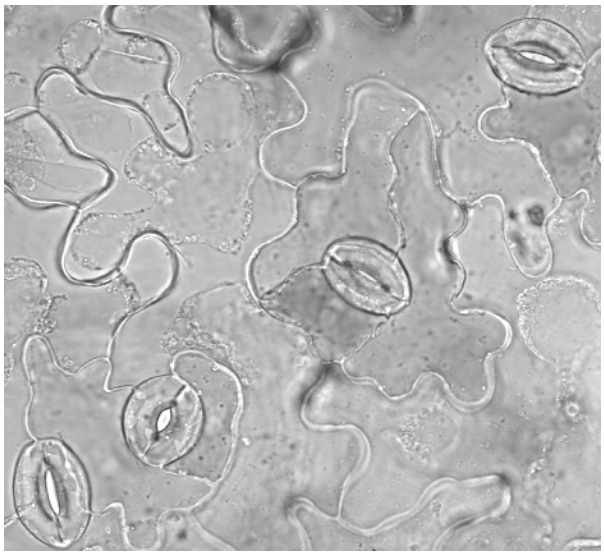
THE ROLE OF HT1 PROTEIN KINASE IN RED LIGHT-INDUCED STOMATAL OPENING



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Abstract

Red light and blue light induce stomatal opening in plants via separate pathways. It is unclear to what extent photosynthetic processes, if any, of guard cells or underlying mesophyll are involved in the red light-induced stomatal response. The HT1 protein kinase is a negative regulator of high CO_2-induced stomatal closure and the *ht1* mutant responds to blue light and ABA. If the red light signal is transduced via a change in photosynthetic activity, this mediator signal could be an altered intercellar [CO_2]. Due to the stomatal CO_2 insensitivity in *ht1*, this mutant was analyzed for its role in red light-induced stomatal opening.

Gas exchange measurements of stomatal conductance showed a lack of functional red light stomatal opening in *ht1-1* and *ht1-2*, while ht1 alleles exhibited stomatal opening under blue light. In order to examine photosynthesis processes, measurements of photosynthetic assimilation, several fluorescent parameters and a net carbon assimilation assay were performed. The results show that mesophyll photosynthesis is largely functional in *ht1-2* both for light-dependent and Calvin Cycle-dependent reactions. Despite red light illumination, fusicoccin induced H^+-ATPase activity and stomatal opening in *ht1* mutant as examined by microscopy-based bioassays. To assay whether other red-light triggered processes are functional in *ht1* mutants, the de-etiolation response was investigated. The wild-type response of *ht1* suggested that an impaired phytochrome B performance is not the cause for an impaired red light stomatal response. In addition, a method to perform guard cell protoplasts isolation was established for use in future cell-specific applications. Putting the findings in perspective led to a model in which HT1 functions within guard cells as a positive mediator of red light and low CO_2 control of stomatal opening, where HT1 acts prior to H^+-ATPase activation.

Contents

Abbreviations

ABA	abscisic acid
AtALMT12	*Arabidopsis thaliana* aluminium-activated malate transporter family
βCA1/ βCA4	BETA CARBONIC ANHYDRASE 1 and 4
Col-0	*Arabidopsis thaliana* Columbia ecotype
Ci	intercellular CO_2
[Ci]	intracellular CO_2 concentration
CAs	carbonic anhydrases
$[Ca^{2+}]_{cyt}$	cytoplasmic calcium concentration
CAM	Crassulacean acid metabolism
CO_2	carbon dioxide
$[CO_2]$	atmospheric carbon dioxide concentration
CRY1/2	cryptochromes 1and 2
DCMU	3(3,4-dichlorophenyl)-1-1-dimethylurea
EPF1/EPF2	EPIDERMAL PATTERNING FACTOR 1 and 2
GC	guard cell
GCPs	guard cell protoplasts
GMC	guard mother cell
gs	stomatal conductance
HCO_3^-	bicarbonite
HT1	HIGH LEAF TEMPERATURE 1
HRL	high pure red light
IRGAs	infra-red gas analyzers
K^+_{in} channel	K^+ inward-rectifying voltage-gated channel
K^+_{out} channel	K^+outward rectifying voltage-gated channel
LED	light emitting diode
LCF	Leaf Chamber Fluorometer
MCPs	mesophyll cell protoplasts
NPQ	non-photochemical quenching
OST1	protein kinase OPEN STOMATA 1
$PhiCO_2$	the CO_2 assimilation of photosynthesis at a given light intensity
PhiPSII	the quantum yield of photosynthesis
PHOT1/2	phototropin 1 and 2
PSII	photosystem II
q_P	the photochemical quenching
R-type	rapid anion channels
Rubisco	ribulose bisphosphate carboxylase/oxygenase
SBPase	sedoheptulose-1,7-bisphosphatase
S-type	slow anion channels
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
TMM	TOO MANY MOUTHS

1. Introduction

1.1. General back-ground to rising CO_2 levels

Carbon dioxide plays a vital role as the source of inorganic carbon in plant photosynthesis. The CO_2 concentration in the atmosphere is increasing due to pollution from fossil fuel burning and deforestation which influences the global climate. The enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco) uses either CO_2 for carbon fixation or oxygen in photorespiration (Taiz & Zeiger, 2006). Increasing CO_2 levels will therefore affect photosynthetic efficiency, together with any changes in temperature and fresh water availability.

Increasing [CO_2] triggers environmental changes on a global ecological level. One of the consequences is a drop in the ocean CO_2 uptake potential related to an increase of the bicarbonate to carbonate ratio in water (Malhi et al., 2002). Other studies have found that increased [CO_2] promotes accumulation of plant biomass (reviewed in Hetherington and Raven, 2005). CO_2 elevation could also lead to changes in biodiversity (LaDeau & Clark, 2001). Annual measurements of atmospheric [CO_2] by C. David Keeling have shown a steady increase in mean atmospheric CO_2 concentration from about 315 parts per million (ppm) in 1958 to 392 ppm as of December 2011 (http://www.esrl.noaa.gov/gmd/ccgg/insitu.html). The established CO_2 rising plays a vital role in global climate change. Carbon dioxide is one of the several most predominant greenhouse gases which are increasingly trapping solar heat and warming the global climate. Estimation of the future emission scenarios predicts a great temperature increase leading to sea-level rise due to melting of glaciers and an increased spread of diseases etc (Leaf et al., 2003). As a consequence of global warming the agricultural productivity, farm incomes and global food security will be strongly affected (Battisti & Naylor, 2009). Increased CO_2 concentrations will reduce plant transpiration and affect the land-ocean H_2O distribution which will lead to continental run-off of fresh water (Otto-Bliesner, 1995; Betts et al., 2007). In addition, climate change is together with population growth and economic development causing a decrease of the world's fresh water resources (Schiermeier, 2008).

1.2. Stomatal function and movement

Most leaf transpiration results from the diffusion of water vapor through the stomatal pore which is surrounded and formed by a pair of specialized epidermal cells, named the guard cells. Stomata can be found in leaves of all vascular plants and also in some more primitive plants, such as liverworts and mosses. The guard cells can shrink or swell which leads to stomatal closing or opening respectively, in order to regulate transpirational water loss and optimize photosynthesis (Roelfsema & Hedrich, 2005; Schroeder et al., 2001; Kim et al., 2010). Guard cells integrate signals from the leaf environment and convert them into the appropriate turgor pressure changes. Stomatal function is much determined by the structural features of the guard cells. Guard cells are very small and contain chloroplasts. The cell wall of guard cells have a highly specialized structure, which is up to 5 μm thick while typical epidermal cell walls are only 1-2 μm, which enables cell stability under the large turgor pressure changes. In addition, cellulose microfibrils in guard cell walls are, unlike normal cells, organized to allow cell size flexibility. In some plant species, guard cells associate with subsidiary cells which are epidermal cells that surround the guard cells and aid the stomatal movements (Heldt et al., 2005; Taiz & Zeiger, 2006). Stomatal pores occupy between 0.5 and 3% of the leaf surface (Morison, 2007).

The water movement into/out of guard cells is driven by osmosis. An increase in the osmotic potential in guard cells (GC), which is caused by accumulation of solutes (mainly K^+ salts), leads to a decrease in the water potential. To compensate for the decreased water potential, the turgor pressure rises due to an inflow of water causing swelling of the guard cells. and the guard cells to swell (Roelfsema, 2004). The increase in volume of both guard cells causes opening of a stomatal pore. Several environmental factors induce stomatal opening. Stomata in C_3 and C_4 plants open in response to high humidity and low CO_2. Stomatal conductance overall depends on CO_2 availability and photosynthetic activity as well as water humidity at the leaf surface. Stomatal opening is also induced by light, independently by blue and red light, signaling for the plant the need for photosynthesis and CO_2 uptake. In addition to direct light regulation, stomatal movements during a night and day cycle are controlled by circadian rhythms ensuring that stomatal opening is initiated prior to the break of dawn (Gorton et al., 1989; Tallman, 2004). In C_3 and C_4 plants, darkness promotes stomatal closing. In contrast, CAM-plants do not close stomata in darkness in order to preserve water, instead CO_2

is accumulated by converting into organic molecules (eg malate) during the night. Stomata also close in response to drought, via abscisic acid (ABA) and elevated ozone (Acharya & Assmann, 2009). In addition, stomatal closure is induced by elevated CO_2, since less opening is required for efficient CO_2 uptake.

1.3. Long-term adaptations to balance water and carbon uptake

Individual plants regulate the balance between photosynthesis and water availability by opening and closing stomatal pores to altered [CO_2] within minutes. In the long-term, when leaves are exposed to changes in [CO_2] the number of stomata is also altered (Woodward, 1987; Beerling et al., 1998). In an experiment where only mature leaves were exposed to altered [CO_2] it was shown that the newly developing leaves adapted their stomatal density to the conditions of the mature leaves (Lake et al., 2001).

Decreased [CO_2] will lead to more newly formed stomata, whereas relatively higher [CO_2] will induce the formation of fewer stomata. Based on the [CO_2]-stomatal density relationship, the fossil record can be used as an indicator of pre-historic atmospheric CO_2 levels. It would then be possible to investigate the atmospheric temperature fluctuations in correlation with changing CO_2 concentrations over millions of years. In one such study, data from the leaves of living Ginkgo trees and from the fossil leaves of four groups of Ginkgo relatives were compared (Kürschner, 2001). By calibrating the stomatal density to [CO_2] in experiments using living Gingko specimens, it was possible to reconstruct long-term trends in the changing levels of atmospheric CO_2 from the fossil record. The data obtained were compared to atmosphere temperature levels chaining through 300 million years. It demonstrated that the periods of low CO_2 corresponded to the cold periods of Earth's climate, whereas warming trends were accompanied by increased CO_2 levels.

Each step of the guard cell development is highly organized and regulated (Nadeau, 2009). In dicots like Arabidopsis, stomatal formation begins with asymmetric divisions of protodermal cells to generate meristemoids which differentiate into a guard mother cell (GMC), which is the immediate precursor of the paired guard cells. Transition from GMC to guard cells and the underlying genetic control of the transition are conserved processes in stomatal formation, which is regulated by the transcriptional factor FAMA (Ohashi-Ito & Bergmann, 2006; Hachez et al., 2011). Stomatal development is regulated by two classes of signals which

have opposing effects: the negative signals and a positive signal. Two putative negatively regulating signalling factors are known- EPIDERMAL PATTERNING FACTOR 1 and 2 (EPF1 and EPF2) which are sensed by cell-surface receptors TOO MANY MOUTHS (TMM) (Nadeau, 2009). A novel secretory peptide designated as stomagen acts as a positive intercellular signalling factor (Sugano et al., 2010). Stomagen is a 45-amino-acid, cysteine-rich peptide generated from a 102-amino-acid precursor protein, STOMAGEN. It has stomata-inducing activity in a dose-dependent manner. A genetic analysis showed that TMM is epistatic to STOMAGEN, indicating that stomatal development is regulated by competitive binding of stomagen and EPF1 or EPF2 to TMM. STOMAGEN is predominantly expressed in immature organs (leaves, stems and flower buds). In leaves it is expressed in inner tissues (the mesophyll) but not in the epidermal tissues where stomata develop. Being produced in mesophyll cells, stomagen is secreted to the apoplast, providing a positive regulation of stomata differentiation thereby linking photosynthetic tissue needs to stomatal density (Sugano et al., 2010). It remains to be shown whether changes in [CO_2] can ultimately alter stomagen availability and hence the formation of stomata.

1.4. **Short-term stomatal adaptation to changing CO_2 concentration**

CO_2 concentration is one of the factors that can trigger either stomatal opening or closing depending on its relative amount. How are these changes perceived and transmitted into stomatal movements? Physiological [CO_2] shifts do not affect cytosolic pH in *Vicia faba* guard cells (Brearley et al., 1997) nor in *Arabidopsis thaliana* based on ratiometric pH indicator studies (Xue et al., 2011). In the later steps following perception, low CO_2-induced stomatal opening involves guard cell H^+-ATPase activation which leads to proton efflux and a subsequent hyperpolarization of the plasma membrane. The hyperpolarization of the plasma membrane causes activation of the inward-rectifying voltage-gated K^+_{in} channels and triggers the influx of the solutes followed by water uptake leading guard cells' swelling (Assmann et al., 1985). High [CO_2] on the contrary, induces anion channels and activation of K^+_{out} efflux channels in guard cells (Brearley et al., 1997; Roelfsema et al., 2004) and triggers chloride release causing depolarization that ultimately leads to the loss of guard cell turgor and stomatal closing (Hanstein and Felle, 2002). The efflux of anions from the guard cells is accompanied by slow (S-type) and rapid (R-type) anion channels. A recently isolated R-type anion channel

in guard cells is one member of the *Arabidopsis thaliana* aluminium-activated malate transporter family, AtALMT12 (Meyer et al., 2010). The voltage-dependent AtALMT12 functions to release malate^{2-}, an organic osmolite, from the guard cells to the apoplast.

The role of intracellular free calcium ions ($[Ca^{2+}]_i$) in CO_2 signal transduction has been shown for several plant species (Young et al., 2006; Kim et al., 2010). Increased $[Ca^{2+}]_i$ in guard cells causes activation of slow acting (S-type) anion channels, down-regulation of inward K^+_{in} channels and proton ATPases. Therefore, high $[Ca^{2+}]$ provides a central mechanism mediating stomatal closing and inhibition of opening (Schroeder & Hagiwara, 1989; Chen et al., 2010; Xue et al., 2011). Interestingly, $[Ca^{2+}]_i$ also plays an active role in low $[CO_2]$-induced stomatal opening (Young et al., 2006), although the exact function of this mechanism is not yet known.

Within the last few years, a number of genes and mutants mediating CO_2 control of guard cells movements have been discovered. A genetic screen for *Arabidopsis* mutants that have altered CO_2 responses was conducted by analyzing leaf temperature changes monitored by leaf thermography. This screen isolated two allelic mutations in HIGH LEAF TEMPERATURE 1 (HT1), *ht1-1* and *ht1-2*, that alter the mutants' ability to control stomatal movements in response to CO_2 (Hashimoto et al., 2006). The *ht1–2* recessive mutation causes a constitutive high-$[CO_2]$ stomatal closure phenotype based on which the HT1 protein kinase was assigned a role as a negative regulator of high $[CO_2]$-induced closing. *ht1* mutants maintain the responsiveness to both ABA and blue light suggesting the possible function of HT1 upstream of the convergence of the CO_2- and ABA-induced stomatal closure pathways (Hashimoto et al., 2006). In *Arabidopsis,* disruption of two carbonic anhydrases, BETA CARBONIC ANHYDRASE 1 (βCA1) and BETA CARBONIC ANHYDRASE 4 (βCA4), leads to strongly impaired stomatal CO_2 responses (Hu et al., 2010). *ca1/ca4* double mutants show impaired CO_2 closing and opening responses while exhibiting functional ABA-induced closing. It is known that carbonic anhydrases (CAs) reversibly catalyse CO_2 into bicarbonate ions and protons and the data in this study supports an early role of CA in the perception of altered $[CO_2]$. Furthermore, high $[HCO_3^-]$ was shown to trigger activation of S-type anion channels in guard cells (Hu et al., 2010). Progress has also been made in the identification of the anion channels. The voltage-independent SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) anion channel confers S-type, not R-type, anion current activities in response to both high CO_2 and ozone (Negi et al., 2008; Vahisalu et al., 2008). Recent permeability studies have characterized

SLAC1 as an anion-selective channel with preference to nitrate (NO_3^-) and chloride (Cl^-) (Geiger et al., 2009).

Findings from the laboratory of JI Schroeder (Xue et al., 2011) have revealed an additional role for the protein kinase OPEN STOMATA 1 (OST1), previously assigned to the ABA signal transduction pathway (Mustilli et al., 2002; Yoshida et al., 2002; Vlad et al., 2009), in the control of CO_2-induced stomatal movements. Both low CO_2-induced stomatal opening and high CO_2-induced stomatal closing were impaired in *ost1* as compared to the wild-type. Co-expression studies in *Xenopus oocytes* had previously shown that OST1 activates SLAC1 anion currents (Geiger et al., 2009; Lee et al., 2009). In the recent study, elevated HCO_3^- acts as an intracellular signaling molecule activating SLAC1-mediated anion channels (Xue et al., 2011) but OST1 loss of function alleles show a strong impairment of the HCO_3^- activation of anion channels. It indicates that the OST1 protein kinase is a major regulator of high $[CO_2]$-induced stomatal closing through CO_2 activation of anion channels in guard cells. The HCO_3^--induced anion channel activity was enhanced in *ht1-2* compared to wild-type which fits well with the role of HT1 as a negative regulator of the high $[CO_2]$-induced stomatal closing response (Hashimoto et al., 2006; Xue et al., 2011). The intracellular Ca^{2+} sensitivity to S-type anion channel activation was increased by HCO_3^- in *ht1* and wild-type guard cells. Other studies have also shown a link between CO_2 and ABA to enhanced $[Ca^{2+}]_i$ sensitivity of stomatal closing mechanisms (Young et al., 2006; Siegel et al., 2009). Based on the new OST1

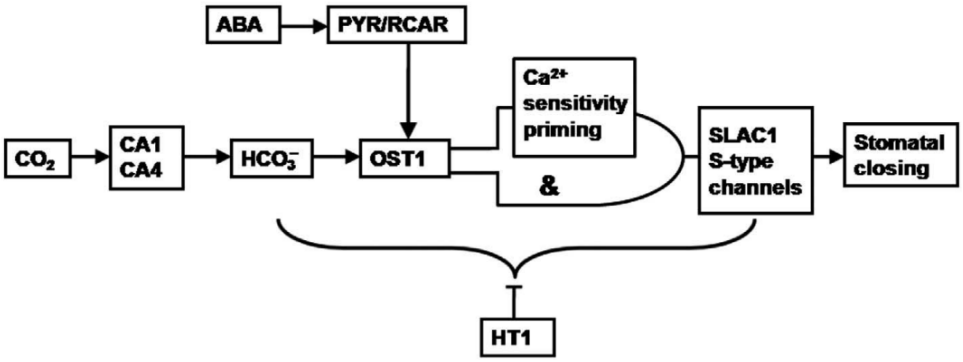

Figure 1. A model for high CO_2 signaling pathway in control of stomatal closing (Reprinted with permission from Nature Publishing Group; Figure 9 in Xue et al., 2011). CO_2 is first protonated by CAs into bicarbonate. CA1/CA4, OST1 and SLAC1 then function as positive mediators of high $[CO_2]$-induced stomatal closing. HT1 protein kinase functions as a negative regulator which continues to require $[Ca^{2+}]_i$.

findings, a new model for high [CO_2] control of gas exchange in plants was suggested (Fig. 1). However, the model does not consider that mutations in HT1 did not affect ABA-induced stomatal closing (Hashimoto et al., 2006). Therefore, it could be argued that HT1 be placed between HCO_3^- and OST1, but at the same time HT1 has an effect on Ca^{2+} sensitivity.

1.5. Light regulation of stomatal movements

In addition to environmental factors, stomata are controlled by an endogenous 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). Stomatal opening is directly driven by light, depending on its wavelength. Blue light stomatal response is rapid and photosynthesis-independent saturating at lower fluences (around 50 μmol $m^{-2}s^{-1}$) (Zeiger, 2000). Red light photosynthesis-dependent response saturates at high fluences similar to PAR (photosynthetic active radiation). Blue light is most efficient in driving stomatal opening but red light acts as a required background for the rapid opening (Vavasseur & Ragavendra, 2005). Stomata in *Arabidopsis* open fast in response to a weak blue light under a strong red light background, whereas they are almost closed under pure blue light illumination in the absence of red light (Willmer & Fricker, 1996).

Blue light in guard cells is perceived by phototropins, cryptochrome and possibly also the chloroplast carotenoid zeaxanthin, which has been suggested to act as a blue light sensor (Kinoshita et al., 1995). The blue light receptors phototropins PHOT1 and PHOT2 are receptor kinases associated with the plasma membrane (Briggs & Christie, 2002). The cryptochromes CRY1 and CRY2 function in addition to PHOT1 and PHOT2 and regulate the blue-light induced stomatal opening (Lin et al., 1998; Mao et al., 2005). The autophosphorylation of PHOT receptors, stimulated by blue light, causes the binding of 14-3-3 proteins (Kinoshita et al., 2003). The binding of 14-3-3 proteins keeps PHOT receptors active and triggers the activation of H^+-ATPase through phosphorylation (Kinoshita et al., 1995; Shimazaki et al., 2007). Activation of H^+-ATPase then mediates extrusion of H^+ leading to increases of the inside-negative electrical potential across plasma membrane (Assmann et al., 1985; Shimazaki et al., 1986) and therefore membrane hyperpolarization. Blue light has been shown to inhibit S-type anion channels, thereby further contributing to membrane hyperpolarization (Marten et al., 2007). The hyperpolarization leads to activation of inward-rectifying voltage-gated K^+ channels

(K^+_{in}) which take up K^+ (Schroeder et al., 1987; Assmann & Shimazaki, 1999; Roelfsema et al., 2001). Several genes encoding K^+_{in} channels in Arabidopsis have been isolated, for example KAT1 (potassium channel in *Arabidopsis thaliana* 1), AKT1, AKT2/3 (potassium transport 2/3), AtKC1 and KAT2 (Heldt, 2005; Roelfsema & Hedrich, 2005; Shimazaki et al., 2007). Blue light induces starch degradation in guard cells (Fig. 1, Vavasseur & Raghavendra, 2005) in order to provide ATP to energise the proton pumps and osmolites to induce opening. Organic anions such as $malate^{2-}$ is produced via starch degradation to balance the negative charge of K^+. The accumulation of solutes such as K^+ leads to a lowered water potential that results in water uptake. An increase in turgor pressure then leads to guard cell swelling and stomatal opening (Roelfsema et al., 2005). The blue light-induced H^+-ATPase activation and effect on K^+ uptake in guard cells is summarized in Fig. 2.

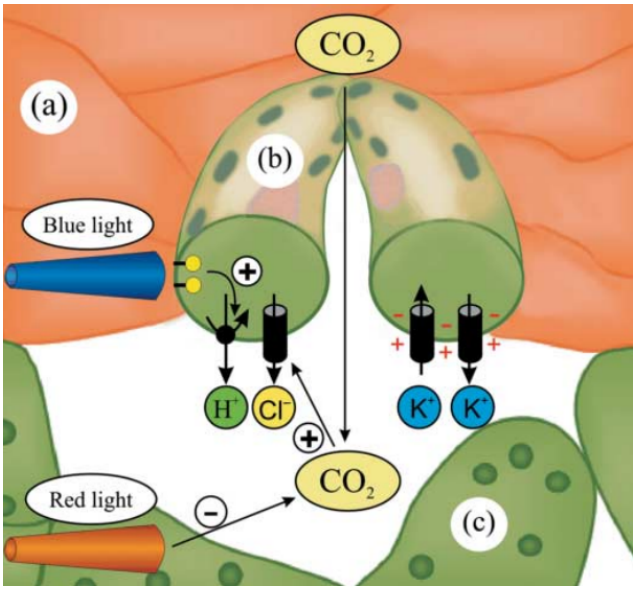

Figure 2. Light-induced stomatal opening signaling pathways. Pavement cell of epidermis (a), guard cell (b), mesophyll cell (c). Red light leads photosynthesis and a reduction in CO_2 concentration inside the leaf, resulting in deactivation of guard cell anion channels. Blue light induces PHOT1 and PHOT2 mediated activation of H^+-ATPase. Stimulated by either of these pathways, the guard cells hyperpolarize and take up K^+ which causes them to swell, leading to opening of the stomatal pore. CO_2 provides a negative feedback mechanism as its increased influx through the opening pore can promote closure of the stomata via activation of guard cell anion channels. Figure reprinted by kind permission from John Wiley & Sons, Inc. (Figure 9 in Roelfsema & Hedrich, 2005).

It has been shown that blue light also triggers Ca^{2+} fluxes via the phototropin-activated Ca^{2+}-permeable channel (Stoelze et al., 2003; Harada et al., 2003). $[Ca^{2+}]$ elevation is dependent on membrane hyperpolarization and takes place after activation of H^+-ATPase (Harada et al., 2009). It is known that increase of cytosolic $[Ca^{2+}]$ contributes to ABA-induced stomatal closure by activation of anion channels and inhibition of the plasma membrane H^+-ATPase and K^+_{in} channels, which contradicts the fact that Ca^{2+} is involved in stomatal opening. However, the Ca^{2+} elevation induced by blue light does not show $[Ca^{2+}]_{cyt}$ oscillations which are necessary for the maintenance of stomatal closure (Shimazaki et al., 2007). The exact role of Ca^{2+} in stomatal opening responses is yet to be revealed.

1.6. Red-light induced stomatal opening – dependent or independent of photosynthesis?

The opening response of stomata to red light requires a high light intensity and continuous illumination (Willmer & Fricker, 1996; Shimazaki et al., 2007). The stomatal opening response to red light is saturated at fluences similar to those for photosynthesis saturation and is affected by an inhibitor of photosystem II (PSII) DCMU (3(3,4-dichlorophenyl)-1-1-dimethylurea), which indicates that the response is photosynthesis-dependent (Sharkey & Raschke, 1981; Tominaga et al., 2001; Messinger et al., 2006). Blue-light stomatal opening is not affected by DCMU (Schwartz & Zeiger, 1984). Overall, relatively little is known with regards to the turgor driving changes leading up to red light stomatal opening. Red light may induce activation of H^+-ATPase in the plasma membrane, generating a negative membrane potential that drives the accumulation of K^+. The pump activation could be the result of an increase in ATP concentration in the cytosol, originating from photophosphorylation in guard cell chloroplasts. Earlier studies support a proton pump activation by red light (Schwartz & Zeiger, 1984; Serrano et al., 1988; Olsen et al., 2002), although more recent red light investigations do not confirm H^+-ATPase activation (Shimazaki et al., 2007). The stomatal opening in response to red light is driven by accumulation of K^+ and sucrose. Sugars could be produced in guard cells from a combination of starch degradation during the day, photosynthesis, and import from the apoplast. Estimations have shown that guard cell CO_2 fixation is only 2-4% of that of mesophyll cells (Outlaw & De Vlieghere-He, 2001), suggesting that photosynthesis-derived sucrose cannot provide enough osmotica to drive stomatal opening. Unlike blue light, red light does not induce starch degradation in the guard

cells (Fig. 1 Vavasseur and Raghavendra, 2005), and therefore import of sucrose from the apoplast is likely to occur.

There is a debate whether red light induces stomatal opening via photosynthetic CO_2 fixation in the mesophyll, that leads to a decrease in intercellular [CO_2] (Ci) of the leaf, which is in turn sensed by the guard cells (Fig. 2). Or could guard cell photosynthesis directly trigger stomatal movements? With the entire set of photosynthetic reactions performed in the guard cell chloroplasts (Shimazaki & Zeiger, 1987; Lawson et al., 2002), a signal to induce stomatal opening based on guard cell photosynthesis is possible. There are a few studies showing red light effects in either isolated epidermis or guard cell protoplasts, although many of these studies show unclear responses (Tallman and Zeiger, 1988; Shimazaki et al., 2007). For example, red light-activation of a guard cell plasma membrane H^+-ATPase in isolated epidermis or protopasts have been observed (Schwartz and Zeiger, 1984; Serrano et al., 1988; Olsen et al., 2002) but the same effect has not been detected in protoplasts or intact leaves (Taylor and Assmann, 2001; Roelfsema et al., 2001). In an experiment aimed at understanding the requirements for mesophyll cells in the red light stomatal opening response, red light was applied to a single guard cell of an intact leaf and it did not induce plasma membrane hyperpolarization nor stomatal opening (Roelfsema et al., 2002). Conversely, when red light was projected on a large area including both guard cell and the surrounding mesophyll, hyperpolarization followed and induced an opening response. Stomatal responses have been studied in albino norflurazon-treated *Vicia faba* plants (GC chloroplasts were nonfunctional due to impaired carotenoid biosynthesis after norflurazon treatment) and in albino leaf patches of variegated leaves of *Chlorophytum comosum* (GC chloroplasts functional) (Roelfsema et al., 2006). There was no stomatal opening in response to red light in either albino leaf representative, although the plants did respond to blue light as well as low [CO_2] and ABA. The above studies from the Hedrich group all support the role of a functional mesophyll in transducing the red light signal into stomatal movements, irrespective of the photosynthetic capacity of GC. The red-derived signal from the mesophyll that triggers the stomatal response may therefore be Ci.

What other evidence is there for a photosynthesis-dependent red light induced signaling, and what may be the nature of a signal from the mesophyll to stomata? Studies on transgenic tobacco with reduced amounts of cytochrome b_6f complex or Rubisco, with lowered CO_2

assimilation, has shown that the stomatal opening red light-induced response is independent of the simultaneous guard cell or mesophyll photosynthetic rate (Baroli et al., 2008). However, reduction of sedoheptulose-1,7-bisphosphatase (SBPase) activity in tobacco led to a an increased rate of stomatal opening to red light (Lawson et al., 2008). Interestingly, the quantum efficiency of PSII electron transport in SBPase anti-sense guard and mesophyll cells was reduced, indicating that photosynthetic operating efficiency depends on the regeneration capacity of the Calvin Cycle. Under red light and mixed blue/red illumination, the transgenic plants had significantly more opened stomata than the wild-type at all Ci levels. The closing response to elevated [CO_2] was not affected. Therefore, the authors concluded that photosynthetic electron transport is responsible for the regulation of the light and CO_2 stomatal movements control. Further support for the role of photosynthetic electron transport in control of stomatal aperture comes from studies in cocklebur (*Xanthium strumarium*), where Ci-induced changes to stomatal conductance depended on the balance between Rubisco- to electron transport-limited photosynthesis (Messinger et al., 2006). Findings of a Ci-independent red light-induced stomatal opening was also given in the same study, since red light caused stomatal opening while Ci levels were kept constant (Messinger et al., 2006). Therefore, the major red light stomatal response was not due to mesophyll-induced reductions in Ci. It was caused either by a Ci-independent signal from the mesophyll or by processes in the guard cells themselves.

What is the evidence for a red light-induced photosynthesis-independent pathway in the control of stomatal movements? Phytochrome B has been suggested to mediate red-induced stomatal opening due to the reversibility of the response under far-red light observed in orchids and *Arabidopsis thaliana* (Talbott et al., 2002, 2003). Furthermore, recent investigations show moderately reduced stomatal apertures of a *phyB* mutant and a hypersensitive stomatal response in *PHYB*-overexpressing plants which suggests a positive role for PHYB in stomatal signaling (Wang et al., 2010). In summary, the literature supports the existence of both photosynthesis-dependent and -independent pathways with the former possibly involving Ci or photosynthetic electron transport as a mediator signal. The contribution of several pathways that collectively control stomatal movements induced by red light cannot be ruled out.

2. Aim of Study

Red light has been proposed to induce stomatal opening via photosynthetic CO_2 fixation in the mesophyll that leads to a decrease in intercellular [CO_2] of the leaf. The lowered [Ci] would then be sensed by the guard cells leading to stomatal opening. The primary goal of this project was to improve the knowledge on the link between red light and low [CO_2]-induced stomatal movements. HT1 is a previously described protein kinase with a role as an inhibitor of high CO_2-induced stomatal closing and stomata of the *ht1* mutant are insensitive to altered [CO_2] (Hashimoto et al., 2006). The aim was to test if the red light-induced stomatal opening was functional in *ht1* and whether photosynthetic activities in mesophyll and guard cells were intact. In this study, the function of *ht1* compared to wild-type was set out to be tested by the following:

- Study the red and blue light effects on stomatal conductance and carbon assimilation
- Investigate status of the photosynthetic machinery, both in light-dependent reactions and the Calvin Cycle
- Examine other red light-induced physiological responses in *ht1*
- Establish a method to isolate guard cell protoplasts for cell-specific Real-Time PCR and metabolite analyses.

3. Comments on the methodology

3.1. Methods to measure stomatal responsiveness

Guard cell turgor changes is one way to optimize the balance between transpiration and photosynthesis in plants. Several different methods can be used to study guard cell behaviour. Stomatal gas exchange recordings is one option which makes it possible to monitor stomatal responsiveness in intact plants in real-time. The LiCOR 6400XT Portable Photosynthesis System (LI-COR Inc., NE, USA) has been used as powerful tool to measure stomatal conductance and other transpirational and photosynthetic parameters. Another approach that has been employed is microscopy-based stomatal aperture measurements. The latter method enables the direct investigation of the guard cells by measuring the width and length of the stomatal pores under any given condition. Because stomatal movements are subject to circadian

regulation, the order of analysis was consistently swapped between genotypes throughout each day of experiments.

3.2. Gas exchange measurements

The Li-COR 6400XT Portable Photosynthesis System (Li-COR Inc., NE, USA) is an open system that permits measurement of photosynthesis and transpiration of an individual intact leaf, while changing or keeping desirable environmental conditions such as CO_2 concentration, light intensity, humidity, and temperature stable (Fig. 3).

Figure 3. The Li-COR 6400XT Portable Photosynthesis System and its main components. Image used by kind permission from © Li-COR, Inc.

The main principle of the Li-COR is based on the measurement of gaseous concentration differences. The loss of water through stomata (transpiration) can be measured as the amount of H_2O leaving a leaf to the surrounding air. In a similar manner, the amount of CO_2 that diffuses in through stomata and is fixed in photosynthesis is a read-out of carbon assimilation, i.e. photosynthetic activity. Differences in $[CO_2]$ or $[H_2O]$ are measured by two independent infra-red gas analyzers (reference and sample IRGAs) placed in the sensor head of the instrument (Fig. 4).

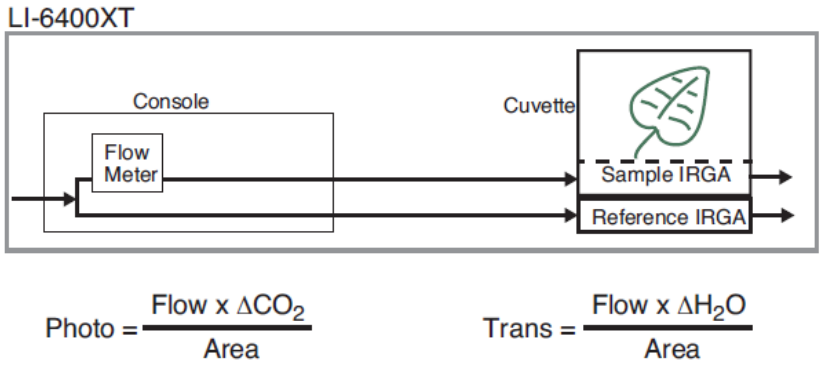

Figure 4. A schematic overview of the gas exchange system Li-COR 6400XT. The measurements of photosynthesis and transpiration are based on the differences in CO_2 and H_2O in an air stream that is flowing through the leaf cuvette. Image used by kind permission from © Li-COR, Inc.

The sensor head is attached to a leaf chamber where a leaf is placed. When Li-COR operates, atmospheric air enters the machine and is given its appropriate environmental settings and goes through a tube system passing the leaf chamber thereby exposing the pre-set environmental conditions to the leaf. IRGAs are capable to accurately measure [H_2O] (signal noise 0.04 mmol mol^{-1}) and [CO_2] (signal noise 0.2 µmol mol^{-1}) in the air flow within and outside the leaf chamber. One of the main parameters from a gas exchange experiment is stomatal conductance (gs) - leaf conductance to water vapor (mmol H_2O m^{-2} s^{-1}). Stomatal conductance is computed based on the molar concentration of water vapor (mmol H_2O mol^{-1} air), the leaf temperature T_l (°C) and the total atmospheric pressure P (kPa) while excluding the boundary layer conductance, see manufacturers instructions for full description (www.licor.com). Stomatal conductance depicts the amount of H_2O that is lost from a leaf under a given environmental condition, in other words stomatal opening width. It is important to point out that when different genotypes are compared, stomatal density between the leaves must be similar in order to accurately compare gs values as a measure of stomatal opening.

The Li-COR 6400XT is equipped with a Leaf Chamber Fluorometer (LCF) 6400-40 (LI-COR Inc., NE, USA) which is a light emitting diode (LED)-based fluorescence/light source attachment. It contains a variety of LEDs (blue, far-red and red) and two detectors to enable fluorescence experiments. Red and blue illuminations can be given separately and in different intensities which has been used extensively in the light studies presented in this study. LCF gives the possibility to measure stomatal conductance, the assimilation rate of photosynthesis and fluorescent parameters such as PhiCO_2, PhiPSII, q_p and NPQ. The LCF analyzer head was

not originally intended for *Arabidopsis thaliana* plants and therefore, a major constraint in the presented work has been obtaining healthy and large enough leaves. Satisfactory leaves were achieved by prolonged growth and keeping stable growth conditions with respect to amount and frequency of water supply, light intensity, light regime and air humidity.

3.3. Microscopy-based stomatal bioassays

Stomatal bioassays allow the direct measurement of the stomatal aperture through investigation of epidermal tissue, containing the guard cells, under the microscope. One of the techniques to prepare a leaf sample for stomatal bioassay is Blended leaf. A leaf is excised from a plant and placed in a Petri dish containing Stomatal buffer (see Material and Methods for contents) with the leaf petiole submerged. After the appropriate incubation, the leaf is blended in a commercially available blender in incubation buffer. The mixture is filtered through a 200 μm nylon mesh to remove the disrupted mesophyll cells. The epidermal sections remaining on the mesh are immediately placed on an object glass for microscope analysis. In this technique the stomata from both the upper and lower sides are investigated.

Another technique used in this study was Exposed epidermal layer. The leaf is placed firmly with the guard cell-rich lower side facing down onto a cover slip coated with medical adhesive glue. Using a razor blade, the upper epidermal layer and mesophyll cells of the leaf are scraped away, exposing the lower epidermal layer, containing the guard cells, left stuck to the glue. A sample obtained by either of these two techniques can be analyzed under the microscope with a digital camera attached. Using the DeltaPix software, measurements of the width of individual stomatal pores are made. A clear image can be obstructed by the medical adhesive glue or mesophyll fragments that block the view of the epidermal tissue. Young healthy plants, approximately 4 weeks-old were used for the stomatal bio-assays. Due to the risk of biased results, the sample identity must be unknown to the person conducting the experiment. Blinding is therefore performed for the plant genotypes and/or treatment identity, as single-blinded or double-blinded depending on the nature of the experiment.

3.4. Guard cell protoplast isolation

In order to investigate the cellular or biochemical attributes of guard cells, different techniques can be used to achieve cell-specific samples. Guard cells can be collected using tedious laser ablation techniques or by obtaining purified guard cell protoplasts (GCPs) by flow cytometry (FACS) or isolation using size-excluding meshes (Assmann & Wang, 2001; Schroeder et al., 2001; Pandey et al., 2002). Although GCPs are required for downstream analyses of for example transcripts or metabolites, *Arabidopsis* guard cells are relatively difficult to isolate. The small height of the plant combined with a small guard cell size require large amounts of tissue for sufficient amounts of protoplasts. Many studies have been conducted on GCPs isolated from *Vicia faba* (Kruse et al., 1989; Kinoshita & Shimazaki, 1999; Talbott & Zeiger, 1998; Jacob et al., 1999) and *Commelina communis* (Fitzsimons & Weyers, 1983), species that have different leaf morphology characteristics compared to *Arabidopsis* (Pandey et al., 2002). The mesophyll cells of *Arabidopsis thaliana* adhere more strongly to epidermal fragments than those of *Commelina* and *Vicia*. As a consequence, epidermal fragments from *Arabidopsis* usually contain larger contamination of mesophyll cells adhering to them, which is a significant obstacle for downstream applications. Therefore, only GCPs samples with less than 3% of mesophyll cell protoplasts are included for downstream analyses.

The GCPs isolation is based on two consecutive enzymatic digestions of leaves which enable a release of guard cells protoplasts. The main constrain of the method is the difficulty to get a sufficient amount of the pure protoplasts. The major contaminants are mesophyll cell protoplasts (MCPs) and chloroplasts. RNA isolation from GCP material is the application that is currently being developed in order to perform Real-Time PCR analyses. For the RNA isolation the TRIZOL reagent (Invitrogen, Sweden) was used accordingly to manufacturers protocol. The constraints in the early isolations were high polysaccharide contamination and low RNA yield and therefore a number of minor modifications were introduced. To improve the RNA precipitation step glycogen was introduced. Air drying of the final RNA pellet improved the yield as compared to vacuum-drying. Following these modifications, RNA has been successfully obtained and used in cDNA synthesis and finally gene transcripts have been amplified in RT-PCR (Marian Plaszczyca, data not shown).

4. Materials and Methods

4.1. Plant growth conditions and mutant genotyping

All *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype (Col-0). Wild type, *ht1-1* and *ht1-2* mutant plants were grown in an *Arabidopsis* cultivation room at 21°C, 50% humidity with 8 h light / 16 h dark photoperiod time regime at ~100 µmol m^{-2} s^{-1}. To confirm the point mutations in *ht1-1* and *ht1-2*, the primers HT1-F (CACACAAGGAAGAGACAAGGGC) and HT1-R (GGAGGTTTCCTTGTGACATGTAC) were used to amplify a 300 bp PCR fragment from plant genomic DNA which was then sequenced.

4.2. FluorCam chlorophyll fluorescence measurements

Individual Col-0 and *ht1-2* leaves of 6-7 weeks age were pre-treated with 700 µmol m^{-2} s^{-1} red light for 2 h using Li-Cor 6400XT Portable Photosynthesis system equipped with the Leaf Chamber Fluorometer (LI-COR Inc). Following the red light exposure, the Open FluorCam 701 MF (Photon Systems Instruments, Brno, Czech Republic) Imaging Fluorometer was used for chlorophyll fluorescence measurements according to the manufacturers – Fv/Fm 15 min wait protocol.

4.3. Gas exchange measurements

Stomatal conductance measurements were made using a Li-COR 6400XT Portable Photosynthesis system equipped with the Leaf Chamber Fluorometer (LI-COR Inc., NE, USA). The default conditions used were 14 mmol H_2O $m^{-2}s^{-1}$ humidity, 21 ºC temperature, 100 µmol m^{-2} s^{-1} light intensity (10 % blue light and 90 % red light) and 400 µmol CO_2 mol^{-1} air concentration. Healthy, large leaves of 6-7 weeks-old plants were used.

For analyses of blue-light responses, a measured leaf was kept at a light intensity of 90 red + 10 blue µmol $m^{-2}s^{-1}$ for 30 min and then 90 red + 80 blue µmol $m^{-2}s^{-1}$ for 90 min. For analyses of red-light responses, a measured leaf was kept at the light intensity 90 red + 10 blue µmol $m^{-1}s^{-1}$ for 30 min and then 490 red + 10 blue µmol $m^{-2}s^{-1}$ for 60 min. Specific light quality stimulation was obtained by using light-emitting diodes (LEDs) with a maximum of

470 nm ± 10 nm half-bandwidth for blue light and 630 nm ± 10 nm half band-width for red light (Li-COR 6400XT Leaf Chamber Fluorometer, LI-COR Inc. NE, USA).

Fluorescence light response curves were run with the following red light intensities; 100, 300, 500, 700, 1000, 1300, 1600, 2000 μmol $m^{-2}s^{-1}$. The fluorescence parameters $PhiCO_2$, PhiPSII, q_P and NPQ were generated.

A - Ci curves were performed on the following CO_2 values; 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 μmol CO_2 mol^{-1} air with 1-2 min waiting time between each step. The assimilation rate (A) was measured at each point against the intercellular CO_2 (Ci) concentration thus creating an A-Ci plot.

4.4. Stomatal aperture measurement

Middle-sized leaves from 5- to 6-week-old plants were detached and incubated in Stomatal buffer (10 mM MES, 10 mM KCl, 50 μM $CaCl_2$; pH 6.15, adjusted by TRIS) containing 10 μM Fusicoccin (Sigma Aldrich, Sweden) or DMSO mock for 1.5 h. LED lamps (Photon Systems Instruments, Czech Republic) with 100 μmol $m^{-2}s^{-1}$ white light or 500 μmol $m^{-2}s^{-1}$ red light were used. Following incubation, each leaf was disrupted in Stomatal buffer using a commercial blender. The blended mixture was filtered through a 200 μm nylon mesh and the remaining epidermal sections were investigated under a microscope. The stomatal apertures were measured using DeltaPix software (DeltaPix InSight, Denmark). Approximately 20 stomata were measured per sample.

4.5. Stomatal density measurements

Abaxial (lower) leaf epidermis samples from the sixth or seventh leaf of five-week-old plants were used for stomatal density measurements. Ten random parts (each part consisted of 0.15 mm^2) from five leaves per genotype were counted for determination of stomatal density.

4.6. De-etiolation in white light

For hypocotyl measurements, seeds were first sterilized in 95% ethanol for 10 min, followed by 3 washes in 70% ethanol. Seedlings were grown in one-half Murashige and Skoog basal salt mixture (MS; Sigma) agar, pH 5.7 without sucrose. All seeds on plates were

vernalized in darkness at 4°C for 3 days. The de-etiolation experiment was carried out under white light conditions in a growth cabinet (Percival Scientific Inc., USA) at 130 umol m-2s-1 photon light fluency and 22°C temperature. After 6 days, seedlings were captured on digital images and hypocotyl lengths were analyzed using the ImageJ program. The experiments were performed three times using plates containing 15 seeds each of Col-0, *phyB-9*, *ht1-1* and *ht1-2* with all 4 genotypes represented on each row (3 rows, 5 seeds/genotype).

4.7. Guard cell protoplasts isolation

For small-scale isolations 12-16 large healthy 5-6 week-old leaves of *Arabidopsis thaliana* were used, and large-scale isolations required 100 leaves. The main veins were cut out and the leaves were blended. For the first digestion the blended leaves' mixture was placed in the Enzyme solution 1 (0.7% Cellulasine cellulose; Merck Chemicals Ltd, 0.1 % Polyvinylpyrrolidone, 0.25 % Albumin from bovin serum, 0.5 mM Ascorbic acid, dissolved in 55% (v/v) basic solution (5 mM MES-TRIS, pH 5.5, 0.5 mM $MgCl_2$, 10 μM KH_2PO_4, 0.5 mM ascorbic acid, 0.55 M sorbitol, 0.5 mM $CaCl_2$) and 45% (v/v) distilled water) and incubated at 27°C in a shaker (140 rpm) for 3.5 h in darkness. For the second digestion, filtered epidermal fragments obtained after the first digestion were placed in the Enzyme solution 2 (1.5% Onozuka RS Cellulase; Fisher Scientific, 0.02% Pectolyase Y-23; Fisher Scientific, 0.25% BSA, 0.5 mM Ascorbic acid, in basic solution, pH 5.5; before use, the pH is reduced to pH 3.5 for 5 min, using HCl, to inactivate contaminating proteases) and kept at 19 – 21°C in a shaker (80 rpm) for 2.5 h. After the second digestion GCPs were filtered and centrifuged 3-4 times at 1000 g for 5 min. After the last centrifugation, the supernatant was removed and the sediment containing guard cells resuspended in the basic solution.

5. Results and discussion

5.1. Light responses in *ht1-1* and *ht1-2* are impaired in response to red light and functional in response to blue light

The HT1 protein kinase acts as a negative regulator of CO_2-induced stomatal closing (Hashimoto et al, 2006). *ht1* mutants lack CO_2 stomatal opening under low CO_2 and show a constitutive high CO_2 stomatal closing response with very closed stomata at ambient CO_2. The

mutants were shown to open stomata in response to blue light and to the phytotoxin fusicoccin, both of which signals have been previously shown to induce stomatal opening by activation of the plasma membrane H^+-ATPase (Turner & Graniti, 1969; Kinoshita & Shimazaki, 2001). In addition, the ABA-induced closing response is retained in *ht1-1* and *ht1-2* mutants.

The present study set out to examine the role of HT1 kinase in light-induced stomatal opening. The red light response was investigated by exposing individual intact leaves of *ht1-1*, *ht1-2* and wild-type plants to a red light intensity of 500 µmol $m^{-2}s^{-1}$ for 60 min after a 30 min ambient light pre-exposure of 100 µmol $m^{-2}s^{-1}$. Blue light was constantly given at an additional intensity of 10 µmol $m^{-2}s^{-1}$ in order to mimic normal light conditions for plant growth. Increased red light intensity for 60 min caused a large increase of stomatal conductance in the wild-type ($P<0.0001$) whereas the responses in *ht1-1* ($P=0.04$) and *ht1-2* ($P=0.4$) mutants were

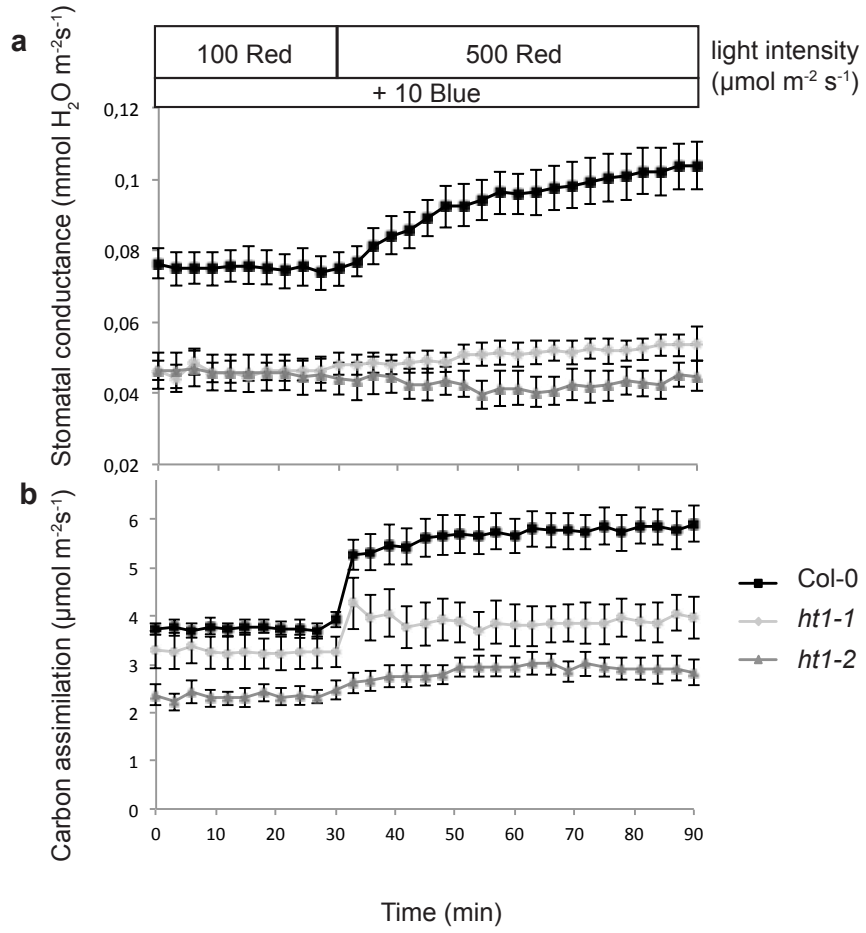

Figure 5. Stomatal and photosynthetic responses to red light. **a**. Red light response of stomatal conductance in Col-0, *ht1-1* and *ht1-2*. **b**. Red light response of CO_2 assimilation during same experiment as above. Data presented are the mean of ± SE ($n>7$).

impaired (Fig. 5a). Meanwhile the photosynthetic assimilation was increased in wild-type plants ($P<0.001$), but overall *ht1-1* and *ht1-2* demonstrated much smaller increases ($P=0.06$) and ($P=0.008$) respectively (Fig. 5b). The relatively larger assimilation increase in *ht1-1* as compared to *ht1-2* may be due to a larger initial stomatal conductance in *ht1-1* that would allow more CO_2 uptake for carbon fixation. The lower stomatal conductance values in *ht1* were not due to differences in stomatal density as measured on the lower abaxial leaf surface (wild-type=97.4±11 and *ht1-2*=99.6±12 stomata/mm^2; means ±SE, n=5), see also Hashimoto et al, 2006.

Previous studies had shown functional blue light responses in *ht1* mutants exposed to 50 µmol $m^{-2}s^{-1}$ of blue light after 30 min dark incubation (Hashimoto et al., 2006). Here, the goal was to assay stomatal blue light responses in plants pre-exposed to light, given the influence of red back-ground light for the blue light response (Wilmer and Fricker, 1996). Various blue light intensities were tested to find the most suited to initiate a well-defined wild-type response (data not shown). In the selected experimental set-up, leaves were initially exposed for 30 min to the irradiance 100 µmol $m^{-2}s^{-1}$ (90 µmol $m^{-2}s^{-1}$ red and 10 µmol $m^{-2}s^{-1}$ blue light). Raising the blue light intensity to 80 µmol $m^{-2}s^{-1}$ for 60 min strongly increased the stomatal conductance in wild-type ($P<0.0001$) as well as in *ht1-1* ($P=0.002$) and *ht1-2* ($p<0.0001$) (Fig. 6a). The stomatal conductance within each genotype increased by 47% for wild-type, 35% for *ht1-1* and 44% for *ht1-2*. Assimilation increases were also statistically significant in all genotypes, wild-type $P<0.0001$; *ht1-1* $P<0.001$ and *ht1-2* $P<0.0001$ and the relative increments were 27%, 28% and 51% respectively. The 51% increase in *ht1-2* carbon assimilation is most likely due to an increased access to CO_2 due to higher stomatal conductance (gs) values (Fig. 6). When comparing the effect of red or blue light on stomatal conductance and assimilation, both *ht1* alleles showed larger increases to blue light irradiation (Fig. 5 and 6). The gas exchange data collectively show that both *ht1-1* and *ht1-2* lack functional stomatal responses to red light (Fig. 5), while *ht1* alleles show stomatal opening under the blue light (Fig. 6). Any impairment to the Calvin cycle machinery in the *ht1* background is suggested to be minor, based on the 1.5-fold increase in photosynthetic carbon assimilation in *ht1-2* plants with increased gs levels.

The experiments above were performed in both *ht1-1* and *ht1-2* back-ground (Fig. 5 and 6). Our data together with previously performed bio-assays, gas exchange measurements, germination assays in Hashimoto et al., 2006 consistently show *ht1-2* as a stronger allele compared to *ht1-1*. Due to its stronger phenotype, the *ht1-2* mutant was selected for several of

the analyses described below. As comparison, only *ht1-2* was selected for patch clamp analyses in Xue et al., 2011.

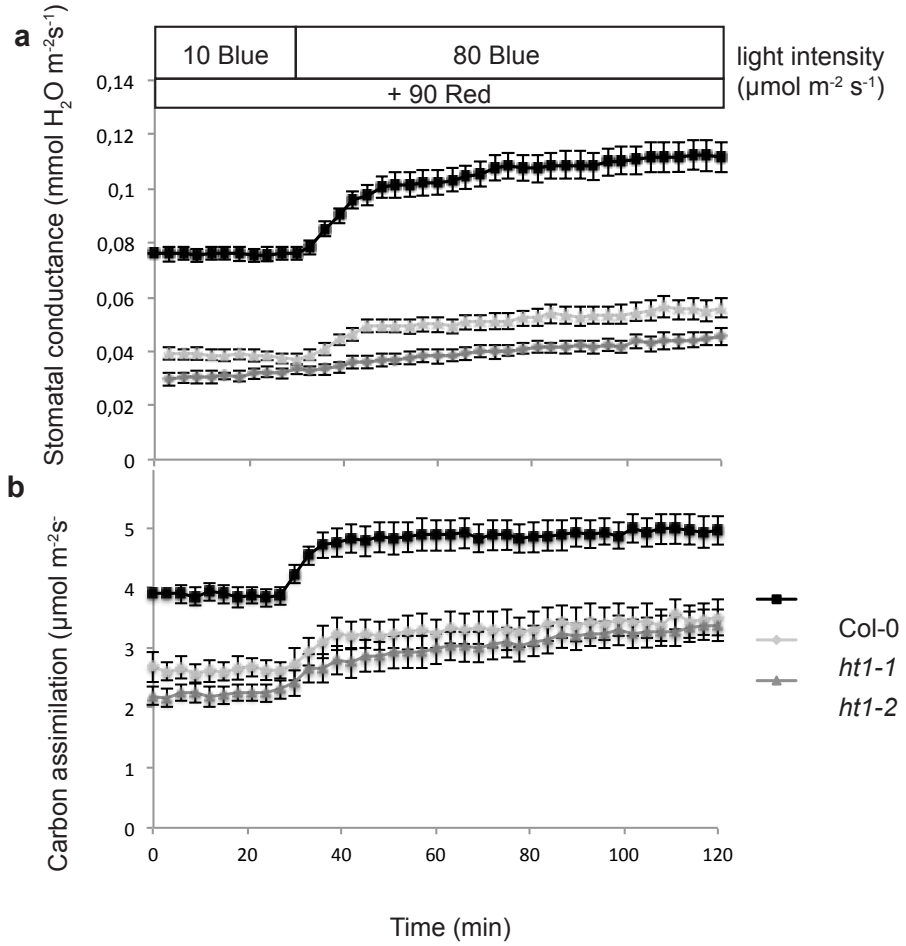

Figure 6. Stomatal and photosynthetic responses to blue light. **a**. Blue light response of stomatal conductance in Col-0, *ht1-1* and *ht1-2*. **b**. Blue light response of CO_2 assimilation during same experiment as above. Data presented are the mean of ± SE (n > 5).

5.2. The photosynthetic apparatus is functional in the *ht1-2* mutant

Red light triggers photosynthesis in plants. Since the red light stomatal response is impaired in *ht1* we wanted to examine whether the photosynthetic machinery was functional. Using an open gas exchange measurements system, light curve experiments were performed in which several photosynthetic parameters were measured simultaneously. The quantum yield of photosynthesis (PhiPSII) shows the fraction of absorbed photons that are used for

photochemistry for a light-adapted leaf. PhiPSII is relatively high at low light intensities and decreases as the light incident on a leaf increases. The quantum yield of photosynthesis in both wild-type and *ht1-2* mutant showed a predicted response pattern with no significant differences (Fig. 7). The CO_2 assimilation of photosynthesis at a given light intensity ($PhiCO_2$) decreased along with a drop in photosynthetic efficiency. Both genotypes demonstrated an overall decrease of $PhiCO_2$ with elevated intensities (Fig. 7). At 500 $m^{-2}s^{-1}$ light intensity the $PhiCO_2$ of the *ht1-2* mutant dropped compared to wild-type, but the difference was not statistically significant ($P>0.05$). Furthermore, at higher light intensities the $PhiCO_2$ performance in *ht1-2* was indistinguishable from that of the wild-type. The photochemical quenching (q_P) of fluorescence includes photosynthesis and photorespiration. It tends to be largest in low light, where leaves use light more efficiently. Conversely, non-photochemical quenching NPQ of fluorescence includes heat dissipation. NPQ is highest at the high light intensities, reflecting a plant protection mechanism to avoid over-energisation of the thylakoid membranes. q_P and NPQ measurements in both wild-type and *ht1-2* showed an expected normal response without significant differences (Fig. 7). According to the fluorescence data, the photosynthetic machinery is functional in *ht1-2* both for light-dependent and Calvin Cycle-dependent reactions. Therefore, the impaired red light-induced stomatal opening of *ht1* mutants is not due to an affected photosynthesis.

5.3. Effects of high red light intensity treatment on Col-0 and *ht1-2* mutant

In order to study photosynthetic performance after strong red light illumination, individual leaves were exposed to 30 min darkness followed by high pure red light (HRL) of 700 $m^{-2}s^{-1}$ for 2h. The quantum yield of PSII (Fv/Fm) was measured using an Imaging Fluorometer. The control leaves of Col-0 and *ht1-2* were not exposed to HRL and showed similar values Fv/Fm values, whereas HRL exposure caused a significant difference between genotypes ($P< 0.01$) (Fig. 8). It can be argued that the lower values of PSII quantum yield in *ht1-2* are due to inadequate red light-induced stomatal opening, which in turn led to insufficient transpirational cooling and insufficient CO_2 supply to the leaf. A lack of carbon supply to the mesophyll coupled to a higher leaf temperature will lead to imbalances in photosynthesis where excess energy will ultimately damage the photosystems. The HRL experiment demonstrates

that a non-functional stomatal red light response can severely affect plant photosynthesis and health.

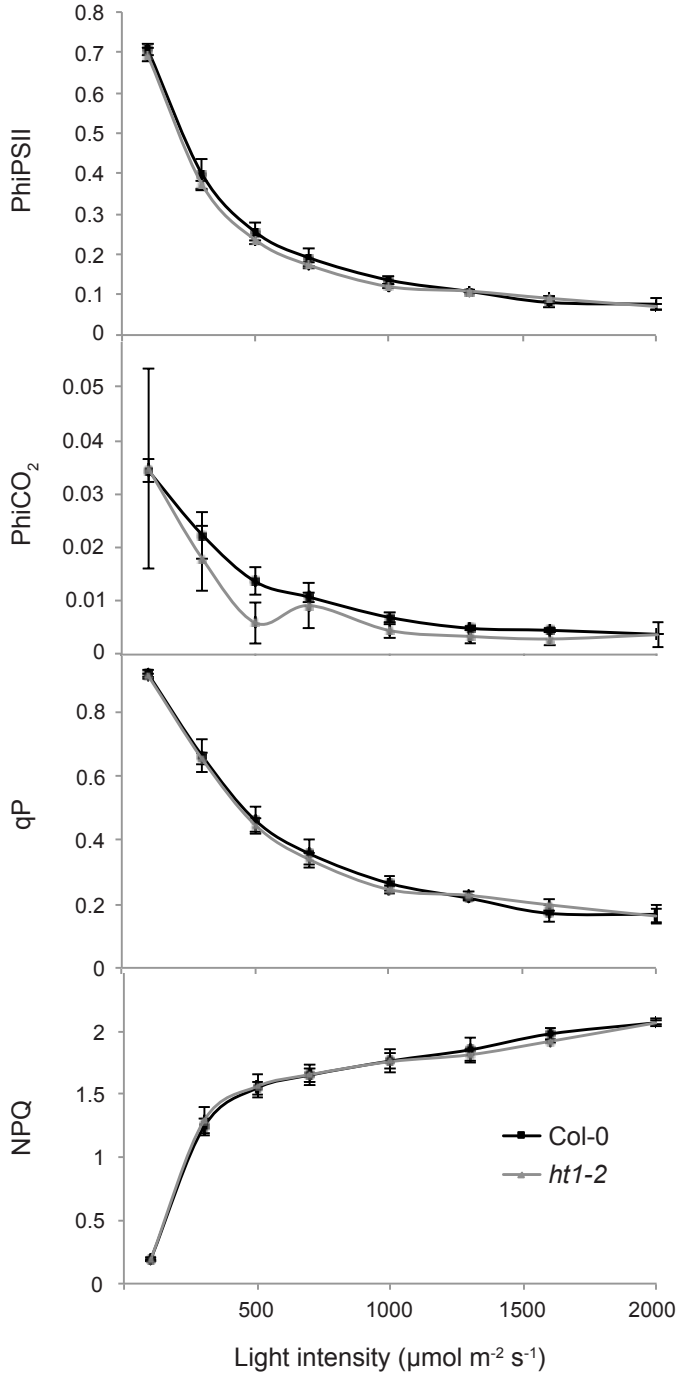

Figure 7. Photosynthetic responses to increasing light conditions as measured by Fluorescence in Col-0 and *ht1-2*. The light quality was consistently 90% red and 10% blue light. **a**. PhiPSII **b**. $PhiCO_2$ **c**. qP and **d**. Non photochemical quenching (NPQ). Data presented are the mean of ± StDev (n = 3).

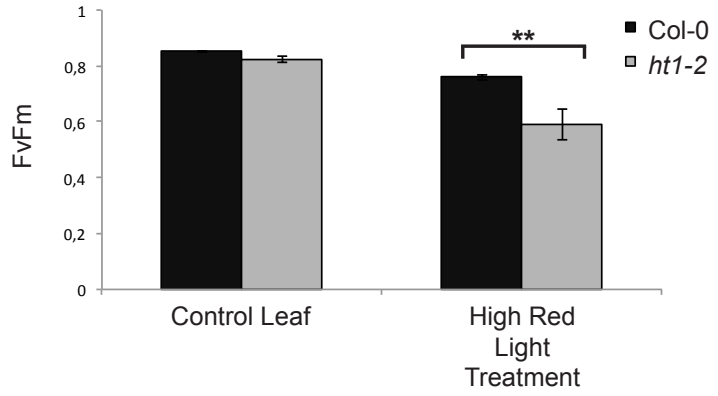

Figure 8. Comparison of maximum quantum yield of PSII (Fv/Fm, 15 min dark adaptation) using an Imaging Fluorometer in Col-0 and *ht1-2*. One leaf per plant were pre-exposed to 1 h darkness followed by 2 h high red light (700 µmol $m^{-2}s^{-1}$) using Li-COR 6400 XT. The remainder of each plant was kept in white light and did not receive any red light treatment. Data presented are the mean of ± SE (n=9). ***P*<0.001, Student's *t*-test.

5.4. Net carbon assimilation response to changing intercellular CO_2 concentration [Ci]

An alternative way to study the Calvin Cycle reactions of photosynthesis is to assay the net carbon assimilation under different Ci levels thus creating an A-Ci plot. A-Ci measurements allow, among other factors, the identification of the CO_2 compensation point where photosynthesis and respiration are in balance and the net carbon assimilation is zero. Both Col-0 and *ht1-2* show an increase in assimilation rate along with increasing CO_2 concentrations (Fig. 9). The compensation point of wild-type was 100 µmol mol^{-1} Ci compared to 200 µmol mol^{-1} Ci in *ht1-2*, meaning the mutant required twice as much carbon to reach balance between respiration and photosynthesis (Fig. 8). The lower A values in *ht1-2* may be due to the Calvin Cycle operating at decreased capacity at low Ci levels. Alternatively, the low stomatal conductance values of *ht1-2* might deprive the plants from getting enough CO_2 to drive the Calvin Cycle. Such deprivation would be even more noticeable at low CO_2 levels. The curve is plotted against Ci, not supplied CO_2, and Ci is based on calculations from parameters measured by the gas exchange analyzer system. In general, the lower the gs, the less reliable the calculated Ci values are (Li-Cor Inc. technical expert, personal communication). Therefore, the plotted low Ci values in *ht1-2* may not reflect the true intercellular CO_2 concentrations. Opposite to *ht1-2,* Col-0 has functional stomatal opening response with decreasing Ci levels

and therefore more accurate low Ci values. The experiment could be repeated with longer incubation times at each [CO_2] to allow for more opening and an increased stability. However, due to the inadequate low CO_2 stomatal response in *ht1* (Hashimoto et al., 2006), it is unlikely the result would be different. In other words, when the stomata of a mutant are restricted, an A-Ci curve will not be able to distinguish technical limitations from findings of biological relevance at low [Ci]. Alternatively, the examination of photosynthesis efficiency can be done by mass-spectrometry analyses measuring CO_2 uptake and O_2 evolution in MCPs and GCPs separately/individually (eg. Gautier et al., 1991). At the highest Ci values, no differences in CO_2 assimilation between Col-0 and *ht1-2* were observed (Fig. 9). Plants with impaired photosynthesis have been shown to have consistently lower assimilation levels at both low and high Ci as compared to wild-type (von Caemmerer et al., 2004; Lawson et al., 2008), arguing against a general impaired photosynthetic capacity in *ht1-2*.

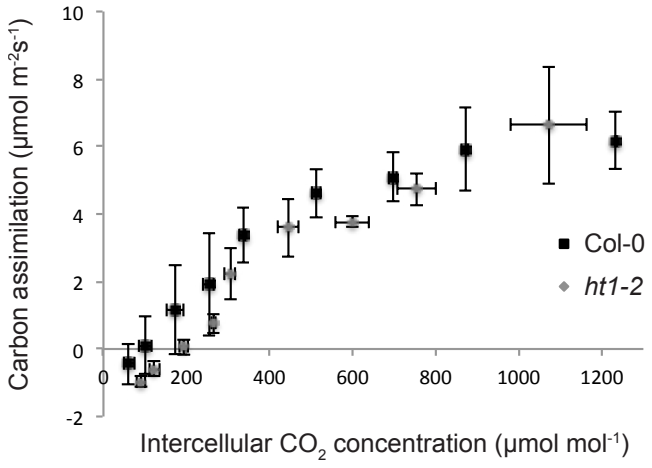

Figure 9. A-Ci curve, response of net carbon assimilation to intercellular CO_2 (Ci) in Col-0 and *ht1-2* measured under 100 red, 10 blue light intensity (µmol $m^{-2}s^{-1}$). Values were allowed to stabilize at a maximum of two min at each CO_2 concentration. Data presented are the mean of ± StDev (n=3).

5.5. Fusicoccin induces stomatal opening in *ht1-2* under high intensity red light

The phytotoxin fusicoccin can induce stomatal opening by the same manner as blue light via activation of plasma membrane H^+-ATPase of guard cells (Turner & Graniti, 1969;

Kinoshita & Shimazaki, 2001). Fusicoccin has been shown to induce opening in *ht1* (Hashimoto et al., 2006). In order to examine whether fusicoccin can induce H^+-ATPase activity in *ht1* despite pure red light illumination, microscopy-based bioassays were performed. Individual leaves, control or treated with fusicoccin, were exposed to pure HRL of 700 µmol mol^{-1} for 90 min. The *ht1-2* mutant showed statistically significant fusicoccin-induced opening responses under HRL exposure as measured both by stomatal ratio ($P<0.001$) and stomatal aperture ($P<0.001$) (Fig. 9). The results show that a lack of HT1 does not cause a general

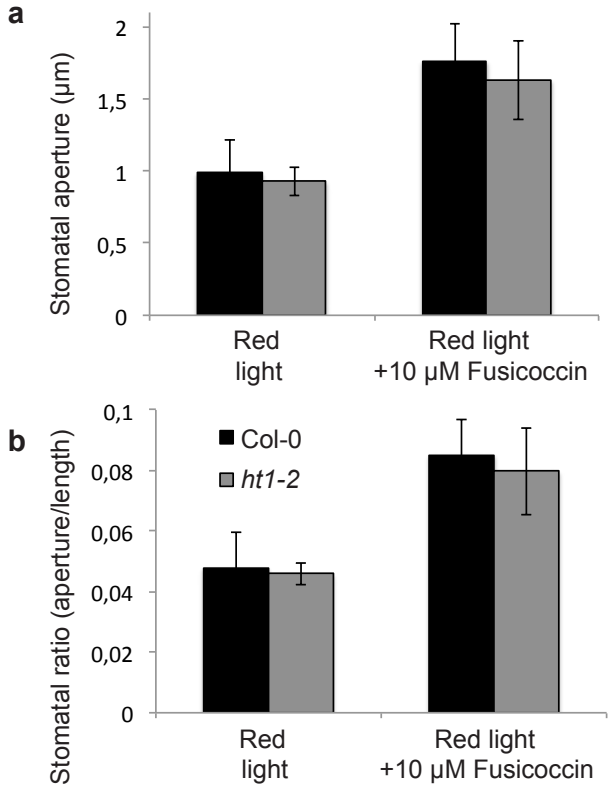

Figure 10. Fusicoccin-induced responses in red light of Col-0 and *ht1-2* leaves. Controls were white and red light-treated mock samples. **a** Stomatal aperture measured in microns **b.** Stomatal ratios calculated as stomatal average divided by total length of each stomata. Data presented are the mean of ± StDev (n>3).

inhibition of plasma-membrane H^+-ATPases under monochromatic red light. Despite recent progress on placing HT1 and OST1 in HCO_3^--mediated activation of SLAC1 (Xue et al., 2011), how red light leads to stomatal opening is not yet fully known (Schwartz & Zeiger, 1984; Tallman & Zeiger, 1988; Taylor & Assmann, 2001; Roelfsema et al., 2001). Is it possible to

place HT1 as a positive regulator in stomatal opening acting prior to H^+-ATPase activation, or on other mechanisms induced by red light and low [CO_2]? At the same time HT1 would inhibit high CO_2-induced stomatal closing. By comparison, blue light stimulates H^+-ATPases, through phosphorylation of their C-termini (Kinoshita & Shimazaki, 1999), and inhibits anion channels (Marten et al., 2007).

5.6. *ht1* mutants show wild-type de-etiolation response in white light

A positive role of phytochrome B in transducing red light-induced stomatal opening was shown in a recent study where over-expression of *PHYB* led to increased stomatal apertures and a *phyB* mutant back-ground had moderately decreased apertures (Wang et al., 2010). Far-red reversibility has also been shown for red light-induced stomatal opening (Talbott et al., 2002, 2003). Red light triggers a multitude of different physiological responses in plants including de-etiolation. The *ht1* mutant alleles were tested for their light-induced de-etiolated growth compared to *phyB* and Col-0. The hypocotyls of six day old *phyB* seedlings were characteristically elongated confirming a disrupted de-etiolation process (Fig. 11). The hypocotyls of *ht1-1* and *ht1-2* plants were similar to wild-type, demonstrating a functional de-etiolated plant growth (Fig. 11). The existence of a non-affected phyB-mediated response in *ht1*

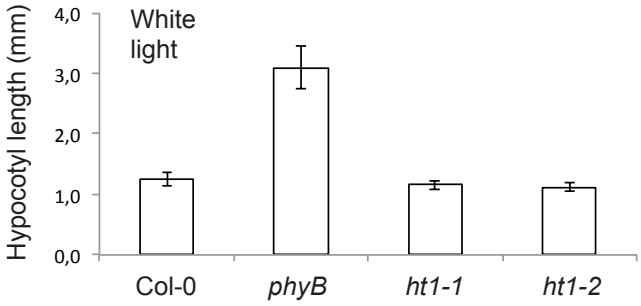

Figure 11. De-etiolation responses in Col-0, *phyB*, *ht1-1* and *ht1-2*. Hypocotyl lengths in white light-grown seedlings were measured. Data presented are the mean of ± StDev (n=3).

suggests that the *ht1* red light stomatal phenotype is not caused by an impaired phyB performance. In previous experiments where Ci levels were clamped, red light illumination could still induce stomatal conductance increases independent of altered [Ci] (Messinger et al.,

2006). Because the red light stomatal conductance response in *ht1-1* and *ht1-2* is severely impaired (Fig. 5), it could be argued that the function of HT1 lies downstream the convergence point of all, if there is more than one, red light pathways in control of stomatal aperture.

5.7. What is the role of HT1 in red light-induced stomatal opening?

The present study set out to understand if the red light response is generated via photosynthetic processes in the mesophyll or from processes located entirely within the guard cells themselves. The support for photosynthetic processes in red light-induced opening comes from studies where the opening to red light is saturated at fluences similar to those for photosynthesis saturation and is inhibited by DCMU (Sharke & Raschke, 1981; Tominaga et al., 2001; Messinger et al., 2006). Analyses of photosynthetic performance did not reveal any large deviations in *ht1* mesophyll photosynthetic electron transport (Fig. 7, 8) or Calvin Cycle efficiency (Fig. 6, 7, 9) as compared to wild-type. The *HT1* gene is expressed specifically and at high levels in guard cells (Hashimoto et al. 2006) and has been shown to function as an inhibitor of high CO_2-induced SLAC1 activation in isolated guard cell protoplasts (Xue et al., 2011). It is therefore plausible that HT1 activity is confined within the guard cells also in transduction of the red light response.

From our data it is conceivable that red light induces photosynthetic activities in the mesophyll and that a lowered Ci signal transfers into the guard cell where HT1 functions (Fig. 12). The inability of *ht1* to open to red light (Fig. 5) and low [CO_2] (Hashimoto et al., 2006) suggests that HT1 is a positive regulator to both signals. Based on the ability of *ht1* stomata to respond to blue light (Fig. 6; Hashimoto et al., 2006), HT1 activity is not involved in transducing blue light-induced stomatal opening. Our working model suggests that the red light pathway initiates photosynthesis in the mesophyll and a drop in Ci, followed by low [CO_2] signaling that requires HT1 activity in the guard cells. This would explain why both red and low [CO_2]-induced opening is impaired in *ht1*. In a model proposed by Roelfsema and Hedrich 2005, red light-induced opening is equal to a down-regulated high-CO_2 stomatal closing response (Fig. 2). A decreased [CO_2] would lead to less activation of anion channels, without affecting H^+-ATPase activity, and this would in turn cause hyperpolarization followed by stomatal opening. However, red light-induced opening is an active process that requires activation of H^+-ATPases to hyperpolarize the guard cell plasma membrane (Schwartz & Zeiger, 1984; Serrano et al., 1988; Olsen et al., 2002). There was no general inhibition of red

light-induced H^+-ATPase activation in *ht1*, as fusicoccin could induce stomatal opening in the presence of pure red light illumination (Fig. 10). Therefore, our model places HT1 prior to H^+-ATPase activity in control of red light-induced stomatal opening (Fig. 12). By comparison, blue light stimulates H^+-ATPases, through phosphorylation of their C-termini (Kinoshita & Shimazaki, 1999) and inhibits anion channels (Marten et al., 2007). Similarly, HT1 functions as

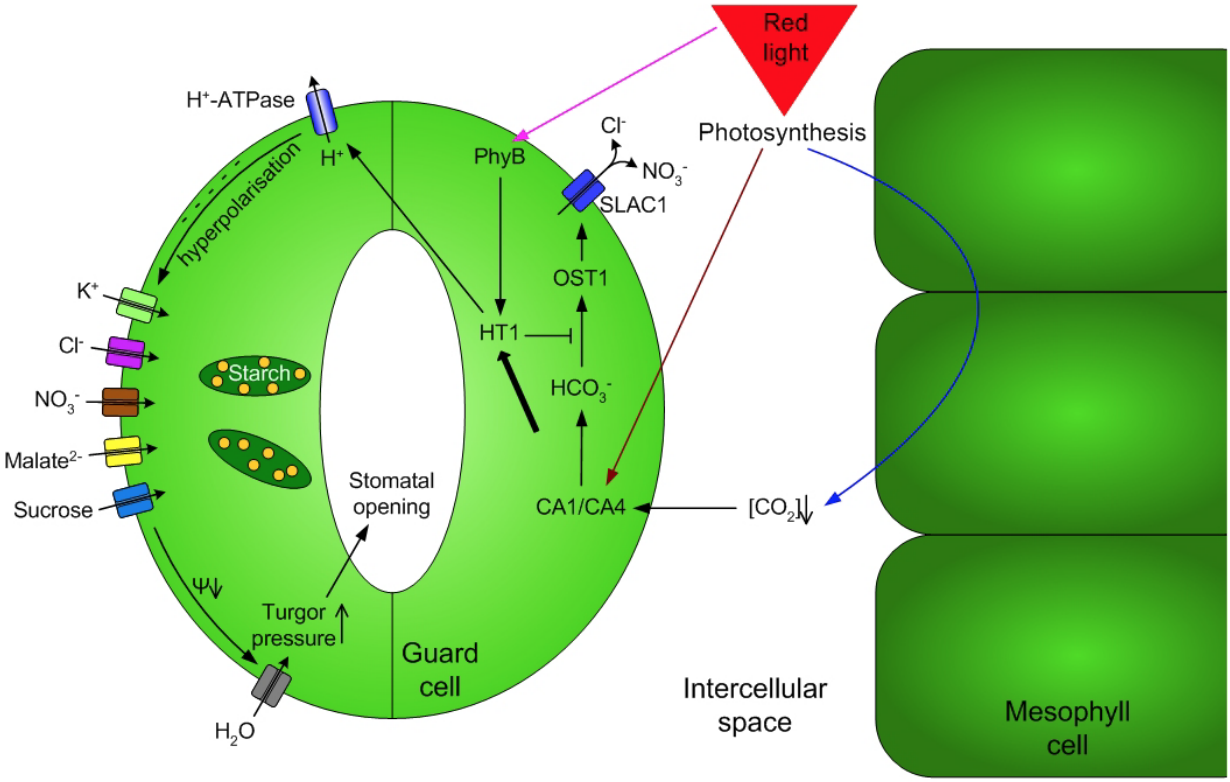

Figure 12. Model of red-light induced stomatal opening. Red-light induced mesophyll photosynthesis causes intercellular [CO_2] to drop (blue arrow) which is in turn perceived by guard cells. Carbonic anhydrases (CA1/CA4) catalyse CO_2 into bicarbonate ions and protons. Red light-induced guard cell photosynthesis (brown arrow) may also act as a signal. HT1 kinase activates H^+-ATPase leading to H^+ extrusion and plasma membrane hyperpolarisation. The latter activates K^+ inward-rectifying voltage-gated channels and therefore K^+ influx. $Malate^{2-}$ and other anions enter the cell together with sucrose. The water potential (ψ) decreases causing water to come into the cytosol. Turgor pressure rises, guard cells swell, stomatal pore opens. Red light activation of HT1 kinase inhibits the function of OST1 protein kinase which is known to stimulate SLAC1 and anion efflux. A photosynthetically independent pathway (pink arrow) is shown in parallell.

an inhibitor of SLAC1 anion channel activity (Xue et al., 2011) and thereby stomatal closing. Whether there is a photosynthesis-independent pathway dependent on phyB that regulate stomatal opening in response to red light is yet to be fully elucidated (Tallman et al., 2002,

2003; Wang et al., 2010). There was a lack of any compensating red light stomatal opening pathways in *ht1* (Fig. 5a), suggesting that the signaling pathways in control of red light-induced opening merge prior to HT1 activity (Fig. 12). The activity of CA1/CA4 and OST1 was placed in the pathway based on a decreased low [CO_2] stomatal responses in *ost1* (Xue et al., 2011) and *ca1/ca4* double mutants (Hu et al., 2010); and their role with HT1 in high [CO_2]-induced control of stomatal closing (Xue et al., 2011). According to our model, HT1 acts both as a positive regulator of H^+-ATPase activation (Fig. 12) and as an inhibitor of anion channel activation. HT1 functions within guard cells where it may act in close contact to membrane-located H^+-ATPases and ion channels in control of guard cell turgor pressure and hence regulate stomatal aperture.

5.8. Developing a guard cell protoplast isolation protocol

For the cell-specific RT-PCR and metabolite analyses, both small-scale (12 - 16 leaves) and large-scale (100 leaves) guard cell protoplasts isolations were optimized for our laboratory conditions based on Pandey et al., 2002; Zhang et al., 2008. Prior to an enzymatic digestion, the leaves were blended in distilled water and filtered. The remaining filtrate consisted of small mesophyll fragments together with epidermal fragments containing guard cells (Fig. 13A). The

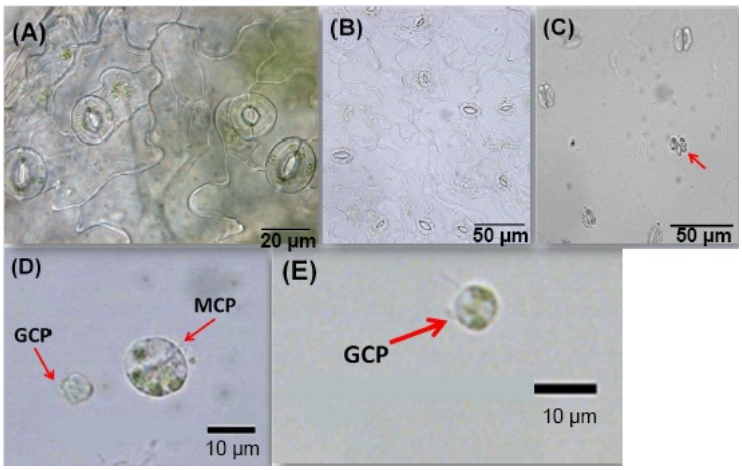

Figure 13. Different stages of enzymatic digestion.
(A) Epidermal peels following blending. **(B)** A peel after the first digestion. **(C)** Underdigested peel following the second digestion. The arrow indicates guard cells in the process of becoming spherical. **(D, E)** Guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP).

first digestion, out of two, enables the epidermal protoplasts to be released from the epidermal fragments leaving epidermal cell walls and intact guard cells. The epidermal protoplasts then burst as a result of the low osmolality (Fig. 13B). The second digestion releases the guard cell protoplasts into solution (Fig. 13D,E) where GCPs and MCPs are distinguishable from each other by the size differences (ØGCP=8µm, ØMCP=20µm). The duration of the second digestion is a crucial factor for getting healthy viable protoplasts. In the case of underdigestion, the protoplasts are still in process of becoming spherical (Fig. 13C). Overdigestion causes the formed protoplasts to collapse.

The expected yield was 2.7×10^6 GCPs per 100 leaves (Pandey et al., 2002) which was interpolated to approximately 4.3×10^5 for 16 leaves. The yield obtained in this study with the small-scale isolation protocol was 5.5×10^5 GCPs and with the large-scale isolation 2.08×10^6. The major constraint of this technique is getting sufficient amounts of pure protoplasts, which can be affected by many factors such as the age and overall health status of the plants, the freshness and concentration of the enzyme solutions, the duration of the digestions, the size of nylon meshes used for the filtrations in between each digestion, the conditions of the centrifugations at the last step of the isolation. All these factors were found to influence the results of the isolation in our hands. In order to optimize the procedure, the effect of an overnight-digestion has been examined. Here, the first digestion was extended to approximately 16 h overnight under a lower temperature of 19°C and with lower rotation speed, 80 rpm. The modified protocol resulted in 6-fold larger GCP yield ($3.65x10^6$ vs 5.5×10^5 GCPs) obtained per 16 leaves (personal communication, Marian Plaszczyca). It is important to point out that the analyses the GCPs are intended for may affect whether or not to include the longer overnight digestion protocol. For example, an overnight digestion, albeit at a lower temperature and slower rotation, may induce alterations in the metabolome or transcriptome where the latter can be avoided by adding transcriptional inhibitors but where changes in the metabolite profile are difficult to avoid. With functional protoplast isolation techniques in place, it is now possible to utilize them for downstream applications such as qPCR and metabolite analyses.

7. Conclusions

The present study has provided the first genetic evidence of a link between red light, a photosynthesis-derived drop in Ci and low [CO_2]-induced stomatal opening (Fig. 12). The *ht1* mutant is not affected in its photosynthetic capacity and does not induce stomatal opening to red light nor to low [CO_2], while it responds to blue light. Red light-induced de-etiolation was normal in *ht1*, suggesting that the red light stomatal phenotype was not due to a general phyB-deficient signaling. Based on the specific expression of *HT1* in guard cells, it is suggested that HT1 acts within the guard cells prior to H^+-ATPase activation and ion channel regulation to transduce red light-induced stomatal opening. Tools to prepare guard cell protoplasts have now been developed that will be used in future cell-specific analyses on guard cells versus mesophyll cells.

8. Future prospects

To further characterize the photosynthetic activity in the *ht1* mutant, **guard cell** measurements will be performed using **PAM microscopy**. The resolution on the fluorescence signal emitted from *Arabidopsis* guard cell protoplasts has not been strong enough in pilot experiments using a Walz set-up at the Department of Botany (Marian Plaszczyca, data not shown). By capturing individual stomata on leaf samples in the field of view, the fluorescence signal will include both guard cell and back-ground mesophyll. In order to assay the light reactions with cell-specificity, it is however the best option available. This method has the potential to unveil large deviations in photosynthetic efficiency of PSII between genotypes.

To characterize the **stomatal red light response in *ost1***, a mutant recently shown to respond poorly to low CO_2 (Xue et al., 2011; Mustilli et al., 2002), using gas exchange measurements. OST1 functions as a positive mediator of high CO_2 signal in the same pathway as HT1 in control of stomatal closing.

To screen for any transcriptional differences in genes with a role in low CO_2-induced stomatal opening between ***ht1* and wild-type guard cell protoplasts** using **Real-Time PCR**. Genes could include *CA1/CA4*, *OST1, KAT1, ABI1, SLAC1* and *AtALMT12*.

Measure **metabolites of guard cell protoplasts** in collaboration with Dr Thomas Moritz at Umeå Plant Science Center, in order to identify differences in metabolite composition between different genotypes affected in the control of stomatal aperture. In particular, differences in malate composition and Calvin Cycle metabolites would be of interest to examine.

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