

New Insights into the Regulation of Stomatal Movements by Red Light, Carbon Dioxide and Circadian Rhythms

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Cover: A magnified epidermal layer with guard cells from an *Arabidopsis thaliana* leaf

(photo: Anastasia Matrosova)

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Abstract

Stomata are small adjustable pores formed by pairs of guard cells that enable gas exchange between leaves and the atmosphere, thus directly affecting water loss and CO₂ uptake in plants. The current work focuses on the regulation of stomatal movements by red light, carbon dioxide and the circadian system and attempts to uncover molecular mechanisms that control guard cell function.

The signaling pathway that underlays stomatal opening in response to red light is yet to be fully elucidated. Here, the HIGH LEAF TEMPERATURE 1 (HT1) protein kinase, known as a negative regulator of high CO₂ stomatal closure, is shown to be a key component of stomatal signaling in response to red light (Paper I). It was demonstrated that HT1 is epistatic to the positive regulator of ABA- and high CO₂-induced stomatal closure OPEN STOMATA1 (OST1) protein kinase both in red light- and CO₂-induced signal transduction in guard cells (Paper I). A photosynthesis-induced drop in intercellular [CO₂] as well as processes originating in the photosynthetic electron transport chain (PETC) have been proposed to signal the guard cell response to red light. Investigation of the effect of PETC inhibitors on stomatal conductance in *Arabidopsis thaliana* ecotypes Col-0 and Ely-1a has suggested the redox state of plastoquinone (PQ) pool to be involved in the regulation of stomatal movements (Paper II).

The full mechanisms that link the regulation of stomatal movements to the circadian clock are yet unknown. The blue light receptor, F-box protein and key element of the circadian clock ZEITLUPE (ZTL) was here shown to physically interact with OST1 protein kinase (Paper III). Furthermore, *Arabidopsis thaliana* mutant plants and *Populus* transgenic lines that lack the activity of ZTL or OST1 demonstrated similar phenotypes, affected in stomatal movement control (Paper III). The work supports a requirement of both ZTL and OST1 in the regulation of guard cell turgor and suggests a direct link between the circadian clock and OST1 activity.

Keywords: stomatal opening, red light, plastoquinone, redox regulation, circadian clock.

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Dedication

To my family

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Matrosova A, Bogireddi H, Mateo-Penas A, Hashimoto-Sugimoto M, Iba K, Schroeder JI, Israelsson-Nordström (2015). The HT1 protein kinase is essential for red light-induced stomatal opening and genetically interacts with OST1 in red light and CO₂-induced stomatal movement responses. *New Phytologist* 208 (4), 1-12.
- II Mateo-Penas A, Matrosova A, Israelsson-Nordström M. Redox-status of the plastoquinone pool determines stomatal movements in plants: lessons from the two *Arabidopsis thaliana* ecotypes Col-0 and atrazine-resistant Ely-1a (manuscript).
- III Jurca M, Matrosova A, Johansson M, Ibanez C, Kozarewa I, Bako L Webb AR, Israelsson-Nordström M, Eriksson. ZEITLUPE interacts with OPEN STOMATA 1 and reveals a clock-regulated stomatal aperture control (manuscript).

Paper I is reproduced with the kind permission of the publisher.

Other papers by the author not included in this thesis:

Ahmad I, Matrosova A, Svennerstam H, Holmlund M, Nincovic V, Israelsson-Nordström M, Ganeteg U. The Lysine Histidine Transporter 1 regulates leaf C/N balance in Arabidopsis (manuscript).

The contribution of Anastasia Matrosova to the papers included in this thesis was as follows:

- I Planned and performed the majority of the experiments. Optimized and executed all gas exchange assays as well as flowering time and de-etiolation experiments. Participated in the gene expression analyses. Performed the metabolite extraction and participated in the data analyses of the metabolomics and ABA concentration analyses. Involved in the manuscript preparation including all figures and tables.
- II Planned and executed all gas exchange experiments and water loss assays. Participated in the data analyses, manuscript writing and figures preparation.
- III Planned and participated in all stomatal response-related experiments conducted at UPSC and performed the radicle emergence assay. Participated in the data analyses.

Abbreviations

$[Ca^{2+}]_{\text{cyt}}$	cytoplasmic calcium concentration
[Ci]	intracellular CO ₂ concentration
[CO ₂]	atmospheric carbon dioxide concentration
ABA	abscisic acid
ABI	Abscisic Acid Insensitive
ABRE	ABA-responsive elements
AHA1	Arabidopsis H ⁺ -ATPase 1
AREB1/ABF2	ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTOR 1
AtALMT12	<i>Arabidopsis thaliana</i> aluminium-activated malate transporter family
BLUS1	BLUE LIGHT SIGNALING1
bZIP	basic leucine zipper transcriptional factors
CA	carbonic anhydrase
CAM	Crassulacean acid metabolism
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CDPKs	calcium-dependent protein kinases
<i>Ci</i>	intercellular CO ₂
CO ₂	carbon dioxide
Col-0	<i>Arabidopsis thaliana</i> Columbia ecotype
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
CRSP	CO ₂ RESPONSE SECRETED PROTEASE
CRY1/2	cryptochromes 1 and 2
DBMIB	2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
EBI	EARLY BIRD
ELF	EARLY-FRLOWERING
EPF	EPIDERMAL PATTERNING FACTOR
<i>gca2</i>	growth controlled by abscisic acid

GI	GIGANTEA
GMC	guard mother cell
GORK	GUARD CELL OUTWARD RECTIFYING K ⁺ CHANNEL
g_s	stomatal conductance
H ₂ O ₂	hydrogen peroxide
HAB	Hypersensitive to ABA
HCO ₃ ⁻	Bicarbonate
HT1	HIGH LEAF TEMPERATURE 1
IRGAs	infra-red gas analysers
K ⁺ _{in} channel	K ⁺ inward-rectifying voltage-gated channel
K ⁺ _{out} channel	K ⁺ outward-rectifying voltage-gated channel
KAT1	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1
LCF	Leaf Chamber Fluorometer
LED	light emitting diode
LHY	LATE ELONGATED HYPOCOTYL
LOV	light, oxygen or voltage
LUX	LUX ARRHYTMO
MAPK	mitogen-activated protein kinase
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NO	nitric oxide
NPQ	non-photochemical quenching
O ₃	Ozone
OST1	protein kinase OPEN STOMATA 1
PCL1	PHYTOCLOCK 1
PhiCO ₂	the CO ₂ assimilation of photosynthesis at a given light intensity
PhiPSII	the quantum yield of photosynthesis
PHO1	phosphate transporter
PHOT1/2	phototropin 1 and 2
PP2C	2C-type protein phosphatase
PQ	plastoquinone
PRR	PSEUDO RESPONSE REGULATOR
PsbO	PHOTOSYSTEM II SUBUNIT O
PSII	photosystem II
PYR/PYL/RCAR	pyrabactin resistance1/PYR1-like receptors/regulatory components of ABA receptors
q_P	photochemical quenching
QUAC1	quick anion channel

RHC1	RESISTANT TO HIGH CARBON DIOXIDE 1
ROS	reactive oxygen species
R-type	rapid anion channels
Rubisco	ribulosebisphosphate carboxylase/oxygenase
SBPase	sedoheptulose-1,7-bisphosphatase
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
SLAH3	The SLAC1 homolog 3
SNF	Superfamily of sucrose-nonfermenting kinase
SnRK	SNF-related protein kinases
S-type	slow anion channels
TMM	TOO MANY MOUTHS
TOC1	TIMING OF CAB EXPRESSION 1
ZTL	ZETLUPE

1 Introduction

1.1 The structure and function of guard cells

The gas exchange between plants and the environment depends on specialized epidermal cells called guard cells. Pairs of guard cells form small pores called “stomata” which literally means “mouth” in Greek due to the stomatal function that allows for water diffusion and CO₂ uptake between plants and the atmosphere. All vascular plants as well as some more primitive plants contain stomata. Guard cells are able to integrate environmental and endogenous signals and convert them into the appropriate turgor pressure changes. Thus the guard cells shrink or swell which results in opening or closing of the stomatal pore (Roelfsema and Hedrich, 2005; Kim et al., 2010). Such stomatal movements facilitate the regulation of water loss through transpiration and the optimization of photosynthesis in response to changing environments.

Guard cells are relatively small with a length of 10 – 80 μm and a width between 9 – 50 μm. The function of guard cells is much determined by their structural features. Stomata can form a kidney or dumb-bell shape. Kidney-shaped guard cells are mainly characteristic for dicots, whereas the dumb-bell-shape is prevalent for most monocots (grasses). Stomata in monocots are arranged in regular arrays whereas in dicots the distribution among other epidermal cells is random. The cell wall of guard cells has a highly specialized structure. Some places of the cell wall are substantially thickened, up to 5 μm, as compared to 1-2 μm thickness in other epidermal cells. This enables cell stability under the large turgor pressure changes that drive stomatal movements. Another distinct feature of the cell wall of guard cells is the alignment of cellulose microfibrils which are radially distributed, this allows for cell size flexibility. Some plant species have additional epidermal cells called subsidiary cells that surround guard cells. Subsidiary cells provide a cushion for the adjacent epidermal cells while guard cells expand or contract. (Heldt et al., 2005; Taiz and Zeiger, 2006). Approximately 0.5 to 3% of the

leaf surface is filled by stomatal pores, where the number of stomata on the abaxial (lower) side of the leaf is usually higher compared to that of the adaxial (upper) side. The abaxial guard cells are often bigger and more opened than the adaxial ones (Willmer and Fricker, 1996). Moreover, the guard cells from the lower surface of the leaf are more sensitive to environmental factors and provide the major part of the leaf gas exchange (Lawson et al., 2003). Compared to other cell types, guard cells have a high metabolic activity provided by an abundance of mitochondria. On the contrary, the amount of chloroplasts in guard cells is lower and their size is smaller as compared to mesophyll cells. The guard cell chloroplasts have low chlorophyll content, limited thylakoid structures and contain few granal stacks. The efficiency of electron transport flow and Calvin cycle in guard cells is therefore lower than that in mesophyll cells (Vavasseur and Raghavendra, 2005). As a result, guard cells possess high rates of respiration and limited photosynthetic capacity. However, photosynthesis in guard cells is functional, although at a lower efficiency than that of mesophyll cells (reviewed in Lawson 2009). A recent study has shown that guard cell photosynthesis plays an important role in the guard cell turgor maintenance (Azoulay-Shemer et al., 2015).

Stomatal movements result from the transport, accumulation and release of osmotically active solutes in guard cells (Lawson and Blatt, 2014). K^+ and Cl^- act as the main inorganic ions, and malate²⁻ and sucrose as the main organic ions. Malate is synthesized in the guard cell cytosol and functions as a key organic solute during stomatal movements. The transport of osmolytes against their concentration gradient across guard cell vacuolar and plasma membrane is driven by H^+ -translocating transporters such as H^+ -ATPases (Roelfsema and Hedrich, 2005). An accumulation of the solutes increases the guard cell osmotic potential that in turn leads to a drop in water potential. The consequent water inflow to the cell causes a rise in the turgor pressure (Roelfsema, 2004). Guard cells swell, thus increasing the guard cell volume, leading to opening of the stomatal pore. Mature guard cells lack plasmodesmata (Willmer and Sexton., 1979). Therefore, transport of water and solutes goes through aquaporins and ion channels situated in the plasma membrane and the vacuole (Roelfsema and Hedrich, 2005) (Fig 1).

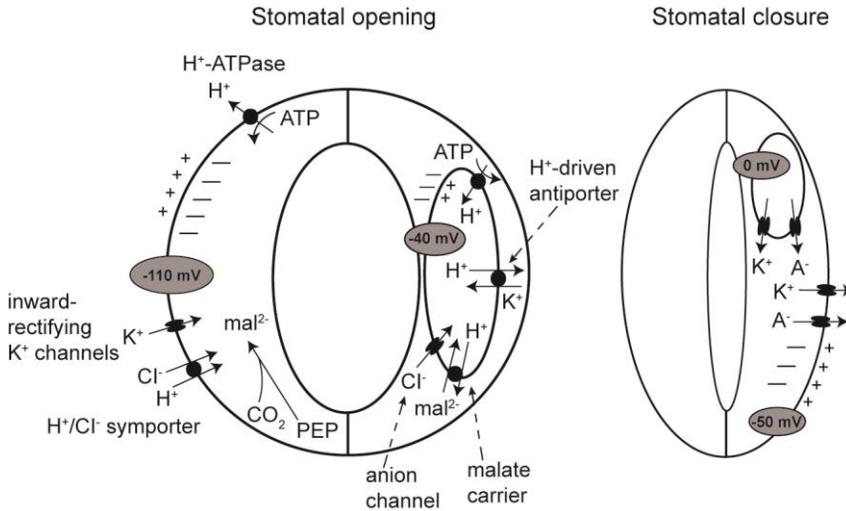


Figure 1. General model of ion fluxes during stomatal opening and closure. Stomatal opening is induced via activation of plasma membrane H^+ -ATPase. The protein provides H^+ extrusion outside of guard cell which leads to decreased membrane potential (-110 mV) and hyperpolarization. The consequent activation of inward-rectifying K^+ channels provides K^+ influx. As one of the counter anions, Cl^- enters guard cell by symport with H^+ , whereas malate is produced in the cytosol. The electrochemical proton gradient across vacuolar membrane is provided by V-Type ATPases which transfers H^+ inside the vacuole lumen. Anion channels transport Cl^- inside the vacuole along the vacuolar electrical potential (-40 mV). A malate carrier maintains cytoplasmic levels of malate decreased. An H^+ -driven antiporter takes up K^+ against the vacuolar membrane potential. During stomatal closure, K^+ efflux through outward rectifying channels causes vacuolar membrane depolarization (0 mV) which is accompanied by Cl^- extrusion through an anion channel. Consequent activation of plasma membrane anion channels provides anion efflux from cytoplasm and depolarization of plasma membrane (-50 mV). Due to membrane potential change, K^+ outward-rectifying channels are activated and release K^+ .

The stomatal conductance at a given time point is a function of the density, size and degree of opening of the stomatal pores. Both the amount and the size of the stomatal aperture in turn depend on environmental conditions. It has been shown that stomatal density and size can be negatively correlated (reviewed in Lawson and Blatt et al, 2014). Stomatal opening is induced by abiotic factors such as low carbon dioxide concentration ($[\text{CO}_2]$), high atmospheric humidity and light. The regulation of guard cell movements by light is complex and depends on the wavelength: red and blue light induce opening of stomata independently. Additionally, opening and closing of stomata during a 24 hour cycle is regulated by a circadian clock as to anticipate the transitions between light and dark. This provides an induction of stomatal opening in the morning before the break of dawn and closing in the evening before dusk (Webb, 2003). In addition, light and darkness alone may induce

stomatal opening and closing respectively in C₃ and C₄ plants. Guard cells of Crassulacean acid metabolism (CAM) plants are closed during the day and opened in darkness at night to prevent water loss. The transpiration rate at night is decreased due to lower atmospheric air temperature and relatively higher humidity, than during the day. During the night in CAM plants CO₂ is taken up and converted into malate in the vacuoles and during the day it is released in chloroplasts and used in photosynthesis in a water conserving manner. Stomatal closing can be induced by elevated ozone and drought conditions and is mediated through abscisic acid (ABA) signaling (Acharya and Assmann, 2009). Elevated [CO₂] also causes stomata to close as sufficient amount of CO₂ can be taken up while minimizing the water loss.

1.2 Regulation of stomatal development

An altered water availability, temperature, light, wind speed and [CO₂] can affect stomatal apertures within minutes. Altered environmental conditions can also induce long-term changes in stomatal density that in turn determines the limits for maximum stomatal conductance. By optimizing plant water loss and CO₂ uptake, stomata aid in determining the water use efficiency of the plant as well as the maximum rate of photosynthesis, leaf temperature, resistance to heat stress and nutrient uptake through promotion of root mass flow (Haworth et al., 2011). Large differences in CO₂ concentration and light intensity within plant communities may therefore affect the development of stomata. The number of newly developed stomata is greater at relatively lower [CO₂] while at relatively higher [CO₂] less stomata is formed. A higher light intensity also increases stomatal index and density (Lake et al., 2001). It was shown that the newly developing leaves adapt their stomatal density to the conditions of the mature leaves exposed either to altered [CO₂] or shaded light. This indicates that both light and CO₂ can regulate stomatal development through long-distance signaling. Moreover, the responses triggered by light and CO₂ are correlated, which is of a significant ecological importance (Lake et al., 2001). The relationship between [CO₂] and stomatal density has been used to analyse fossil plants to predict the historical fluctuations in the atmospheric temperature in correlation with CO₂ changes throughout millions of years (Kürschner, 2001). By calibrating the stomatal density in fossil leaves to [CO₂] in experiments using living Ginkgo specimens, it was possible to reconstruct long-term trends in the changing levels of atmospheric CO₂ from the fossil record. The data obtained were compared to atmosphere temperature levels changing through 300 million years. It demonstrated that the periods of low CO₂ corresponded to the cold periods of Earth's climate, whereas warming

trends were accompanied by increased CO₂ levels. The increasing levels of atmospheric [CO₂] contribute to tremendous environmental changes including global warming, changes in biodiversity and decreased fresh water resources. The reduction of stomatal conductance under elevated [CO₂] will reduce plant transpiration that in turn may cause a continental run-off of fresh water (Betts et al., 2007). Despite such negative ecological effects, elevated atmospheric [CO₂] also leads to increased biomass production and has been suggested to positively impact the water-use efficiency of forests through reduced stomatal conductance levels, which ultimately preserves water availability under desiccation (Keenan et al., 2013). A recently introduced global-scale database, of stomatal conductance from field-grown plants, confirms a relationship between climate and *g_s*, and therefore water use efficiency (Lin et al., 2015). This extensive database can now be used as a tool in establishing various ecosystem productivity models.

In almost all plant species, at least one epidermal cell separates mature stomata from each other. This rule of stomatal patterning is important to maintain normal guard cell function. The activity of genes involved in control of stomatal development ensures that the need for gas exchange and the proper function of stomata are fulfilled. Each consequent step in stomatal development is highly organized and regulated by a number of transcriptional factors and mitogen-activated protein kinases (MAPK) (Nadeau, 2009; Dow and Bergmann, 2014). In *Arabidopsis*, stomatal development is initiated by a series of asymmetric divisions of the epidermal precursor cells, protodermal cells, which lead to formation of meristemoids. A meristemoid cell then transitions into a guard mother cell (GMC). Through a symmetric division GMC directly forms a pair of guard cells. The main transcription factor that controls this step is FAMA, named after the goddess of rumor (Ohashi-Ito and Bergmann, 2006). The small peptides EPIDERMAL PATTERNING FACTOR 1 and 2 (EPF1 and 2) act as negative regulators of stomatal development. They function as ligands of the TOO MANY MOUTHS (TMM) transmembrane receptor (Nadeau, 2009). In contrast, the secretory mesophyll-derived peptide stomagen is a positive intercellular regulator of guard cell development (Sugano et al., 2010). Stomagen (45 amino acids) is derived from a precursor protein STOMAGEN (102 amino acids) and induces stomatal formation in a dose-dependent manner. It has been shown that TMM is epistatic to STOMAGEN (Sugano et al., 2010). This indicates that control of stomatal development is dependent on a competitive binding of EPF1 or 2 and stomagen to TMM. STOMAGEN is highly expressed in immature organs such as leaves, flower buds and stems. It is expressed in inner tissues (the mesophyll) of immature leaves but not in epidermis where guard cells develop. Stomagen is

produced in mesophyll and then secreted to the apoplast to induce stomatal development. Thus the photosynthetic tissues of a leaf regulate their own function by controlling the number of stomata in the epidermis (Sugano et al., 2010).

The stomatal development in leaves is reduced under elevated atmospheric CO₂ as mentioned in the beginning of this section. This process is regulated by carbonic anhydrases (CA1 and CA4) as well as by the secreted protease CO₂ RESPONSE SECRETED PROTEASE (CRSP) (Engineer et al., 2014). The pro-peptide EPF2 is cleaved by the CRSP protease to form a mature EPF2 ligand that represses stomatal development. The *Arabidopsis thaliana* β-carbonic anhydrase double mutant *calca4* shows increased stomatal development at high CO₂ concomitant with down-regulation of EPF2 (Engineer et al., 2014). Guard cell initiation is also inhibited by phytohormones, at least ABA and brassinosteroids (reviewed in Dow and Bergmann, 2014). Thus the development of guard cells is coordinated with accordance to both exogenous and endogenous cues.

1.3 Stomatal closing

A number of environmental factors lead to stomatal closure that protects plants from water loss and therefore minimizes negative effects of these often unfavorable conditions. Drought, [CO₂], decreased relative humidity and elevated atmospheric ozone (O₃) all lead to stomatal closure. Drought conditions induce production of the phytohormone ABA which in turn triggers the stomatal response. High atmospheric [CO₂] leads to reduced stomatal apertures, which enables plants to receive enough carbon dioxide for photosynthesis while minimizing the water loss. Decreased relative humidity slows down the rate of transpiration which in turn serves as a signal for stomata to close (Xie et al, 2006). Ground-level O₃ enters plants through open stomata and becomes degraded into reactive oxygen species (ROS) in the apoplast which causes an oxidative burst. The consequences are stomatal closure and a photosynthetic reduction that prevents further uptake of O₃ (Kangasjärvi et al., 2005). The factors leading to stomatal movements can trigger a fast physiological response taking place within minutes, involving activation and/or inhibition of ion channels, and a more long-term response generating changes on the transcriptional level. Several abiotic factors, leading to stomatal closure, activate pathways that merge at the level of ABA signal transduction in guard cells (Xue et al., 2011, Merilo et al., 2013). The ABA signaling network is complex and is of great importance in regulation of stomatal function and coordination of plant adaptation to stress.

1.3.1 Abscisic acid

The phytohormone ABA is ubiquitous in plants and is found in all photosynthetic organisms (Finkelstein et al., 2013). It regulates different aspects of plant growth and development such as embryogenesis, seed maturation, dormancy and seed germination, stress tolerance and stomatal movements (Koornneef et al., 1998). Under drought conditions, the increased concentration of ABA provokes changes that lead to stomatal closure, a reduced transpiration and drought stress adaptations. ABA controls guard cell function through both induction of stomatal closure and inhibition of stomatal opening (Wang et al., 2010; Yin et al., 2013). ABA produced under water stress causes dephosphorylation of aquaporins which limits water loss and reduces the hydraulic conductance of the leaf, thus possibly triggering or contributing to stomatal closure (Pantin et al., 2013). Additionally, ABA induces intracellular accumulation of protectants such as small hydrophilic proteins dehydrins which confer desiccation tolerance (reviewed in Battaglia et al., 2008).

ABA is derived from carotenoid precursors. The early steps of ABA biosynthesis take place in plastids and the final steps in the cytosol (Finkelstein, 2013). The process of ABA formation is enhanced during oxidative stress conditions (high light, low CO₂), when violaxanthin is converted into zeaxanthin to prevent over-oxidation of the photosynthetic reaction centers (Havaux and Niyogi, 1999). Zeaxanthin is an ABA precursor and facilitates the dissipation of excess energy and therefore prevents light damage of the photosystem II during oxidative stress conditions (Heldt et al, 2005). In other words, when oxidative stress induces the conversion of violaxanthin to zeaxanthin, the formation of ABA also increases.

During recent years, important achievements were made in the understanding of ABA signal transduction in guard cells. Several components of ABA signaling pathways have been identified by genetic screens and a number of ABA-deficient mutants were revealed. The main signaling components include a key positive regulator of ABA signaling in guard cells OPEN STOMATA 1/ SNF-related protein kinase 2.6 (OST1/SnRK2.6) and the negative regulators the protein phosphatases of the 2C-type protein phosphatase (PP2C) family. Other components include calcium-dependent protein kinases (CDPKs), mitogen-activated protein kinases (MPKs), reactive oxygen species, anion channels and cytosolic calcium ion concentration ([Ca²⁺]_{cyt}). Some of the above-mentioned signaling components will be discussed in more detail below.

The identification of the ABA receptor has been accomplished only in recent years due to redundancy of the receptor family. Eventually a yeast-two

hybrid screen (Ma et al, 2009) and a screen for resistance to pyrabactin (Park et al, 2009) led to the identification of the cytosolic ABA receptors designated as pyrabactin resistance1/PYR1-like receptors/regulatory components of ABA receptors (PYR/PYL/RCAR) (Gonzalez-Guzman et al., 2012). These receptors belong to the START domain superfamily consisting of 14 proteins in *Arabidopsis*. Several studies on the crystal structure of ABA receptors uncovered the mechanisms of their function. The receptor has an open ligand binding cavity where ABA binds causing a conformational change of the receptor (Raghavendra et al, 2010). ABA bound to PYR/PYL/RCAR physically interacts with PP2C protein phosphatases and forms a co-receptor complex (Raghavendra et al., 2010). The PYR/PYL/RCAR proteins at least partially regulate stomatal closing responses also to other environmental signals than ABA, such as high CO₂, O₃, darkness and low humidity (Merilo et al 2013). This may suggest that these abiotic conditions to some extent merge at the level of ABA signaling. In addition, a possible plasma membrane ABA receptor has been proposed.

Many of the ABA signaling components downstream of the ABA receptor have been revealed. Several of them are also involved in other guard cell signaling pathways leading to stomatal closure. For example, drought and other abiotic stresses cause increased production of reactive oxygen species (ROS) and an oxidative burst. Under presence of ABA, hydrogen peroxide (H₂O₂) synthesis, mediated by NADPH oxidases, rises in guard cells. H₂O₂ induces rapid production of nitric oxide (NO), cytosolic alkalization and the elevation of [Ca²⁺]_{cyt} via activation of plasma membrane Ca²⁺-permeable channels, all of which mediate stomatal closure. NO and H₂O₂ are important second messengers in guard cell ABA signaling which downregulate K⁺_{in} channels (Garcia-Mata et al., 2003). Elevated [Ca²⁺]_{cyt} in turn activates efflux guard cell anion channels (Hedrich et al., 1990).

The efflux of anions from guard cells is achieved by slow-activating (S-type) and rapid-activating (R-type) anion channels. The voltage-independent SLOW ANIONCHANNEL-ASSOCIATED 1 (SLAC1), with permeability to chloride (Cl⁻) and nitrate (NO₃⁻), confers anion current activities to multiple signals including ABA, high CO₂, Ca²⁺, ozone and darkness and therefore plays a pivotal role in stomatal closing responses (Vahisalu et al., 2008; Negi et al., 2008). The SLAC1 homolog 3 (SLAH3) is a voltage-dependent S-type plasma membrane anion channel that conducts nitrate. Both SLAC1 and SLAH3 contribute to the release of anions in guard cells in the presence of ABA. A possible R-type anion channel in guard cells has been identified as a member of the aluminum-activated malate transporter family (ALMT) of *Arabidopsis thaliana*, AtALMT12 (Meyer et al., 2010) or QUAC1 (quick

anion channel 1). The voltage-dependent vacuolar QUAC1 functions to release malate anions from guard cells into the apoplast and facilitates stomatal closure. Recently it has been shown that phosphate efflux through the phosphate transporter PHO1 anion channel also plays a role in the stomatal response to ABA (Zimmerli et al., 2012). The release of anions results in depolarization of the guard cell plasma membrane. Depolarization in turn leads to an inhibition of inward-rectifying K⁺ channels (KAT1, K⁺_{in}) and proton pumps of the plasma membrane as well as an activation of outward K⁺ channels such as GUARD CELL OUTWARD RECTIFYING K⁺ CHANNEL (GORK) (Osakabe et al., 2014). This causes a reduction in the osmotic pressure and guard cell turgor and hence leads to stomatal closing.

A number of key phosphatases and protein kinases regulate ABA-induced stomatal closure by protein phosphorylation/dephosphorylation events that enable a fast response on protein activity and anion channel activation. As mentioned earlier in the text, PP2C protein phosphatases contribute to ABA perception complex with PYR1/PYL1/RCAR. Among them are ABSCISIC ACID INSENSITIVE 1/2 and HYPERSENSITIVE TO ABA 1 (ABI1/2 and HAB1) that function as negative regulators of ABA-induced stomatal closing (Raghavendra et al, 2010). A major positive regulator in guard cell ABA signal transduction is the protein kinase OPEN STOMATA 1 (OST1/SnRK2.6/SnRK2E) (Mustilli, 2002; Youshida, 2002; Vlad, 2009). It belongs to a superfamily of sucrose-nonfermenting kinases (SNF) found in yeast. It is highly expressed in guard cells but also in vascular tissues (Hrabak et al, 2003; Fujii et al, 2007). ABA is sensed by the PYR/PYL/RCAR receptor that then binds and therefore inhibits PP2C (ABI1/2) phosphatase activity. In the absence of ABA, PP2Cs instead inhibits OST1 protein kinase by dephosphorylation (Vlad et al., 2009). The formation of a PYR/PYL/RCAR – PP2C heterotrimeric receptor complex therefore facilitates OST1 activation (Ma et al., 2009; Fujii et al, 2009). By using protein-protein interaction assays, it has been shown that OST1 and ABI1 interact with SLAC1. OST1 activates SLAC1 anion currents by phosphorylation. ABI1 prevents activation of SLAC1 by direct phosphorylation (Brandt et al., 2013) as well as through dephosphorylation of OST1 (Geiger et al., 2009). OST1 also activates R-type anion channel QUAC1 in guard cells (Imes et al., 2013). Alternatively, SLAC1, as well as SLAH3, is directly activated by Ca²⁺-activated CDPKs, (Brandt et al., 2012) which in the presence of ABA are released from ABI1 inhibition and activated by elevated [Ca²⁺]_{cyt}.

In addition to stomatal closure activation, ABA also inhibits light-induced stomatal opening. It has been suggested that OST1 kinase regulates both of these processes. In the presence of ABA, SnRk2.6/OST1 downregulates the

inward-rectifying K^+ channels (KAT1) by direct binding and phosphorylation (Sato et al., 2009; Acharya et al., 2013). Another study showed an ABA-induced inhibition of blue light-induced proton pump phosphorylation and the absence of ABA-inhibited proton pump phosphorylation in the *ost1-3* mutant (Yin et al., 2013). Thus, it has been suggested that ABA inhibits guard cell plasma membrane H^+ -ATPase phosphorylation through OST1 activity. The inhibition of stomatal opening by ABA is also coupled to down-regulation of cytosolic Ca^{2+} , NO and ROS production as well as cytosolic alkalization (Yin et al., 2013).

Drought stress causes alterations in ABA-induced gene expression and many of these genes are also regulated by light and the circadian clock (reviewed in Fujita et al., 2011). ABA triggers activation of guard cell-expressed transcription factors that bind genes containing ABA-responsive elements (ABREs) within their promoters. The transcription factor that binds ABRE motifs is named ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTOR 1 (AREB1/ABF2). ABA-activated SnRK2 kinases are necessary for control of gene expression via phosphorylation of basic leucine zipper (bZIP) transcriptional factors including ABI5 and AREB1 (Fujita et al., 2011) during seed development and germination. Other transcription factors such as MYBR (MYB-recognition site) and MYB44 are expressed in guard cells and regulate light-induced stomatal opening and ABA-induced closure, respectively (reviewed in Kim et al., 2010).

1.3.2 Carbon dioxide

Carbon dioxide in the atmosphere is the major source of carbon on Earth. CO_2 is used in photosynthesis, as a substrate of the enzyme ribulose biphosphate carboxylase-oxygenase (Rubisco), and is produced in respiration. Rubisco utilizes CO_2 for fixation of carbon (carboxylase reaction) or oxygen (oxygenase reaction) that leads to photorespiration. Prior to the industrialization the concentration of carbon dioxide in the atmosphere was controlled by photosynthetic organisms. The additional industrial sources, such as deforestation and fossil fuels burning, lead to an increase of CO_2 in the atmosphere (Hetherington and Raven, 2002). This does not only affect the efficiency of photosynthesis but leads to environmental changes on a global level. The latter include ocean acidification, accumulation of plant biomass, biodiversity changes, global warming, decline of fresh water resources in the world and an altered agricultural productivity.

Stomata open in response to relatively low intercellular CO_2 concentration (C_i) and close at higher $[C_i]$, rather than to ambient $[CO_2]$ (Mott, 1988). In a plant, the concentration of $[C_i]$ can reach up to 600 ppm during night due to

respiration activities and drop to 160 ppm during photosynthesis in the light (Engel et al., 2015). The level of C_i in leaves is in turn affected by photosynthesis, respiration and increasing atmospheric $[\text{CO}_2]$. High $[\text{CO}_2]$, like ABA, induces activation of anion channels as well as K^+_{out} channels in guard cells (Brearley et al., 1997; Roelfsema et al., 2004). This causes plasma membrane depolarization which eventually leads to closing of the guard cells. Physiological $[\text{CO}_2]$ shifts do not affect cytosolic pH as shown for *Vicia faba* (Brearley et al., 1997) and *Arabidopsis thaliana* guard cells (Xue et al., 2011).

The mutant alleles *abi1-1* and *abi2-1* were isolated in a screen based on their ABA insensitivity and they show a degree of stomatal CO_2 insensitivity (Webb and Hetherington 1997). Similarly, the mutant *growth controlled by abscisic acid* (*gca2*) is impaired in ABA-induced stomatal closure (Allen et al., 2001), as well as in $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate modulation by high $[\text{CO}_2]$ and CO_2 -induced stomatal closing (Young et al., 2006). Based on the above mentioned examples, ABA and CO_2 signaling pathways may merge in the control of stomatal aperture.

Meanwhile, several mutants have been identified that show stomatal insensitivity to $[\text{CO}_2]$ changes but retain functional ABA responses. A high throughput infra-red leaf thermography set-up was used in a genetic screen for *Arabidopsis* mutants with altered stomatal responses to CO_2 . As a result the two allelic mutations in HIGH LEAF TEMPERATURE 1 (HT1), *ht1-1* and *ht1-2*, with impaired ability to regulate $[\text{CO}_2]$ -induced stomatal movements, were isolated (Hashimoto et al., 2006). Homozygous plants carrying the recessive mutation *ht1-2* demonstrate a constitutive high- $[\text{CO}_2]$ stomatal closure phenotype. Thus it has been concluded that HT1 protein kinase negatively regulates high $[\text{CO}_2]$ -induced stomatal closing. The plants lacking HT1 activity retain functional blue light responses. They also close in response to ABA which suggests that HT1 protein kinase possibly acts upstream of the point where ABA- and CO_2 -induced stomatal closure pathways merge and/or downstream of ABA signaling close to anion channel activation. HT1 is highly expressed in guard cells but not in mesophyll cells in leaves (Hashimoto et al., 2006). The carbonic anhydrases are enzymes that catalyse the reverse process of CO_2 and water into bicarbonate ions and protons. In *Arabidopsis*, plants that lack the activity of the carbonic anhydrases BETA CARBONIC ANHYDRASE 1 and 4 (βCA 1 and 4) have strongly disrupted responses to CO_2 and demonstrate high constitutive stomatal conductance (g_s) (Hu et al., 2010). The double mutant *ca1/ca4* do not close or open stomata in response to CO_2 while exhibiting functional closing in the presence of ABA. The data shown by Hu et al., 2010 support an early role of CAs in the perception of altered $[\text{CO}_2]$, upstream of HT1 function. It has also been shown that increased

[HCO₃⁻] induce guard cell S-type anion channel activation (Hu et al., 2010), which was confirmed in more recent studies (Xue et al., 2011; Tian et al., 2014). In *ht1-2* mutant the S-type anion channel activity induced by HCO₃⁻ is enhanced (Xue et al., 2011). This is in accordance with the HT1 function as a negative regulator of stomatal closing response to high [CO₂] (Hashimoto et al., 2006). The loss-of-function mutation in *SLAC1* consequently causes both ABA and high [CO₂] insensitivity (Negi et al., 2008; Vahisalu et al., 2008; Merilo et al., 2013). OST1 plays a key role in activation of *SLAC1* anion currents (Geiger et al., 2009; Lee et al., 2009) – a crucial step in high [CO₂]- and ABA-induced stomatal closure. Therefore, OST1 would constitute an appropriate merging point for several pathways in control of stomatal aperture.

Very recently, a new RESISTANT TO HIGH CARBON DIOXIDE 1 (RHC1) MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-like protein has been identified to act as an important regulator of high [CO₂]-induced stomatal closure (Tian et al., 2015). By using BiFC assay in *Arabidopsis* protoplasts and yeast two-hybrid analysis physical interactions of RHC1 with CAs and HT1 have been established. Gas exchange measurements of stomatal conductance revealed a constitutive high CO₂, *ht1-2*-like phenotype in the *rhc1/ht1-2* double mutant. This indicates that HT1 is epistatic to RHC1 and functions downstream of RHC1 in high [CO₂]-induced stomatal closing. Unlike *rhc1/ht1-2*, the *rhc1* guard cells are impaired in activation of S-type anion current by bicarbonate which indicates that RHC1 negatively regulates HT1 and is required for S-type anion channel activation. RHC1 interacts with CAs and is suggested to function downstream of them as a HCO₃⁻ sensor. According to the new findings, HT1 in turn inhibits OST1 under ambient [CO₂]. During increased [HCO₃⁻], RHC1 has been shown to interact with HT1 and inhibit it, whereby OST is released from HT1 inhibition and subsequent *SLAC1* activation can occur. On the basis of this knowledge a new model for CO₂-induced stomatal closing has been proposed (Figure 1).

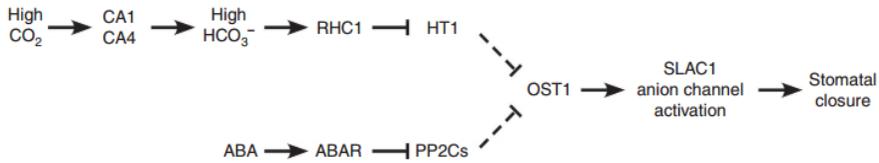


Figure 2. A model for high $[\text{CO}_2]$ - and ABA-induced stomatal closure signalling pathway. Low CO_2 conditions keep $[\text{HCO}_3^-]$ at reduced levels which facilitates HT1 interaction and inhibition of OST1. Thus, SLAC is maintained inactive and stomata open. Under elevated $[\text{CO}_2]$, the intracellular levels of HCO_3^- increase which is sensed by RHC1 causing its interaction with HT1. Consequently, HT1 is recruited to the plasma membrane and releases OST1 inhibition. As a result, SLAC1 is phosphorylated and activated by OST1 which causes anion efflux from guard cells and leads to stomatal opening.

1.4 Stomatal opening induced by light

Blue and red light regulate stomatal opening through different signaling pathways (Shimazaki et al., 2007). The red light (620 – 750 nm) stomatal response is photosynthesis-dependent and saturates at higher intensities similar to photosynthetic active radiation (PAR). The blue light (450 – 495 nm) stomatal response is fast, photosynthesis-independent, saturates at lower fluencies ($\sim 50 \mu\text{mol m}^{-2}\text{s}^{-1}$) (Zeiger, 2000) and is more efficient than that of red light. Interestingly, a red light background is necessary to enhance the effect of blue light on stomatal opening processes (Assman, 1988; Vavasseur and Ragavendra, 2005). A weak blue light illumination under a strong red light background induces rapid stomatal opening in Arabidopsis, whereas no opening is triggered in the absence of red light (Shimazaki et al., 2007).

1.4.1 Blue light-induced stomatal opening

Blue light in guard cells is perceived by the blue light receptors phototropins (PHOT1 and 2), cryptochromes (CRY1 and 2) and possibly also the chloroplast carotenoid zeaxanthin (Kinoshita et al., 2001; Mao et al., 2005; Zeiger and Zhu, 1998). Zeaxanthin is postulated as a photoreceptor chromophore for stomatal opening (Zeiger and Zhu, 1998), but the apoprotein for this receptor remains yet unknown. The blue light receptors PHOT1 and PHOT2 belong to a family of light-activated receptor kinases associated with the plasma membrane. Phototropins contain two photosensory light, oxygen or voltage (LOV) domains at the N-terminus and a serine/threonine domain at the C-terminus. Blue light brings about photoexcitation of the LOV domains that leads to autophosphorylation of the phototropin protein (reviewed in Christie 2007). The autophosphorylation induces the binding of 14-3-3 proteins

(Kinoshita et al., 2002) that maintain PHOTs in an active state. CRY 1 and CRY2 have been shown to act additively with phototropins to regulate guard cells opening under blue light (Mao et al., 2005). A recent study has identified the novel protein kinase BLUE LIGHT SIGNALLING 1 (BLUS1) which is directly phosphorylated by phototropins (Takemiya et al., 2013). The *blus1-1* and *blus1-2* mutant allele plants are impaired in blue light-induced stomatal opening, suggesting BLUS1 phosphorylation is essential in this process. Another positive regulator in blue light stomatal signaling is the type 1 protein phosphatase (PP1). It has been shown that BLUS1 acts upstream of PP1 in the blue light-induced signaling pathway which eventually leads to plasma membrane H⁺-ATPase activation (Takemiya et al., 2013). Plasma membrane H⁺-ATPases in guard cells are activated via phosphorylation of a penultimate threonine in the C-terminus of the protein by a serine/threonine kinase which is yet unknown (Kinoshita and Shimazaki, 1999). It has been shown that 14-3-3 protein binds to the phosphorylated C-terminus of the pump and therefore keeps it in an active state (Kinoshita and Shimazaki, 1999). Activation of H⁺-ATPase drives extrusion of H⁺ from the guard cells leading to the increase in the inside-negative electrical potential across plasma membrane (Assmann et al., 1985; reviewed in Roelfsema and Hedrich, 2005 and in Shimazaki et al., 2007). The effect of hyperpolarization is enhanced by blue light-induced inhibition of plasma membrane anion channels (Marten et al., 2007). As a consequence of hyperpolarization, inward-rectifying voltage-gated K⁺ channels (K⁺_{in}) are activated (Schroeder et al., 1987; Assmann and Shimazaki, 1999). In *Arabidopsis*, KAT1 (potassium channel in *Arabidopsis thaliana* 1) is a major gene, among several, that encode K⁺ influx channels (reviewed in Shimazaki et al., 2007). It has been shown that in *Commelina communis* starch degradation occurs during the day in guard cells upon blue light irradiation, in contrast to mesophyll cells where starch builds up during the night (Vavasseur and Raghavendra, 2005). The release of stored energy during the day fuels the guard cell proton pumps with ATP and provides osmolites that facilitate stomatal opening (Shimazaki et al., 2007). However, this may not apply for all the species as in *Arabidopsis* guard cells accumulate starch during the day and degrade it at night (Stadler et al., 2003). Malate²⁻ produced from starch degradation is one of the organic anions which, together with inorganic Cl⁻ and NO₃⁻ ions, accumulate in the guard cells and act as counter ions of K⁺. The water potential decreases upon solute accumulation in the cytoplasm resulting in guard cell water uptake, the raise of turgor pressure and stomatal pore opening (Fig. 2) (Roelfsema and Hedrich 2005; Vavasseur and Raghavendra, 2005; Shimazaki et al., 2007).

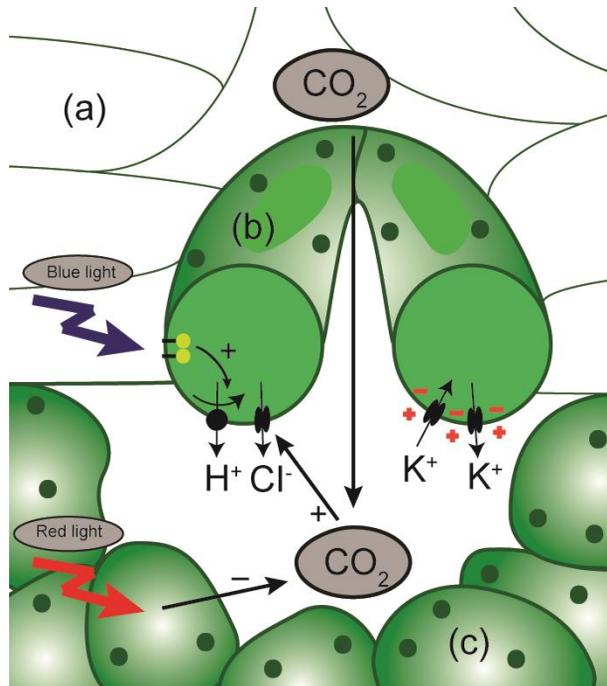


Figure 3. An overview of signaling pathways involved in red and blue light-induced stomatal opening. (a) Epidermal pavement cell, (b) guard cell and (c) mesophyll cell. Red light induces photosynthesis and decreases the [CO₂] within the leaf, thereby deactivating anion channels in guard cells. Blue light is perceived by phototropins and activates H⁺-ATPase. Both red and blue light cause hyperpolarization of the guard cell with consequent K⁺ uptake, turgor increases and stomatal opening. When more CO₂ is taken up, an activation of guard cell anion channels will lead to stomatal closing, thus providing a negative feedback mechanism. Figure adapted from Roelfsema and Hedrich, 2005.

The activation of plasma membrane H⁺-ATPase acts as a driving force in light-induced stomatal opening responses. Overexpression of guard cells H⁺-ATPase leads to enhanced stomatal opening and photosynthesis as well as plant growth (Wang et al., 2014). Overexpression of *PATROL1*, a gene that controls the translocation of AHA1 H⁺-ATPase to the plasma membrane, increases stomatal opening and enhances both CO₂ assimilation rate and plant growth (Hashimoto-Sugimoto et al., 2013). These findings show that the activity of plasma membrane H⁺-ATPase in control of stomatal opening can be regulated by different ways other than phosphorylation of a penultimate threonine (Kinoshita and Shimazaki, 1999). It remains to be shown whether blue and/or red light can induce H⁺-ATPase activity in ways other than the penultimate threonine phosphorylation.

1.4.2 Red light-induced stomatal opening

Red light triggers stomatal opening less efficiently than blue light does (Sharkey and Raschke, 1981). Thus, high light intensity and a continuous illumination are required for an effective red light-induced opening response (Willmer and Fricker, 1996; Shimazaki et al., 2007). Red light-evoked guard cell swelling is likely to be mediated by photosynthesis, since it saturates at red light fluencies similar to those for photosynthesis. Moreover, the red stomatal response is blocked by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor of photosystem II (PSII) (Sharkey and Raschke, 1981; Tominaga et al., 2001; Messinger et al., 2006). In comparison, DCMU does not inhibit blue light-induced stomatal opening (Schwartz and Zeiger, 1984). The photosynthetic CO₂ fixation by guard cells was estimated to be 2-4 % of mesophyll cells and such limited carbon fixation could not produce a sufficient amount of osmolites to initiate stomatal movements (Willmer and Fricker, 1996). Additionally, degradation of starch does not occur under red light (Vavasseur and Raghavendra, 2005). Thus the most likely source of sugars is from the apoplast via transport into guard cells.

The mechanism that induces a K⁺ uptake and drives stomatal opening by red light is yet to be elucidated. Several studies based on patch clamp and stomatal bioassay techniques have shown a red light-induced activation of H⁺ pumps in isolated protoplasts and epidermis (Schwartz and Zeiger, 1984; Serrano et al., 1988; Olsen et al., 2002). The proton pump identity/ies could include member/s of the plasma membrane H⁺-ATPases, possibly activated by an increased amount of cytosolic ATP produced during photophosphorylation in guard cell chloroplasts. At least functional guard cell photosynthesis is required for maintaining turgid guard cells at ambient light conditions (Azoulay-Shemer et al., 2015). Several patch clamp- and immunohistochemistry-based studies conducted in recent years, using intact leaves, epidermis and protoplasts, did, however, not reproduce an activation of H⁺-ATPase by red light (Roelfsema et al., 2001; Taylor and Assmann, 2001; Hayashi et al., 2011). Due to the unclear results obtained in these investigations, an unambiguous involvement of H⁺-ATPase in the stomatal red light response is yet to be elucidated.

Red light drives photosynthesis and leads to a reduction in intercellular CO₂ (C_i) in leaves and this decreased [C_i] has been suggested to induce stomatal opening (Fig. 2; Heath, 1950; Roelfsema et al., 2002). Whether it is the photosynthesis in mesophyll and/or guard cells that mediates red light-induced stomatal opening is the matter of an ongoing discussion. Despite a lower quantum efficiency of the photosynthetic electron transport chain (PETC) in guard cells compared to mesophyll cells, the photosynthetic machinery is entirely functional (Lawson et al., 2002). Several studies have been conducted

to understand the role mesophyll cells in red light-induced stomatal opening. In electrophysiological studies where a beam of red light was projected at a single guard cell (Roelfsema et al., 2001; Taylor and Assmann, 2001), an expected hyperpolarization of the plasma membrane was not recorded. On the contrary, in gas exchange measurements where red illumination covered both guard cells and surrounding mesophyll cells the intercellular CO₂ decreased and guard cell swelling was observed (Shimazaki et al., 2007; Roelfsema et al., 2002). To address the issue further, norflurazon-treated (Nf) *Vicia faba* and variegated *Chlorophytum comosum* plants were studied for their stomatal responses (Roelfsema et al., 2006). Due to inhibition of biosynthesis of carotenoids by norflurazon, both GC and MC in treated *Vicia faba* contained non-photosynthesizing chloroplasts whereas they were functional in GCs of albino leaf patches in *Chlorophytum comosum*. Red light-induced stomatal opening was absent in either representative of albino leaves, both lacking functional chloroplasts in mesophyll cells, while the responses to blue light, low [CO₂] and ABA were normal. These studies provide evidence for a role of mesophyll cells in transferring the red light signal to guard cells, independently of an active guard cell photosynthesis. Interestingly, a recent study confirm that guard cell photosynthesis does not influence stomatal responses to CO₂ and ABA, but is required to provide sufficient guard cell turgor (Azoulay-Shemer et al., 2015).

Tobacco plants with reduced content of cytochrome *b₆f* complex or RuBiSCO showed decreased CO₂ assimilation while stomatal opening under red light was intact, thus the red light response did not depend on mesophyll or guard cell photosynthetic rate (Baroli et al., 2008). Another study examined stomatal responses and photosynthetic capacity in transgenic tobacco plants with reduced content of sedoheptulose 1,7-bisphosphatase (SPBase), an enzyme involved in the control of Calvin Cycle regeneration capacity (Lawson et al., 2008). The stomatal opening to red light was increased in the antisense plants at all levels of C_i while the CO₂ assimilation was lowered and the response to high [CO₂] was functional. It is noteworthy that the transgenic plants also showed a decreased quantum efficiency of PSII electron transport in guard and mesophyll cells. This suggests that photosynthetic operating efficiency relies on the regeneration capacity of carbon fixation. The study concluded that light- and CO₂-regulated stomatal movements are controlled by photosynthetic electron transport processes. This is supported further by a study in cocklebur (*Xanthium strumarium*) (Messinger et al., 2006), where C_i -evoked changes to stomatal conductance were dependent on the balance between PET capacity and photosynthetic carbon reduction reactions. Additionally, when C_i was kept constant stomata had normal responses to red

light, indicating that red light-induced stomatal opening can be regulated without a photosynthetic reduction of C_i . These authors concluded that stomatal responses to red light can be mediated by a C_i -independent signal or be due to processes originating in guard cells themselves (Messinger et al., 2006).

The redox state of the photosynthetic electron transport chain has been suggested to participate in the control of stomatal movements (Busch, 2014). High light conditions lead to an over-reduction of the PETC forcing energized electrons to merge with oxygen produced in photosynthesis, leading to ROS production and consequent photosynthetic inhibition. When the light intensity exceeds the photon utilization capacity of the chloroplast, appropriate changes in gene expression are induced to provide protection from oxidative damage. It has been established that the redox status and the presence of ROS can act as signaling components to regulate gene expression and protein function in several physiological processes, including stress acclimation, hormonal responses, metabolism, growth and development (Shigeoka and Maruta, 2014). For example, redox modulation regulates the activity of a vacuolar type of H^+ -ATPase that is involved in various physiological processes in plant cells (Siedel et al., 2012). Oxidative stress also occurs in the mitochondria and peroxisomes where oxygen is formed during respiration and photorespiration. Some of the components of the PETC, such as plastoquinone (PQ) and the thioredoxin/ferredoxin system, act as redox sensors that perceive the energy flow changes between the PETC and the Calvin cycle of photosynthesis (reviewed in Vener et al., 1998). The redox state of the PQ pool in particular has recently been suggested to play an important role in the red light-induced regulation of stomatal opening (Busch, 2014).

Photosynthesis-independent stomatal opening to red light has been shown in orchids and *Arabidopsis* and suggested to be perceived through phytochrome B, due to stomatal closure under far-red illumination (Talbot et al., 2002 and 2003). The *phyB* mutant displays smaller stomatal apertures while *PHYB*-overexpressing plants exhibited extremely opened stomata upon red illumination, supporting a positive function of PHYB in red light-induced signaling in guard cells (Wang et al., 2010). In conclusion, current data supports evidence of both photosynthesis-independent and -dependent pathways in control of stomatal opening to red light.

1.5 The stomatal movements and circadian clock

1.5.1 The circadian system

The environment of most living organisms is subject to rhythmic changes. In order to synchronize seasonal and daily timing of metabolism, gene expression and physiological processes with changing environmental conditions, most of the living organisms have an endogenous circadian clock with a period of approximately 24 hours. In plants, the circadian clock mainly has been elucidated in *Arabidopsis thaliana* (*Arabidopsis*) and shown to regulate such processes as stomatal opening, photosynthesis, transport of starch and cotyledon movement (Hotta et al., 2007). These metabolic and physiological processes are subordinated to an endogenous circadian clock and therefore display a daily rhythm even in the absence of the environmental signals. In plants as well as in mammals, the circadian clocks of different tissues are synchronized with each other (Hotta et al., 2007; Nagel and Kay 2012). For example, in plants the clock of shoot apex influences the circadian rhythms of root tissues (Takahashi et al., 2015). Also, a recent study has suggested that vascular and mesophyll tissues asymmetrically regulate each other, where the vasculature clock controls the gene expression and physiological responses of neighboring mesophyll cells (Endo et al., 2014).

The process of circadian regulation can be divided into: 1) input pathways which transduce the environmental signals to synchronize the internal clock with local time; 2) the central oscillator which provides the periodicity of the clock and 3) the output pathways which couple the activity of the oscillator to the observable rhythms (Hotta et al., 2007). As a general circadian clock theme, positive and/or negative interactions between clock components form autoregulatory interlocked transcription-translation feedback loops (Dunlap, 1998). Therefore, circadian clock proteins are able to regulate their own expression throughout the circadian period.

Input of clock-controlled processes. The endogenous clock responds and entrains (resets) to the daily cycles of light, darkness and temperature. A number of photoreceptors are involved in circadian regulation by light. The transduction pathway of the light signal involves the red/far-red mediating phytochromes (phys) and the blue light mediating cryptochromes (crys) (Somers et al., 1998). ZEITLUPE (ZTL) is one of the key elements of the circadian clock (Somers et al., 2000; Kevei et al., 2006). Similarly to phototropins, ZTL protein contains a flavin-binding LIGHT, OXYGEN OR VOLTAGE (LOV) domain at its N-terminus and therefore acts as a photoreceptor together with phys and crys. ZTL also contains six C-terminal KELCH repeats which facilitate the protein interactions at the LOV domain

(Kevei et al., 2006). Furthermore, ZTL contains an F-box domain with E3 ligase activity and is known to act as a post-translation regulator of protein degradation in the Arabidopsis clock.

Temperature perception by the circadian clock system is yet poorly understood, but allows entrainment to the plant's environment. Atmospheric temperature changes affect levels of expression of circadian clock genes such as *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *GIGANTEA (GI)* and *LATE ELONGATED HYPOCOTYL (LHY)* (Gould et al., 2006) which function is discussed below. Through such regulation, the oscillator is buffered against temperature changes, an ability called temperature compensation. It allows the period of the circadian clock to be stably maintained through a range of physiological temperatures (e.g. 12-27°C; Gould et al., 2006). Importantly, low temperature is also received by the clock and necessary for promoting cold response and freezing tolerance (Eriksson and Webb, 2011).

Circadian oscillator. A number of plant circadian clock components have been characterized. The first described Arabidopsis clock mutant *timing of cab expression1* also known as *pseudo-response regulator1 (toc1/prr1)* was identified in a luciferase imaging screen based on its short-period phenotype (Millar et al., 1995; Eriksson and Millar, 2003). TOC1/PRR1 is a principal component of the Arabidopsis central oscillator with a peak of expression at ~12 h after dawn (Zeitgeber time (ZT) 12) (Somers et al., 1998). It is a member of a larger family of clock proteins; the PSEUDO-RESPONSE REGULATORS including e.g. TOC1/PRR1, PRR3, PRR5, PRR7 and PRR9 (Eriksson and Millar, 2003).

On the opposite end to TOC1/PRR1, in the morning, the light induced MYB-like transcription factors CCA1 and LHY are other key components of the circadian oscillator in Arabidopsis (Schaffer et al., 1998; Wang and Tobin, 1998).

The molecular mechanisms of the core circadian clock are based on transcriptional feedback regulation of the TOC1, CCA1 and LHY (Alabadi et al., 2001). Mechanistically, TOC1 acts as a DNA-binding transcriptional repressor of CCA1 and LHY which in turn act to repress TOC1, together forming a core oscillator loop (Huang et al., 2012). Several, additional components of the clock have been identified and allocated to the evening or morning loops in accordance with the expression time together forming a network of interlocked feedback loops enforcing a robust oscillator function (Dixon et al., 2014). PRR9, 7 and 5 (expressed in this order, from morning to evening) bind and repress *CCA1* and *LHY* gene expression, similarly to TOC1, whereas the CCA1 and LHY positively regulate *PRR7* and *9* gene expression in the morning (Eriksson and Millar, 2003; Nakamichi et al 2010). LUX

ARRHYTMO (LUX) also known as PHYTOCLOCK1 (PCL1) has been shown to play a central role in the generation of rhythms (Hazen et al., 2005; Onai et al., 2005). Together with EARLY FLOWERING 3 and 4 (ELF3 and ELF4) LUX forms an evening complex (EC) of proteins that are the key regulators of clock gene expression at night (Hotta et al., 2007; Nagel and Kay, 2012). GIGANTEA (GI) acts as a negative regulator of EC genes which inhibit expression of *TOC1* (Pokhilko et al., 2012).

Several circadian clock components belong to the E3 ubiquitin-ligase enzyme family and F-box protein family which ubiquitinate proteins and therefore target them for degradation by the proteasome (Seo and Mas, 2015). For instance, ZTL mediates ubiquitination for degradation by the 26S proteasome of TOC1 and PRR5 through direct interaction and therefore regulates stability of the proteins (Mas et al., 2003; Kiba et al., 2007). The function of ZTL is also controlled by proteasome degradation and stabilized via direct interaction with GI under blue light which provides the peak of ZTL expression in the evening (Kim et al., 2007). ZTL-GI interaction also prevents GI degradation and regulates its nuclear and cytoplasmic distribution (Kim et al., 2013). Cytosolic or nuclear localization and timely degradation are important aspects in the regulation of individual circadian clock components. Further, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) ubiquitin E3 ligase is involved in the control of light input to the clock through ubiquitination and subsequent proteasomal degradation of the EC component ELF3. Additionally, GI is targeted for degradation at night by interaction with both COP1 and ELF3 (Kim et al., 2013). Thus, the circadian clock system is regulated by several mechanisms, including ubiquitination and phosphorylation, which provide the fine-tuning of the timing mechanism.

Output of clock-controlled processes. The temporal control of a plant's life is pervasive and impacts gene expression, development, metabolism and physiology. While clock-controlled leaf and petal movements were noted in classical time and in the 18th century by Linnaeus (Eriksson and Millar, 2003), many circadian clock regulated processes are subtle and require elaborate techniques in order to be monitored. For instance, to study the circadian clock-regulated gene expression or cytosolic calcium levels fluctuations, luciferase bioluminescence assay is commonly used. Luciferase (*LUC*) (firefly luciferase and calcium-dependent luciferase apoaequorin) acts as a reporter of the gene of interest expression and used in combination with sensitive experimental tools capable of detecting bioluminescence of the expressed protein (Hotta et al., 2007; Salinas and Sanchez-Serrano, 2006).

Recently a plethora of processes and pathways have been shown to be under control of the circadian clock (Kusakina and Dodd, 2012; Araujo et al., 2011;

Seo and Mas, 2015). Among them is abscisic acid (ABA)-induced signalling as well as other stress related pathways (Araujo et al., 2011). To this end SNF-related protein kinases (SnRKs, see chapter 1.3.3.) have been shown to function as putative mediators of the circadian regulation of the ABA responses (reviewed in Kusakina and Dodd, 2012).

1.5.2 Circadian regulation of stomatal movements

Stomatal aperture is regulated by different factors including the circadian clock. This control may appear as a rhythmic change in aperture under constant conditions or as a rhythmic change in sensitivity to some environmental factor, such as light (Gorton et al., 1989; Tallman, 2004). The period of the rhythm in guard cell opening varies within 24 hours. Due to the fact that mature guard cells lack plasmodesmata and in detached epidermis stomatal movements maintain rhythmicity, it has been assumed that each individual guard cell has its own circadian oscillator. Individual stomata are able to sustain a circadian rhythm independently of mesophyll cells (Gorton et al., 1989).

Due to circadian control of guard cells, plants with C3 and C4 carbon fixation as well as Crassulacean acid metabolism (CAM) plants may anticipate dawn and dusk transitions to promote stomatal opening or closing according to their needs. In *Arabidopsis* (C3 plant) at midday, stomata stop opening and before dusk they initiate closing (Dodd et al., 2006). The stomatal opening response to light is to some extent circadian-regulated and depends on the time of the day. The effectiveness of light to promote stomatal opening is highest early in the day and lowest during the subjective night when stomata are more sensitive to darkness in C3 plants (Gorton et al., 1993). Other stimuli such as potassium anions, fusicoccin and auxin have similar effect on the intensity of stomatal opening during different times of day/night (Hotta et al., 2007). ABA is more effective in promoting stomatal closing in the afternoon than before noon. This allows CO₂ to be taken up in the morning hours while in the afternoon it minimizes the water loss through transpiration (Hotta et al., 2007). The circadian control of C4 metabolism was shown to be generally based on C3 model well described in *Arabidopsis* despite specialized anatomy and enzymatic system (Khan et al., 2010).

The circadian regulation of CO₂ fixation in CAM plants is well studied and based on the rhythmic control of Phosphoenolpyruvate carboxylase (PEPc) phosphorylation. Such regulation enables PEPc to function at night facilitating malate accumulation in the vacuole when stomata are open. The circadian clock components and their function in C3 plants are similar to CAM (Boxall et al., 2005). The exception is *ZTL* gene expression that shows transcriptional oscillation in the CAM ice plant (*Mesembryanthemum crystallinum*) but not in

Arabidopsis (Boxall et al., 2005). Together, this shows that stomatal movements require an integrated, diurnal control as exerted by the circadian system to foresee daily changes in the environment. This provides an efficient gas exchange and facilitates optimal photosynthesis and water status of the plant throughout the day.

2 Aims and Objectives

The goal of this study was to improve the knowledge about the mechanisms regulating stomatal movements induced by red light, carbon dioxide and circadian rhythms. Red light has been proposed to induce stomatal opening through photosynthetic activities where a drop in the intercellular [CO₂] and/or PETC redox state are likely mediators. Our aim was to study any link between red light- and [CO₂]-induced stomatal movements and to address the role of the redox state of PETC in stomatal aperture control. We also attempted to link stomatal signaling to the mechanisms of circadian regulation of guard cell movements.

The objectives of our investigation were as follows:

1. Examine the roles of HT1 and OST1 protein kinase activities in the red light- and CO₂-induced stomatal opening responses.
2. Investigate the involvement of PSII activity and a redox state of PQ pool in the regulation of stomatal movements.
3. Study the involvement of OST1 activity in the circadian control of stomatal apertures.

3 Materials and methods

3.1 Methods to measure stomatal responsiveness

The alteration of guard cell turgor aids the coordination of photosynthesis and transpiration in plants. In order to study stomatal movements, several distinct physiological approaches can be utilized, including bio-assays of stomatal apertures analyzed by microscopy. Estimation of the water loss from a detached leaf, by a decrease in weight with time, is a straight-forward approach to compare relative stomatal conductance changes between plant leaves. The measurement of stomatal CO₂ and H₂O exchange enables a record of real-time stomatal responsiveness of an intact leaf and plant. To monitor stomatal gas exchange, the Portable Photosynthesis System LiCOR 6400XT (LI-COR Inc., NE, USA) has been widely used to estimate stomatal conductance (g_s) as well as chlorophyll *a* fluorescence parameters of photosynthesis. As an alternative, snap-shot stomatal conductance readings of an intact plant can be obtained by a Portable Steady-state Leaf Porometer (Decagon Devices Inc., Pullman, WA, USA). The implementation of several approaches to analyse stomatal conductance allows for a more informative and complete analysis of stomatal behavior.

3.2 Gas exchange measurements

The Li-COR 6400XT provides measurements of stomatal conductance and photosynthesis of intact leaves, while controlling the relative humidity, intensity of light, [CO₂] and temperature, in real-time. The measurements with Li-COR 6400XT are based on the calculation of the differences in gaseous concentrations of CO₂ and H₂O. In essence, stomatal transpiration is calculated based on the amount of H₂O efflux from the leaf to the ambient air. Similarly,

the carbon assimilation is readily estimated as the amount of CO₂ that enters the leaf through stomatal pore and is used up in photosynthesis.

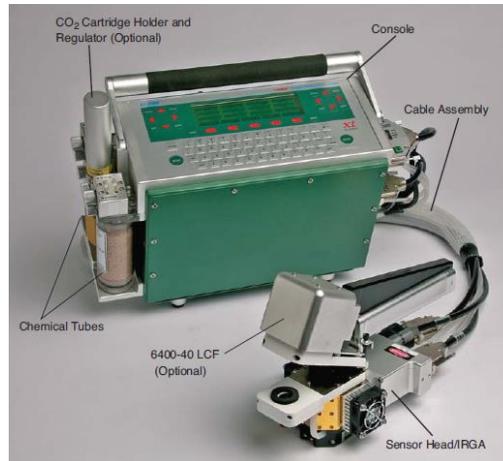


Figure 4. The Li-COR 6400XT Portable Photosynthesis System and its main components. Image used by kind permission from © Li-COR, Inc.

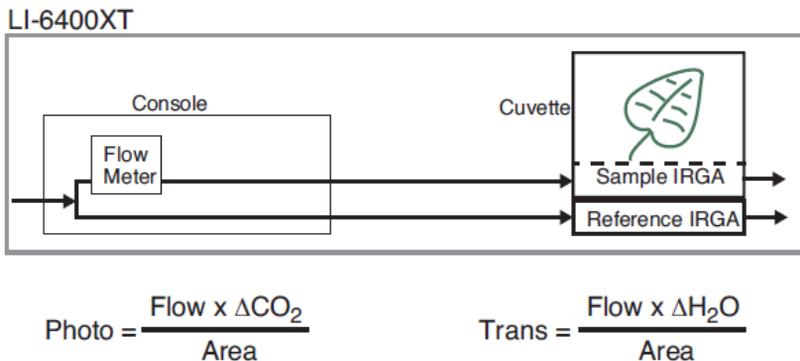


Figure 5. A schematic overview of the gas exchange system Li-COR 6400XT. The measurements of photosynthesis and transpiration are based on the differences in CO₂ and H₂O in an air stream that is flowing through the leaf cuvette. Image used by kind permission from © Li-COR, Inc.

The Li-COR is operated through a controlling console to where the sensor head is connected (Figs 3, 4). A leaf is placed into a chamber attached to the sensor head. The environmental conditions for the incoming air are set up at the beginning of each experiment. Thus, the ambient air circulates in the tubing system of the instrument passing through the chamber and exposes the leaf to

the pre-set conditions. Two infra-red gas analysers (reference and sample IRGAs), located inside the sensor head, are able to precisely detect the concentrations of CO₂ and H₂O (signal noise 0.04 and 0.02 mmol mol⁻¹ respectively) within the leaf chamber and outside of it (Fig. 4). The most widely used parameter of gas exchange measurements in this thesis is stomatal conductance (g_s , mmol H₂O m⁻² s⁻¹) which is calculated taking into account the molar concentration of water vapor within the leaf (mmol H₂O mol⁻¹ air), the total atmospheric pressure P (kPa) and the temperature of the leaf T_l (°C), whereas boundary layer conductance is not included (www.licor.com, see Manufacturer instructions for full description). Stomatal conductance denotes how much H₂O is evaporated from a leaf at certain environmental conditions. In other words, it reflects the width of the stomatal pores. In Li-COR 6400XT, the stomatal conductance is measured from both abaxial (AB, lower) and adaxial (AD, upper) sides of the leaf, however measurements from individual surfaces are also possible. As already mentioned in Section 1.2, stomatal conductance depends on several parameters such as stomatal density, size and degree of pore openness. Thus, when analyzing stomatal conductance of different genotypes/ecotypes, the stomatal density needs to be similar to allow for adequate g_s comparisons.

The Li-COR 6400XT allows gas exchange measurements where light quantity and quality can be controlled in a Leaf Chamber Fluorometer (LCF) 6400-40 (LI-COR Inc., NE, USA). The LCF unit has a light emitting diode (LED)-based source of actinic and fluorescence illumination attached to the sensor head. Red, far-red and blue LEDs as well as two detectors of LCF are together used for fluorescence measurements. The blue and red diodes can also be controlled separately to select illumination of a particular intensity and/or quality. Thus, the LCF unit enables measurements of photosynthetic carbon assimilation rate, several fluorescence parameters (PhiPSII, F_v/F_m , PhiCO₂, NPQ etc) together with stomatal conductance. The LCF analyser head was designed for leaves bigger than those of *Arabidopsis thaliana*, limiting the analysis of this species only to mature, well-expanded and healthy leaves. To achieve suitable leaves of this stature, plants need to be grown for extended periods of time in stable and well-controlled environmental short-day conditions.

3.3 Leaf Porometer measurements of stomatal conductance

The handheld Leaf Porometer SC-1 (Decagon Devices Inc., Pullman, WA, USA) is a portable device that enables stomatal conductance measurements of individual intact leaves. A Leaf Porometer consists of a controller connected to

the sensor head that is clamped onto a leaf to conduct the measurements. The sensor head contains an aluminum sensor block with a diffusion path inside and a detachable desiccant chamber underneath. The desiccant chamber decreases the ambient air humidity providing a pronounced humidity gradient between the outside atmospheric conditions and the leaf-evaporating surface. This humidity gradient constitutes the basis of a porometer measurement. The water vapor concentrations at the leaf surface and at the humidity sensors inside the diffusion path are then detected. The resistance (vapor conductance) between a leaf and the first humidity sensor and between the first and the second sensors are also calculated. These variables are together used to estimate stomatal conductance ($\text{mmol m}^{-2}\text{s}^{-1}$) measured from one side of the leaf (www.decagon.com, see Operator's manual for full description).

A Leaf Porometer enables a fast and effective screening of many plants of interest. The measurements are conducted in the environment where plants are grown to provide an understanding of stomatal function in monitored plants. Unlike Leaf Porometer-based analyses, LiCOR can be used to monitor real-time stomatal conductance responses to manually altered environmental conditions. A complementary application of both techniques in an experiment provides for versatile analyses of stomatal function and ultimately yields more reliable conclusions.



Figure 6. An overview of the Leaf Porometer SC-1. The Porometer instrument consists of a controller unit that is connected to sensor head that is clamped to a leaf. Image used by kind permission from © Decagon Devices Inc., Pullman, WA, USA.

4 Results and Discussion

This study has been carried out to improve the current knowledge on the regulation of guard cell opening responses. The main focus of the investigations was to understand the molecular mechanisms and regulation of red light and CO₂-induced stomatal movements and whether this is dependent on photosynthesis or not. Several aspects of guard cell function have been addressed in the manuscripts presented in this thesis. Paper I investigates the genetic relationship between important guard cell ABA and CO₂ signaling elements, namely the HT1 and OST1 protein kinases, during red and blue light-induced stomatal opening and under various [CO₂]. Paper II addresses the role of the redox state of the PQ pool in the photosynthetic electron transport chain in regulating the red light-induced stomatal opening response. Lastly, Paper III establishes a direct interaction between the key regulator of stomatal movements OST1 protein kinase and the circadian clock protein ZTL, thereby directly linking the clock to stomatal aperture control.

4.1 The role of HT1 and OST1 protein kinases in red light- and CO₂-induced stomatal opening (Paper I)

The HT1 protein kinase is a negative regulator of high [CO₂]-induced stomatal closing, consequently plants lacking HT1 activity show a constitutive high [CO₂] stomatal response and a lack of stomatal opening to low [CO₂] (Hashimoto et al., 2006). The blue light-induced stomatal opening in both *ht1-1* and *ht1-2* mutant alleles remains relatively intact. *OST1* encodes a key protein kinase of ABA signaling. Mutations in this gene cause a reduced stomatal response to ABA (Mustilli et al., 2002; Yoshida et al., 2002; Vlad, 2009), as well as to high and low [CO₂] (Xue et al., 2011; Merilo et al., 2013; Tian et al., 2014). In the present study, the stomatal responses to red light and CO₂ in *ht1-1*, *ht1-2*, *ost1-3* and *ost1-3/ht1-2* mutant plants were investigated.

The complete lack of stomatal opening to red light in *ht1-2* mutant plants led to a conclusion that HT1 has a key function in red light-induced stomatal opening responses, as a positively-acting component. In order to exclude the possibility that HT1 plays a general role in any red light-controlled response, we analyzed several developmental processes induced by red light. It was found that de-etiolation, the time of flowering (Fig. 7, Paper I) and the rate of seed germination (Hashimoto et al., 2016) were intact in plants lacking HT1 activity. Furthermore, a high expression of *HT1* in guard cells has been previously shown (Hashimoto et al., 2006), suggesting HT1 protein kinase function is specific to guard cell signaling.

An impairment of low CO₂ signaling in *ht1* mutant plants could likely be a reason for the disrupted stomatal opening to red light, if red light largely mediates its stomatal control by a photosynthesis-induced drop in C_i. Mutant *ost1-3* plants opened their stomata to red light while stomata of *ost1-3/ht1-2* mimicked the insensitive response of *ht1-2* (Figure 1, Paper I). This indicates that *HT1* is epistatic to *OST1* gene function during red light-induced stomatal opening. In conclusion, the presence of HT1 activity is required for a functional stomatal response to red light illumination, but it does not provide confirmatory evidence of [C_i] as an intermediate step. A functional stomatal opening to blue light in *ht1-1* and *ht1-2* mutant allele plants (Fig. 2 Paper I; Hashimoto et al., 2006) indicates a role of HT1 specifically in the red light stomatal response. Additionally, blue light-evoked stomatal opening in *ost1-3/ht1-2* mimicked the response of *ost1-3* indicating a different contribution of HT1 and OST1 in response to different light regimes. In several real-time gas exchange measurement assays, the stomatal conductance (g_s) in *ost1-3/ht1-2* plants at stable light conditions was intermediate to that of its parental lines, indicating that HT1 and OST1 can additively control stomatal conductance. The OST1 protein kinase mediates an inhibition of plasma membrane H⁺-ATPase phosphorylation in guard cells in the presence of ABA, based on the lack of such a phosphorylation in the *ost1-3* background (Yin et al., 2013). Therefore, it is possible that less H⁺-ATPase inhibition in stomata of *ost1-3* mutant would partly relieve the constitutive high [CO₂] phenotype of *ht1-2* in *ost1/ht1-2* double mutant.

The participation of HT1 in both red light- (Paper I) and low CO₂-controlled stomatal opening (Hashimoto et al., 2006) makes it a candidate gene in control of red light-induced opening mainly dependent on [C_i]-signaling. The CO₂ responses in stomata of *ht1-1*, *ht1-2*, *ost1-3* and *ost1-3/ht1-2* mutants were therefore further addressed in a relatively high and low [CO₂] gas exchange assay. High [CO₂] led to stomatal closing in wild type and *ost1-3* mutant plants, while in *ht1-2* and *ost1-3/ht1-2* stomata did not close (Paper I,

Figure 3). Similarly, low [CO₂]-induced stomatal opening occurred in wild type and *ost1-3* in contrast to the minor stomatal closing observed in *ost1-3/ht1-2* and *ht1-2*. Therefore, based on Paper I, HT1 is epistatic to OST1 gene function in guard cell CO₂ signaling (both to higher and lower levels) and during red light-induced stomatal opening. Stomata of the *ost1-3* mutant are high [CO₂]-insensitive, both by decreased anion channel activation by bicarbonate and less high [CO₂]-induced stomatal closing as detected by gas exchange measurements (Xue et al., 2011; Tian et al., 2015). Tian and co-authors proposed a model where stomatal closing by both elevated [CO₂] and ABA levels are integrated at the level of OST1, where HT1 inhibits OST1 at low [CO₂]. Interestingly, in our study the high [CO₂] stomatal closing response in *ost1-3* is largely functional which suggests the existence of other pathways controlling stomatal response to high [CO₂] which do not involve anion channel activation by OST1 protein kinase. Furthermore, results from Paper I suggests HT1 is epistatic to OST1, not the opposite, raising the need for an expanded CO₂ signaling model. The exact role of HT1 in CO₂-induced stomatal signaling and whether HT1 can promote SLAC1 or other anion channel activity, where OST1 phosphorylation also would be required, remains to be elucidated. Whether HT1 functions at several steps during the signal transduction of stomatal closing, both acting on OST1 protein function as well as more directly on anion channel activity is yet to be elucidated. In comparison, ABI affects both OST1 phosphorylation and SLAC1 activity (Brandt et al., 2012).

4.2 The effect of restricted stomatal apertures in *ht1* mutant plants (Paper I)

HT1 mutation may cause a stomatal dysfunction that in turn alters the photosynthetic capacity and metabolic processes of *ht1* mutant allele plants. Accordingly, we observed reduced chlorophyll *a* fluorescence parameters and a decreased internal concentration of CO₂ (*C_i*) in *ht1-2* (Paper I, Fig. 4, Supplementary Fig. S4). However, the carboxylation capacity and the potential electron transport rate were not affected in the two *ht1* mutant allele plants. A decreased rate of CO₂ assimilation in *ht1* was recovered to wild type levels when [*C_i*] was administered to stable levels (Paper I, Fig. 5). *HT1* mutation did not affect either carboxylation or oxygenation reactions of RuBisCO which together with photosynthetic electron transport chain (PETC) influence the carbon assimilation rates (Farquhar et al., 1980). Furthermore, the metabolite profile analyses did not show any differences in the levels of carbohydrates in *ht1* mutant plants regardless a reduced CO₂ uptake. Therefore, it may be the

restricted stomatal apertures that results in the observed reduced photosynthesis processes of *ht1*. A decreased $[C_i]$ in *ht1* may lead to a lowered $\text{CO}_2:\text{O}_2$ ratio and increased photorespiration processes. However, serine and glycine, which are produced during photorespiration, were not different between the genotypes (Paper I, Table 1). Additionally, ABA content was not increased in the *ht1* mutant plants indicating a lack of oxidative stress caused by restricted CO_2 influx. On this basis it was concluded that HT1 functions specifically in guard cell signaling and that the photosynthetic alterations in *ht1* are caused by restrained *ht1* stomatal apertures, not by reduced photosynthetic activities alone.

4.3 PETC-mediated regulation of stomatal opening to red light is dependent on the redox state of the PQ pool (Paper I, Paper II)

The red light-induced signaling of guard cells is likely to be mediated through photosynthesis (Sharkey and Raschke, 1981; Messinger et al., 2006) where a drop in $[C_i]$ is one of several possible signals (Heath, 1950; Roelfsema et al., 2002), although photosynthesis-independent pathways have also been suggested (Wang et al., 2010). Data in this thesis (Fig. 1, Fig. 3, Paper I) support genetic evidence that red light-induced stomatal opening can be mediated via a photosynthesis-induced low $[\text{CO}_2]$ -dependent signaling, where the function of HT1 protein kinase is crucial. In order to test this hypothesis more widely, we also included experiments with *Arabidopsis thaliana* mutants lacking two crucial guard cell-expressed CO_2 -binding enzymes. Plant carbonic anhydrases function during early $[\text{CO}_2]$ guard cell signaling and *calca4* mutants show slowed stomatal responses to altered $[\text{CO}_2]$ (Hu et al., 2010). In a double mutant lacking the activity of carbonic anhydrases CA1/CA4, red light induced a slowed, but statistically significant, stomatal response as compared to wild type (Paper I, Fig. 8a; Supplementary Fig. 5). The stomatal conductance in this experiment increased even at stable C_i levels. When *cal/ca4* plants were exposed to low $[\text{CO}_2]$, stomata did not open although the level of C_i decreased. Hence, alternative pathways other than reduced $[C_i]$ may participate during stomatal opening to red light. In the literature, such possible mediators include photosynthetic electron transport or its end products (Messinger et al., 2006; Lawson et al., 2008). Busch has proposed the redox status of the PETC components, PQ in particular, to be a signal during stomatal movement responses (Busch, 2014).

The stomatal response to red light has been shown to be reduced upon application of the PETC inhibitor DCMU or antisense reduction of the PSII

protein PsbO, which both lead to a disrupted electron flow upstream PQ function (Messinger et al., 2006; Dwyer et al., 2012). In contrast, red light-induced stomatal opening was increased in transgenic antisense lines where cytochrome *b6f* and SBPase were silenced, and unchanged in Rubisco antisense tobacco lines (Baroli et al., 2008; Lawson et al., 2008). We used a wide range of inhibitors within the PETC (Paper II, Fig. 3) to study any link between photosynthetic capacity and stomatal conductance in the *Arabidopsis thaliana* ecotypes Col-0 and Ely-1a. Ely-1a has been shown to carry a point mutation in the chloroplastic gene *psbA* that encodes the D1 protein of PSII reaction center (El-Lithy et al., 2005). The mutation leads to a reduced efficiency of photosynthesis (*Fv/Fm*, *PhiPSII*) and a specific resistance to the herbicide atrazine in Ely-1a. In Col-0, atrazine negatively affects *Fv/Fm* and *PhiPSII* and stomatal conductance is decreased, while in Ely-1a there is no effect neither on chlorophyll *a* fluorescence parameters nor stomatal closing (Paper II, Fig. 3). Interestingly, in response to DCMU and ioxinyl, which similarly to atrazine block PETC at the donor site of PSII, stomatal closing remains intact in both ecotypes. Stomatal opening to red light, detected by real-time gas exchange analysis, in Ely-1a was not affected by atrazine application while in Col-0 it was slowed as compared to control treatment (Paper II, Fig. 4). DBMIB inhibitor treatment, which blocks the electron flow from PQ towards cytochrome *b6f* complex (keeping the PQ in a more reduced state) lowered the photosynthetic parameters of Col-0 and Ely-1a while stomatal apertures remained unchanged in both ecotypes as measured by a Leaf Porometer (Paper II, Fig. 3). Due to the effects of atrazine on Col-0 and Ely-1a it can be concluded that the PSII activity is involved in stomatal aperture control and mediates the opening to red light independently of C_i . Disruption of PETC by any of the inhibitors used in our study would ultimately result in a reduced ATP, NADPH production and CO_2 fixation in the Calvin cycle leading to an increased $[C_i]$ that would signal stomatal closure. However, the absence of a reduced aperture under DBMIB treatment indicates stomatal movement control by the redox state of PQ pool rather than an altered $[C_i]$. Based on the PETC inhibitor analyses, an oxidized redox state of the PQ pool drives stomatal closure while its reduced state maintains stomata opened.

In paper II, the PQ pool was estimated as more reduced in Ely-1a, where steady-state g_s levels were higher as compared to Col-0. A reduced state of the PQ pool may thus function as one of the factors that contribute to stomatal opening or inhibition of closing. Here, a reduced PQ pool (DBMIB treatment) kept stomata open, without inducing further opening both in Ely-1a and Col-0 (Paper II). Previously, a role of PQ in red light-induced opening has been

suggested (Busch 2014). It may be argued that other factors, in concert with a reduced PQ pool, are required for wider guard cell apertures.

The signaling components that transduce the redox information to the guard cells are yet to be fully elucidated. H_2O_2 and NO are crucial in maintenance of a redox homeostasis and are important in ABA- and CO_2 -induced stomatal movements (Shi et al., 2015). Thus, H_2O_2 and/or NO may function as signaling molecules that transduce the redox status of PQ pool into appropriate stomatal movements. It remains to be shown whether HT1 protein kinase in guard cells may perceive a redox signal and mediate appropriate stomatal movements. HT1 shares high homology to MAPKK kinases (Ichimura et al., 2002) and MAPK pathways can be activated by ROS during oxidative stress (Kovtun et al., 2000; Son et al., 2011).

4.4 Is there a role for H^+ -ATPase activation in the red light-induced stomatal opening mediated by HT1?

In Paper I it was shown that a lack of HT1 activity abolishes stomatal opening to red light. Thus, HT1 protein kinase can ultimately regulate signaling pathways that confer red light-induced stomatal opening responses. Hyperpolarization of guard cell plasma membrane is a crucial process that ultimately leads to stomatal opening (Shimazaki et al., 2007). During blue light-induced stomatal opening, H^+ -ATPase pump activity drives membrane hyperpolarization (Assmann et al., 1985; Roelfsema et al., 2001) which is followed by inhibition of S-type anion channels (Marten et al., 2007). The proton pump function requires an ATP energy supply. Thus, light-induced stomatal opening responses are not only dependent on a passive inhibition of closure, but the result of an active energy-consuming alteration of the cell membrane potential. It has been shown that HT1 prevents activation of anion channel SLAC1 during high $[\text{CO}_2]$ signaling (Xue et al., 2011; Tian et al., 2015). The involvement of H^+ -ATPase activity in the red light-induced stomatal opening has been a matter of debate and a signaling compound that would trigger H^+ extrusion under red light illumination is yet to be elucidated. Several studies have confirmed a red light activation of plasma membrane H^+ -ATPase (Schwartz and Zeiger, 1984; Serrano et al., 1988; Olsen et al., 2002) while others did not (Tylor and Assmann, 2001; Roelfsema et al., 2001). Red light, unlike blue light, does not induce activation of H^+ -ATPase by phosphorylation of a penultimate threonine in the C-terminus of the protein (Kinoshita and Shimazaki, 1999). Alternatively, red light-induced stomatal movements can also be increased by the amount of H^+ -ATPase in the plasma membrane (Hashimoto-Sugimoto et al., 2013; Wang et al., 2014).

In this study, an attempt was made to address a functional interplay between HT1 and plasma membrane H⁺-ATPase activity by examining the process of radical emergence, an important phase during seed germination associated with elongation growth of the embryo hypocotyl (lower hypocotyl and the hypocotyl-radicle transition zone) (Wu et al., 2012). Germination is induced by red light and hypocotyl radicle protrusion, that marks the beginning of seed germination, depends on H⁺-ATPase activity at the plasma membrane (Enriquez-Arredondo et al., 2005). In contrast, ABA inhibits seed germination through suppression of H⁺-ATPase activity (van den Wijngaard et al., 2005; Planes et al., 2014). The plasma-membrane H⁺-ATPase AHA1 is important during elongation growth of the embryo (Enriquez-Arredondo et al., 2005). The genes *AHA1* (At2g18960) and *HT1* (At1g62400) are highly expressed during the globular stage of embryo development in the chalazal seed coat (Arabidopsis eFP browser at bar.utoronto.ca database). A co-localization of *AHA1* and *HT1* gene expression during embryo development may indicate a possibility for the proteins of these genes to be present and function together in other tissues over the course of plant growth.

The phytotoxin fusicoccin activates H⁺-ATPase (Johansson et al., 1993) at the same site as 14-3-3 proteins during blue light-induced stomatal opening (reviewed in Shimazaki et al., 1997). Previously, the application of fusicoccin was shown to restore the radicle emergence of a mutant affected in seed germination (Wu et al., 2012). In the current study, the role of H⁺-ATPase in seed germination was explored using a novel technique, used here for the first time, where ABA-inhibited radicle emergence can be restored by fusicoccin application.

Early radicle emergence in seeds of the strong *htl-2* mutant allele and *ost1-3/htl-2* were slowed as compared to Col-0 at 24 h, whereas *ost1-3* exhibited a more pronounced delay (Fig. 6). The delay in early seed germination processes in *ost1-3* correlates with findings of a recent study (Fig. 1d in Waadt et al., 2015). ABA treatment (0.75 μM) decreased the radicle emergence in *htl-1*, *htl-2* and wild type to similar levels and correlates to the previously described *htl* mutant phenotype (Hashimoto et al., 2006). The *ost1-3* mutant allele was severely affected in radicle emergence under ABA treatment (Fig. 6) in accordance to earlier studies where *ost1-3* germination was decreased during low concentrations of ABA (Yoshida et al., 2002). Addition of the phytotoxin fusicoccin (FC), thus activating plasma membrane H⁺-ATPases, completely restored the ABA-inhibited radicle emergence in Col-0, *htl* and *ost1-3/htl-2* mutant allele seeds to wild-type levels (-ABA), while *ost1-3* was only partially restored. This assay demonstrates that early ABA-inhibited radicle emergence is largely dependent on repressing H⁺-ATPase activity and introduces radicle

emergence analysis as a tool to study the regulation of H⁺-ATPase activity. During radicle emergence, HT1 and OST1 are revealed to positively regulate H⁺-ATPase activity, in contrast to OST1 function in guard cells (Yin et al., 2013). The effect of OST1 on H⁺-ATPase activity may thus differ depending on tissue and biological and developmental context. Interestingly, even during seed germination (Fig. 6) *HT1* gene function is epistatic to *OST1*, as it is during red light and CO₂ responses in guard cells (Paper I). It may be argued that the regulation of red light-induced stomatal opening by HT1 include both the inhibition of high [CO₂]-induced stomatal closure as well as H⁺-ATPase activation, which together drive hyperpolarization of plasma membranes in guard cells.

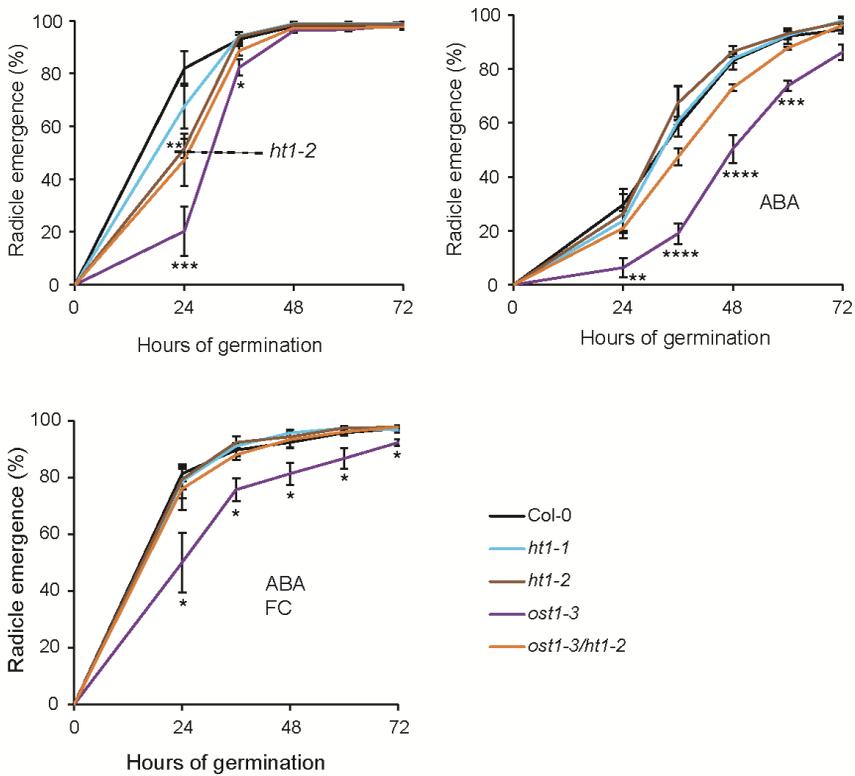


Figure 7. The effect of ABA and fusicoccin on the radicle emergence in the wild type Col-0 and *ht1-1*, *ht1-2*, *ost1-3* and *ost1-3/ht1-2*. Sterilized seeds were plated on ½ MS plates supplemented with or without 0.75 μM ABA and 1 μM fusicoccin, cold-treated for 48 h and consequently transferred to a growth chamber under a light fluency of ~150 μmol m⁻²s⁻¹. After 24 h, the percentage of radicle emergence was estimated at 12 h intervals. Data presented are the mean of ± SE (n=5; 60 seeds per experiment), similar results were obtained in three repeated independent experiments.

4.5 The circadian clock regulates stomatal movements through the light receptor ZTL and the protein kinase OST1 (Paper III).

The OST1 protein kinase is a key regulator of ABA and CO₂-induced stomatal closure (Yoshida et al., 2002; Xue et al., 2011). Work in this thesis has established a role of OST1 also in the regulation of stomatal opening to red light (Paper I). The control of stomatal movements by light and ABA is tightly coordinated by the circadian clock (Gorton et al., 1993; Kusakina and Dodd, 2012; Hotta et al., 2007). The effectiveness of environmental factors in driving stomatal responses depends on the time of the day and therefore on circadian rhythms. For example, ABA initiates stomatal closure more effectively in the evening hours, while light causes more guard cell swelling during the day. The F-box protein ZEITLUPE (ZTL) is a blue light photoreceptor and key circadian clock element which acts both as an input and output circadian component. Mutant analyses showed that ZTL plays a role in regulating stomatal movements and interestingly, a direct interaction between ZTL and OST1 proteins was confirmed (Paper III, Figure 3). An absence of ZTL, similarly to the lack of functional OST1 protein kinase activity, leads to a more opened stomatal phenotype that was shown using different approaches (Paper III, Fig. 1). Similar *ztl-3* and *ost1-3* phenotypes were observed also during early seed germination, with delayed radicle emergence compared to control. A comparable function of ZTL and OST1, based on mutant analysis, thus appears to be conserved in different biological settings of *Arabidopsis thaliana*. The similar mutant phenotypes make it unlikely that OST1 is targeted for degradation through ZTL interaction. A transient expression assay in *ztl/fkf/lkp2* mutant back-ground protoplasts (Paper III, Supplementary Figs. 1, 2) confirmed that overexpressed epitope-tagged OST1 were not degraded by ZTL, while a ZTL-dependent degradation of TOC1 occurred. It is well established that OST1 is a key regulator of ABA signaling and consequently guard cells of *ost1-1*, *ost1-2*, *ost1-3* mutant plants are ABA insensitive (Mustilli et al., 2002; Yoshida et al., 2002). Interestingly, *ztl-3* mutant stomata also showed a degree of ABA insensitivity compared to Col-0 (Paper III, Fig. 2). Thus, both ZTL and OST1 act as positive components in ABA-induced stomatal closure. It can be argued that the higher protein content of ZTL before dusk (Kim et al., 2007) can facilitate the previously established, more effective ABA-induced stomatal closing during evening hours (Correia and Pereira, 1995). A function of ZTL1 in stomatal responses to desiccation and ABA treatment was also confirmed in *Populus* by water loss, stomatal conductance and aperture analyses performed on transgenic, ZTL-RNAi trees (Paper III,

Fig. 4). Together the presented data provide a direct link between the circadian clock and OST1-mediated stomatal aperture control.

5 Conclusions and Future perspectives

Stomatal movements need to be optimally regulated to balance the water use efficiency of plants, a trait crucial for biomass accumulation and stress adaptation. The current study has revealed new aspects on the control of stomatal movements in response to carbon dioxide, red light illumination (Paper I and II) and the circadian clock (Paper III).

The HT1 protein kinase is here established to be a key regulator of red light-induced stomatal opening (Paper I). The low [CO₂]-insensitive *ht1-2* mutant showed a complete lack of stomatal opening to red light, with retained blue light stomatal responses. The *HT1* gene function was found to be epistatic to *OST1* during [CO₂]- and red light-induced stomatal signaling. Based on data in Paper I and II, stomatal opening in response to red illumination may be mediated by processes other than a photosynthesis-derived reduction in C_i, such as a redox signal originating in the PETC or other unknown mechanisms. In a comparative study between Col-0 and the atrazine-resistant Ely-1a ecotype (Paper II), the activity of PETC and its effect on the redox state of PQ pool was found to regulate stomatal movements. It was concluded that stomatal closure is induced by an oxidized state of the PQ pool, while a reduced state maintains stomata open. A reduced PQ pool may still contribute to the stomatal red light response as initially hypothesized, but depend on other unknown signaling. Using several biochemical and physiological approaches, the blue light photoreceptor ZTL was suggested to control stomatal movements through OST1 activity. The physical interaction between ZTL and OST1 uncovers a mechanism by which the circadian clock can control stomatal water loss and desiccation responses via ABA-induced signaling.

Uncovering new mechanisms in the regulation of stomatal movements raises more questions that need to be answered by future investigations. The aim of Paper I was to better understand the role of HT1 in transducing red

light-induced stomatal opening. The function of HT1 as an inhibitor of high [CO₂] signaling helps to explain the lack of red light response in *ht1-2*. However other data suggested the existence of C_i-independent pathways (Paper I) therefore the role of HT1 needed to be expanded. The radicle emergence results introduced H⁺-ATPase activity as a possible downstream target of HT1, therefore prompting more experiments on HT1 function and identification of HT1-interacting partners in the future. In comparison, ABA inhibits stomatal opening, via OST1 inhibition of H⁺-ATPase, and ABA induces stomatal closing, via OST1 activation of anion channels and inhibition of K⁺-in channels (Yin et al., 2013). Another aspect of the stomatal response to red light is the nature of the signal that mediates the guard cell response. A PQ pool with higher redox potential is suggested to positively regulate stomatal opening (Paper II). Red light induces photosynthetic electron transport and Calvin cycle activation that results in C_i depletion. When C_i is low the Calvin cycle activity slows down, the demand for ATP and NADPH is decreased, therefore a higher proportion of PQ is reduced. Thus, the redox state of PQ is likely to signal stomatal opening in order to let CO₂ inside the leaf to support Calvin cycle reactions. How an altered redox status of the PQ pool in mesophyll chloroplasts ultimately would be transduced into the guard cells, situated in the upper and lower epidermis of leaves, remains an interesting topic for future experiments. The HT1 protein kinase shares high homology to MAPKK kinases (Ichimura et al., 2012) and redox-related mechanisms during oxidative stress can activate MAPK cascades (Kovtun et al., 2000; Son et al., 2011). Whether HT1 MAPKK kinase can be activated through a redox signaling originating within the photosynthetic electron transport is yet to be elucidated. Protein kinase activity experiments of HT1 protein where the redox status is controlled could help to resolve this question.

The clock component ZTL and the protein kinase OST1 showed similar mutant stomatal phenotypes and a physical interaction between proteins was established (Paper III). Therefore, a circadian regulation of guard cell turgor via OST1 activities may occur through binding of ZTL to OST1. Interestingly, the content of ZTL protein is high before dusk (Kim et al, 2007) when stomatal sensitivity to ABA is increased (Correia and Pereira, 1995). Whether ZTL acts on OST1 activity, independent of ABA, to govern stomatal movements also deserves further attention. Other clock components such as TOC1 and PRR5 are expressed throughout the day and together with ZTL they provide a continuous diurnal clock control of physiological processes in plants (Hotta et al., 2007; Nagel and Kay 2012). It remains to be shown whether several clock components may regulate OST1 function and what effect that would bring

about on for example the diurnal control of stomatal apertures, stress responses, transcriptional changes and seed germination.

The regulation of stomatal opening by red light relies on sophisticated molecular mechanisms including the interplay between HT1 and OST1 protein kinases. An altered stomatal and photosynthesis phenotype of *Arabidopsis* ecotype Ely-1a offers an opportunity to further examine guard cell function in relation to photosynthetic electron transport and to possibly unravel the signal that drives turgor changes in guard cells. OST1 protein kinase regulates stomatal movements and biochemical evidence in Paper III suggests a link between circadian clock control and stomatal function. Investigations on the role of SnRKs in plant adaptation to stress and to rhythmically changing natural environment are of high importance for understanding plant fitness and improving agricultural yield. Finally, a better understanding of the molecular aspects of stomatal regulation by environmental cues and the circadian clock may lead to effective tools for tailoring plants with C3 metabolism into CAM plants.

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