the first CO_2 insensitive recessive mutant to be identified. Overall, only a few mutants have been identified with altered CO_2 responses (Webb et al., 1997; Young et al., 2006; Hashimoto et al., 2006). SLAC1 was the first guard cell S-type anion channel to be identified at the molecular level and it is an essential component in response to all major factors regulating stomatal closure. SLAC1 regulation will be discussed in more detail below.

4.3 O_3-induced Rapid Transient Decrease in stomatal conductance

ROS are second messengers in guard cell signalling produced in response to ABA (Pei et al., 2000; Zhang et al., 2001), elevated CO_2 (Kolla et al., 2007), darkness (Desikan et al. 2004), ethylene (Desikan et al., 2006), MJ (Munemasa et al., 2007), SA (Mori et al., 2001) and ExtCaM (Chen et al., 2004; Li et al., 2009).

O_3 has been known to induce stomatal closure (Hill and Littlefield, 1969) and to induce ROS production in guard cells (Joo et al., 2005). As described above, O_3 induced RTD already within 5-10 min from the start of exposure, which was followed by recovery to the pre-

exposure level 40 min after the start of O_3 exposure despite of the continuous presence of O_3 (publication I and III). The recovery of stomatal conductance suggested that the decrease in conductance was not due to O_3-induced leaf damage, but because of activation of a signalling cascade by O_3-induced ROS. This demonstrated that O_3 could be used as a tool to study rapid ROS mediated guard cell signalling. It should be noted that Hill and Littlefield (1969) also recorded transient O_3 induced reduction in stomatal conductance followed by recovery to almost pre-exposure level, but in their study O_3 exposure was discontinued after the decrease in stomatal conductance, so it could be argued whether the recovery was due to discontinuation of O_3 exposure (Moldau et al., 1990).

After entering the plant through open stomata, O_3 degrades immediately into ROS. As guard cells respond to O_3 rapidly (publication III), they should be able to perceive ROS directly in the apoplast or alternatively ROS passes through the plasma membrane to trigger downstream responses inside the cell. Interestingly, 3 min of O_3 application was able to induce maximal decrease in stomatal conductance (publication III), and the kinetics of RTD was similar as during the continuous O_3 exposure (publication III). Additional O_3 pulses during the recovery period had no effect on the stomatal conductance (publication III). Responsiveness to O_3 recovered after stomatal conductance had regained the pre-exposure value (publication III). This indicates that RTD is programmed within the first 3 minutes and at the same time temporarily desensitizes guard cells to additional O_3 pulses. Prolonged membrane depolarisation has been shown to deactivate anion channels and for reactivation repolarisation would be needed (Hedrich et al., 1990; Roelfsema and Prins, 1997; Raschke et al, 2003). It could be speculated that anion channel deactivation is one possibility why guard cells are desensitised for O_3 after the initial O_3 pulse. The involvement of K^+ channels in RTD was demonstrated (publication III). Previously it has been shown that K_{out} channels were inactivated after activation, which might contribute to repolarisation after transient depolarisation and short-term K^+ efflux (Pei et al., 1998). K^+_{out} channel inactivation can further contribute to O_3 insensitivity during the reopening phase.

Previously it has been shown that O_3 induces elevation in cytosolic Ca^{2+} within seconds of exposure and a recovery period was needed before the Ca^{2+} elevation could be elicited again (Clayton et al., 1999; Evans et al., 2005).

In addition to O_3, blue light activates a rapid transient cytosolic Ca^{2+} elevation in *Arabidopsis* seedlings, whereas the initial blue light pulse desensitizes them to the subsequent pulses. Responsiveness to blue light was restored within 3-4h (Baum et al., 1999). The similarities between the current work and previous studies suggest the involvement of Ca^{2+} in O_3-induced RTD.

H_2O_2 functions as an intermediate in ABA signalling, and furthermore ROS generation in guard cells is dependent on ABA concentration. Exogenous application of H_2O_2 promoted stomatal closure in a dose-dependent manner (Zhang et al., 2001). In addition, elevation in cytosolic Ca^{2+} was shown to be directly proportional with the applied O_3 concentration

(Clayton et al., 1999; Evans et al., 2005). Similar correlation between the applied O_3 concentration and the extent of O_3-induced RTD was observed (publication III). Collectively, these parallels between O_3- and ABA-induced ROS in guard cells suggest similar mechanisms and the possible involvement of Ca^{2+} in RTD.

Darkness, reduced humidity and elevated CO_2 induced a rapid decrease in stomatal conductance without a recovery in the conductance (publication II), in contrast to the O_3 response (publication II, Hill and Littlefield 1969; Moldau et al., 1990). The existence of different signalling mechanisms for CO_2-, darkness-, humidity- versus the O_3-induced stomatal closure is intriguing. Additionally, the role of Ca^{2+} in RTD would be of interest to address in the future. Another interesting question is whether guard cells are responsive to CO_2, light or reduced air humidity during the period when they are desensitised to O_3.

It should also be noted that O_3-induced stomatal closure and RTD is not restricted only to *Arabidopsis*, but has been observed also in *Phaseolus vulgaris* and *Nicotiana tabacum*. However, the availability of a wide range of different mutants in *Arabidopsis* makes the *Arabidopsis* gas-exchange system and O_3 an elegant tool to studying guard cell signalling. The involvement of different genes in RTD will be addressed in further chapters.

4.4 Regulation of RTD

4.4.1 ROS production

ROS are produced in response to a variety of effectors, like O_3, which induce stomatal closure. To address the role of ROS in the regulation of RTD, confocal microscopy and the ROS sensitive dye H_2DCFDA was used. ROS production was followed in guard cells at the time when the rapid O_3-induced stomatal closure was induced, when stomata had reopened to the pre-exposure level and during the sustained decrease (publication III). A bi-phasic ROS production was detected in guard cells. The first and the second ROS peak coincide with the rapid and sustained O_3-induced decrease in stomatal conductance (publication III). 45 min after the start of exposure, when stomata had reopened, a decrease in ROS levels was seen despite of the continuation of O_3 exposure. This demonstrates the functional importance of ROS in guard cell signalling and additionally suggests the enzymatic control of O_3-induced ROS production in guard cells, rather than direct breakdown of O_3 into ROS. Before, O_3 has been shown to induce a biphasic Ca^{2+} elevation in young *Arabidopsis* seedlings (Clayton et al., 1999; Evans et al., 2005) and ABA was shown to induce a biphasic production of H_2O_2 in *Vicia faba* guard cells (Zhang et al., 2001).

Previously NADPH oxidases encoded by the *AtRBOHD* and *AtRBOHF* genes have been shown to be involved in ROS production in guard cell membranes (Joo et al., 2005; Torres et al., 1998; Torres et al., PNAS 2002; Kwak et al., 2003). RTD was present in the NADPH oxidase single and double mutant plants (publication III); however the O_3-induced first peak of ROS production was significantly reduced in *atrbohD* and *atrbohD atrbohF* mutants. The second peak of ROS production was significantly reduced in the *atrbohD atrbohF* double

mutant plants (publication III). As considerable amount of ROS was detected in the NADPH oxidase mutants during the initial peak of ROS production, it suggests the existence of other ROS sources, for example cell wall peroxidases or chloroplasts. NADPH oxidases have an important role in the second peak of ROS production as the second peak was lower in *atrbohD atrbohF* plants.

OST1 phosphorylates AtRBOHF and possibly regulates its activity (Sirichandra et al., 2009). As *ost1* had a lower initial ROS peak than the second (publication III), it suggests that OST1 could have a role in the regulation of NADPH oxidase mediated first ROS peak production.

Deeper analysis of confocal microscopy images revealed that at the earliest time point ROS production was initiated from chloroplasts, later the signal was seen additionally in other parts of the cell (publication III). Similar conclusion has been drawn by Joo et al. (2005), where O_3-induced ROS production was shown to initiate from chloroplasts, following by NADPH oxidase dependent ROS production in guard cell membranes and later propagating to neighbouring cells. Similarly to O_3, ABA-induced ROS production has been visualised in guard cell chloroplasts significantly earlier than in other parts of the cell (Zhang et al., 2001).

The role of chloroplasts during rapid O_3-induced stomatal closure should be studied further by using inhibitors, for example DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which is a photosynthesis inhibitor or different mutants with modified photosynthesis.

4.4.2 Proteins responsible for RTD

The O_3-induced RTD was completely missing in *abi2-1* and *slac1* (publication I and II). This gave an insight that different genes and proteins previously shown to be involved in guard cell signalling in response to other effectors are involved in the regulation of RTD. A number of mutants shown to be defective in guard cell signalling have been identified (Pandey et al., 2007; Ward et al., 2009). In current study, 59 mutants, which had been shown to be involved in guard cell regulation, and also 12 ecotypes were analysed for RTD in order to dissect molecular components required for rapid guard cell O_3 responses. All the mutants and ecotypes tested for O_3 and the presence or absence of RTD are listed in publication III, supplemental table 1. First, the presence of RTD was observed in 12 different *Arabidopsis* ecotypes, demonstrating the general nature of this phenomenon.

Different hormones are involved in guard cell signalling, therefore mutants shown to be defective in the biosynthesis or signalling of ABA, JA, SA or ethylene, were tested for RTD (publication III). All of them showed WT phenotype for RTD, suggesting that none of these hormones are involved in the initiation of RTD. Another explanation would be that some of the mutants are not “null” mutants and that a small amount of the certain hormone produced would be sufficient to elicit signalling. Furthermore, lack of RTD in the ABA insensitive mutant *abi2-1* implies the involvement of ABA in the fast O_3-induced stomatal closure.

O_3 was shown to induce ROS production in guard cells (publication III). Previously NADPH oxidase subunits and heterotrimeric G protein α and β subunits have been shown

to be responsible for ROS production in guard cells (Kwak et al., 2003; Joo et al., 2005). Interestingly, all NADPH oxidase and G-protein α and β subunit mutants had WT response to O_3. Thus it can be concluded that signalling via heterotrimeric G proteins is not necessary for RTD and that O_3 can mimic NADPH oxidase dependent ROS production or other sources of ROS, possibly in chloroplasts, are functionally important in O_3 induced RTD.

The guard cell anion channel SLAC1 is a central component regulating guard cell rapid O_3 responses after the perception of ROS (publication II). Various other guard cell ion channels have been identified. The guard cell K_{out} channel GORK (Ache et al., 2000) contributes to RTD as in *gork-1* mutant plants the fast O_3-induced stomatal closure was delayed (publication III). This suggests that after O_3 perception and SLAC1 anion channel activation, the guard cell plasma membrane is depolarised, which leads to the K_{out} channel GORK activation. During the recovery phase in stomatal conductance, the hyperpolarisation-activated K_{in} channels play an essential role. In *kincless*, where K_{in} channels are abolished, the rapid O_3-induced stomatal closure was as fast as in the WT, but the recovery in conductance was missing (publication III).

CO_2 is a general factor regulating stomatal aperture. SLAC1 mutants lacked RTD and were also insensitive to elevated CO_2 (publication II). Interestingly, protein kinase HT1 mutants, which have been demonstrated to have a central role in guard cell CO_2 signalling (Hashimoto et al., 2006), had WT O_3 response. This suggests that the HT1 kinase is not involved in the regulation of RTD and that O_3 and CO_2 signalling have at least partially different signalling pathways.

Protein phosphorylation and dephosphorylation has previously been shown to regulate anion channel activity (Schmidt et al., 1995; Grabov et al., 1997; MacRobbie, 1998; Pei et al., 2000). CPK6 and CPK3 have been shown to be involved in ABA and Ca^{2+} activation of guard cell ion channels (Mori et al., 2006); however mutation in these genes did not affect guard cell O_3 responses. Several mitogen activated protein kinases have been shown to be implicated in stomatal regulation. MPK3 plays a role in the perception of ABA and H_2O_2 in guard cells (Gudesblat et al., 2007) and MPK6 is involved in regulating H_2O_2 levels. Both *mpk3* and *mpk6* mutants displayed WT responses to O_3. These results suggest that ABA and Ca^{2+} signalling at least partially diverge from guard cell O_3 signalling. Protein kinase OST1 and protein phosphatase ABI2 play an essential role in RTD regulation as mutations in those genes completely abolished RTD (publication III). OST1 and ABI2 have been shown to be involved in guard cell ABA signalling (Mustilli et al., 2002; Yoshida et al., 2002).

Consequently, the current results suggest that O_3 activates the SLAC1 anion channel via phosphorylation and dephosphorylation events mediated by ABI1, ABI2 and OST1. SLAC1 activation leads to membrane depolarization, which activates the K_{out} channel GORK. K_{in} channels are again central in guard cell reopening after the O_3-induced closure. Recently, OST1 has been shown to phosphorylate and reduce the activity of the K_{in} channel KAT1 (Sato et al., 2009). During RTD OST1 activation is required, thus OST1 could simultaneously activate SLAC1 and inactivate KAT1, leading to faster stomatal closure.

The presence of RTD in several mutants previously shown to be involved in guard cell ABA, CO_2 or Ca^{2+} signalling demonstrate that at least partially those signalling pathways diverge. It should be pointed out that all of the mutants with abolished RTD are also defective in other responses regulating stomatal closure. This demonstrates, that the signalling cascade activated during RTD, is not specific to rapid O_3 responses, but rather uses a subset from a "common" set of regulators. Furthermore, the small number of mutants with abolished RTD suggests that the rapid activation of stomatal closure by O_3 requires only a limited number of components, thus the signalling from ROS sensing to stomatal closure is short and straightforward.

4. 5 The protein kinase OST1 regulates the SLAC1 anion channel

Anion channels are regulated by phosphorylation and dephosphorylation events. Previously it has been shown that OST1 interacts with ABI1 and ABI2 and that PP2Cs are directly implicated in the ABA-dependent activation of OST1 (Mustilli et al., 2002; Yoshida et al., 2006, Vlad et al., 2009). In *abi1–1*, anion channels fail to respond to ABA (Pei et al., 1997), suggesting that both ABI1 and OST1 act in the regulation of anion channel activity. The lack of RTD in *ost1*, *abi1* and *abi2* gave good candidates for possible SLAC1 regulators.

Interaction between SLAC1 and OST1 was demonstrated both by split-ubiquitin membrane yeast two hybrid system (publication III) and bimolecular fluorescence complementation assay in *Nicotiana benthamina* leaves (publication III). Using the TILLING approach (Till et al., 2003), three new SLAC1 mutant alleles were identified, in which putative phosphorylation sites in the N-terminal tail of SLAC1 were mutated (publication III). RTD was absent in plants where Ser120 (*slac1-7*) and Ser146 (*slac1-8*) were mutated to phenylalanine (publication III), demonstrating the importance of these serine residues. Mutation in Ser38 (*slac1-6*) had no effect. *In vitro* phosphoproteomic experiments revealed that OST1 phosphorylates SLAC1 rapidly (publication III), Ser120 being one of the OST1 targets (publication III).

Ser146 was not an OST1 target. However, the strong O_3 insensitivity of the *slac1-8* mutant suggests that Ser146 is the target of some other protein kinase. Alternatively, the O_3 insensitive phenotype of *slac1-8* mutant could be due to conformational change of the SLAC1 protein as in *slac1-8* mutant Ser146 is replaced by phenylalanine, which is a hydrophobic and considerably larger amino acid. Collectively, this data demonstrates that OST1 activates SLAC1 anion channel by phosphorylating the N-terminal tail of SLAC1, Ser120 being an important OST1 target.

Physical interaction between OST1 and SLAC1 has also recently been demonstrated by two other groups (Geiger et al., 2009; Lee et al., 2009) and the functional relation between SLAC1, OST1 and ABI1 has been further confirmed. Co-expression of SLAC1 together with OST1 in *Xenopus* oocytes was required for anion current activation with properties similar to the slow anion channel in guard cells. When co-expressing SLAC1 and OST1 together with ABI1 or PP2CA, the activation of SLAC1 by OST1 is eliminated suggesting that protein

phosphatases counteract the stimulatory effect of OST1 (Geiger et al., 2009; Lee et al., 2009). The biochemical relation between PP2Cs and OST1 has been extensively studied – ABI1, ABI2 and HAB1 interact with OST1 to inhibit its activity by dephosphorylation (Umezawa et al., 2009; Vlad et al., 2009). The ABA recptors RCAR/PYR, in response to ABA, inhibit PP2C activity and convert OST1 to its active form (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). This leads to the phosphorylation of SLAC1 (Klingler et al., 2010).

In addition to OST1, CPK21 and CPK23 activate SLAC1; interestingly also this activation is suppressed by ABI1 (Geiger et al., 2010). Further studies with CPK21 and CPK23 mutant plants could reveal whether they are involved in O_3-induced RTD regulation or in responses to other physiological stimuli.

Recent progress has led to quite good understanding about SLAC1 regulation during stomatal closure. However, after phosphorylation of SLAC1 and stomatal closure, most likely dephosphorylation would be required for stomata to reopen. The identification of a protein phosphatase regulating this event has not been reported. PP2CA could be a possible candidate for further studies as interaction between SLAC1 and PP2CA has been shown (Lee et al., 2009). In addition, the possible role of ABI1 and ABI2 in SLAC1 dephosphorylation should be addressed.

These findings together with the emerging model of ABA signalling through PYR/PYL/RCAR receptor proteins suggest that plant rapid ROS-dependent stomatal closure is controlled by the PYR/PYL/RCAR dependent inhibition of guard cell phosphatases leading to OST1 dependent activation of the guard cell anion channel SLAC1.

4.6 Conclusions and future perspectives

The construction of a novel *Arabidopsis* gas-exchange apparatus, which enables monitoring of eight plants simultaneously in a non-invasive manner, has brought new insight into guard cell signalling (publication I). O_3 induced a rapid transient decrease in stomatal conductance (RTD), which was programmed within the first 3 minutes of exposure, thus further O_3 pulses had no effect on the kinetics of RTD (publication III).

The apparatus proved useful in the identification of molecular components responsible for RTD. The O_3 sensitive mutant *rcd3*, which was later renamed *slac1* (*slow anion channel-associated 1*) according to its function, lacked RTD completely (publication II). SLAC1 was shown to be a central regulator in response to all major factors regulating guard cell aperture: CO_2, light/darkness transitions, O_3, relative air humidity, ABA, NO, H_2O_2, and extracellular Ca^{2+}. SLAC1 encodes the first guard cell plasma membrane S-type anion channel to be identified at the molecular level (publication II). Its central role in response to CO_2, air pollutant O_3 and drought hormone ABA, gives new insights to plant O_3 sensitivity, drought hardiness in plants and plant responses to elevated CO_2. Interestingly, R-type and

Ca^{2+} currents were intact in *slac1* guard cells, which demonstrates that proteins required for these functions are encoded by different genes, which are still undiscovered.

SLAC1 was shown to be regulated by phosphorylation and dephosphorylation events. OST1 phosphorylated Ser120 in the N-terminal tail of SLAC1 (publication III), which led to SLAC1 activation. Recent findings suggest a model for SLAC1 activation (Figure 4). Interestingly, the same players are involved both in O_3 and ABA signalling. However, several questions remain to be resolved: how is O_3 sensed in the plasma membrane? How is the ROS signal passed on to RCAR/PYR receptors leading to inactivation of PP2Cs and release of OST1 and SLAC1 activation? RCAR/PYR receptors need to bind ABA in order to initiate the downstream events. One possibility might be that O_3 activates β-glucosidase AtBG1, which has been shown to hydrolyze glucose-conjugated, biologically inactive ABA to active ABA (Lee et al., 2006), thereby triggering downstream events to SLAC1 activation. After phosphorylation and SLAC1 activation, dephosphorylation and SLAC1 inactivation needs to take place in order for stomata to reopen. The possibility that the same PP2Cs dephosphorylating OST1 would also dephosphorylate SLAC1 should be investigated.

Rapid O_3-induced ROS production in guard cell chloroplasts was demonstrated (publication III). Still, the role of chloroplasts in guard cell signalling during O_3-induced RTD is a topic for further research. Studies with different mutants and inhibitors would be useful. Cytosolic Ca^{2+} concentration elevation takes place in response to several stimuli in guard cells and has been suggested to function as a hub in guard cell signalling. Therefore the role of cytosolic Ca^{2+} in O_3-induced RTD in guard cells and the possible existence of Ca^{2+}-dependent as well as -independent pathways, similarly to ABA signalling, would be topics for further studies.

The crystal structure of SLAC1 would reveal the true nature of the anion transporting pore. Last, but not least I would wish to see the SLAC1 crystal structure be the first reported guard cell ion channel.

In summary the following model for O_3-triggered RTD is proposed (Figure 4).

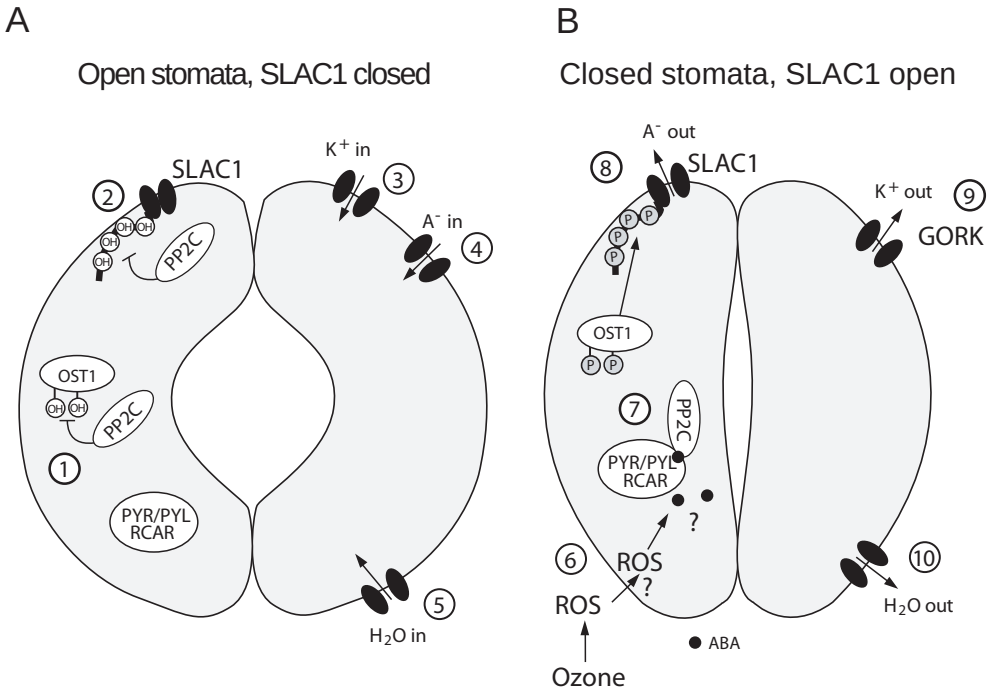

Figure 4 Anion channel regulation and schematic model of ozone-triggered Rapid Transient Decrease in stomatal conductance (RTD).

In open guard cells (left) the PYR/PYL proteins are not bound to PP2Cs, therefore PP2C activity is high. PP2C binds OST1 and SLAC1 thereby inhibiting both autophosphorylation of OST1 (1) and phosphorylation of SLAC1, thus SLAC1 is closed (2). Potassium (3) and anions (4) flow in; this leads to decrease in water potential and water influx (5). Guard cell turgor increases and stomata open. Ozone exposure leads to Reactive Oxygen Species (ROS) production in guard cells (6). Which, via an unknown mechanism, leads to complex formation between PYR/PYL and PP2C, PP2C activity is inhibited (7). This permits the autophosphorylation of OST1 and phosphorylation of SLAC1 N-terminal tail. As a result SLAC1 opens and anions flow out (8). Anion efflux leads to membrane depolarisation and K^+ outward rectifying channel GORK activation (9). Water potential increase drives water efflux from the guard cells (10), guard cell turgor decreases and stomata close (right).

Summary in Estonian
Kokkuvõte

Erinevalt loomadest puudub taimedel võimalus keskkonnatingimuste halvenedes oma asukohta muuta. Seetõttu on taimede jaoks äärmiselt oluline ümbritsevas keskkonnas toimuvatele muutustele kiiresti ja adekvaatselt reageerida. Õhulõhed, mis on mikroskoopilised avad taime lehtedel paarikaupa asuvate sulgrakkude vahel, reguleerivad taime gaasivahetust. Õhulõhed kaitsevad taime põua ajal kuivamise eest vähendades kahe sulgraku vahelist ava. Lisaks on nad esimeseks barjääriks põhilise õhusaaste, osooni vastu; siiski pole õhulõhede käitumist osoneerimise jooksul piisavalt uuritud. Õhulõhede avatust regulaaritakse osmootselt aktiivsete ioonide sisse- ja väljavooga läbi sulgrakkude plasmamembraani kanalite ja transporterite; vaatamata sellele on õhulõhede anioonkanalid jäänud molekulaarsel tasemel identifitseerimata.

Käesoleva töö raames uuriti õhulõhede käitumist osoneerimise ajal, kasutades uudset müürlooga (*Arabidopsis*) gaasivahetuse aparatuuri, mis võimaldab jälgida kogu müürlooga roseti gaasivahetust. Osoon indutseeris Kiire Ajutise õhulõhede Sulgumise (KAS) 10 minuti jooksul, misjärel õhulõhed taasavanesid järgneva 40 minuti jooksul. Sulgumise ulatus oli sõltuv osooni kontsentratsioonist. Kolm minutit osooni oli piisav, et indutseerida KAS täies ulatuses ning järgnev osooni eksposition õhulõhede sulgumise-taasavanemise ajal ei omanud mõju KAS-i kineetikale, demonstreerides, et kogu protsess programmeeritakse esimese 3 minuti jooksul. Selleks, et kindlaks teha molekulaarseid komponente, mis vastutavad KAS-i eest, analüüsiti õhulõhede käitumist osoneerimise ajal 56 erineval müürlooga mutandil, mille geenidefekt mõjutab õhulõhede käitumist. KAS puudus täielikult neljal testitud mutandil: *slac1* (algselt *rcd3*), *ost1*, *abi1-1* ja *abi2-1*.

Kuna osooni poolt indutseeritud KAS puudus osoonitundlikul mutandil *slac1*, oli järgnevaks ülesandeks SLAC1 identifitseerimine ja kirjeldamine. Käesolevas töös näidati, et SLAC1 vastutab õhulõhede reaktsioonide eest kõikidele järgmistele teguritele: CO_2, valguse-pimeduse reaktsioonid, osoon, suhteline õhuniiskus, ABA, NO, H_2O_2 ja rakuväline Ca^{2+}. SLAC1 kodeerib esimest aeglast tüüpi (engl. 'slow type') õhulõhede plasmamembraani anioonkanalit, mis on identifitseeritud molekulaarsel tasemel. Kiiret tüüpi (engl. 'rapid type') anioonkanali töö polnud *slac1* mutantsetel taimedel häiritud.

SLAC1 vajab fosforüleerimist aktivatsiooniks. Näidati, et proteiinkinaas OST1 fosforüleerib mitut aminohapet SLAC1 N-terminaalses sabas ning Ser120 on üks põhilisi OST1 märklaudu, mis osaleb SLAC1 aktivatsioonis. KAS puudumine 2C-tüüpi proteiinfosfataaside *abi1-1* ja *abi2-1* mutantidel annab alust oletada, et nendel valkudel on regulatoorne roll osooni poolt indutseeritud aeglast tüüpi anioonkanalite aktivatsioonis.

Summary in Finnish
Tiivistelmä

Kasvit ovat juurtuneet kasvualustaansa; tämän vuoksi on tärkeää, että ne reagoivat riittävästi ympäristön muuttuviin olosuhteisiin. Ilmaraot, jotka ovat muodostuneet huulisoluparista lehtien päällysketossa, säätelevät kasvien kaasunvaihtoa. Mikä tärkeintä, huulisolut suojelevat kasvia kuivumiselta sateettomissa olosuhteissa rajoittamalla ilmaraon avautumisastetta. Ne toimivat myös ensimmäisenä esteenä merkittävää ilmansaastetta, otsonia vastaan, mutta huulisolujen toimintaa otsonialtistuksen aikana ei ole tutkittu riittävästi. Ilmaraon avautumisastetta säätelevät osmoottisesti aktiivisten ionien sisään- ja ulosvirtaus ionikanavien kautta sekä niitä ympäröivät kuljettajaproteiinit huulisolujen kalvolla; huulisolun solukalvon anionikanavan molekyylibiologinen tausta on kuitenkin jäänyt tunnistamatta.

Tämän tutkimuksen puitteissa huulisolujen toimintaa tarkasteltiin äskettäin rakennetun laitteiston avulla, jossa lituruohon (*Arabidopsis*) ilmarakojen kaasujenvaihtoa voidaan seurata kokonaisesta lehtiruusukkeesta. Otsoni aikaansai nopean ja hetkellisen vähentymisen ilmarakojen johtavuudessa kymmenen minuutin aikana altistuksen alkamisesta, minkä jälkeen ilmarakojen johtavuus palautui neljässäkymmenessä minuutissa. Ilmarakojen johtavuuden vähentyminen oli riippuvainen lisätyn otsonin pitoisuudesta. Kolmen minuutin otsonialtistus oli riittävä aikaansaamaan nopean ja hetkellisen vähentymisen ilmarakojen johtavuudessa, eikä tämän jälkeen lisätty otsoni enää vaikuttanut siihen; tämä osoittaa että koko tapahtumasarja on ohjelmoitu kolmen ensimmäisen minuutin aikana. Jotta ilmarakojen johtavuuden nopean ja hetkellisen vähenemisen molekyylibiologisia osatekijöitä voisi saada selville, 59 erilaisesta lituruohomutantista, jotka liittyvät huulisolujen signalointiin, mitattiin otsonivaste. Neljältä testatuista mutanteista, nimiltään *slac1* (alun perin *rcd3*), *ost1*, *abi1-1* ja *abi2-1*, puuttui nopea ja hetkellinen ilmarakojen johtavuus täysin.

Koska otsoniherkästä slac1-mutantista puuttui nopea ja hetkellinen ilmarakojen johtavuuden vähentyminen, oli tämän tutkimuksen seuraava tavoite tunnistaa ja karakterisoida SLAC1. SLAC1 osoitettiin olevan keskeinen säätelytekijä kaikissa merkittävissä vasteissa, jotka ohjaavat huulisolujen avautumisastetta: CO_2, valo-pimeä siirtymä, otsoni, ilman suhteellinen kosteus, ABA, NO, H_2O_2 ja solunulkoinen Ca^{2+}. Se koodaa huulisolujen solukalvojen hidas-tyyppistä (engl. 'slow type') anionikanava-proteiinia, joka on ensimmäinen osoitettu proteiini tällä molekyylibiologisella tasolla. SLAC1 tarvitsee aktivoituakseen fosforylaation. Proteiinikinaasi OST1:n osoitettiin fosforyloivan useita aminohappoja SLAC1:n N-terminaalisesta aminopäästä. Ser120 oli sen pääkohteista, mikä johti SLAC1:n aktivoitumiseen. Nopean ja hetkellisen ilmarakojen johtavuuden vähentymisen puuttuminen 2C-tyyppisten *abi1-1* ja *abi2-1* fosfataasi-mutanteissa viittaa siihen, että näillä proteiineilla on säätelytehtävä otsonin aikaansaamassa anionikanavien hidas-tyyppisessä aktivoinnissa.

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