Year « 2012 »

Title : The Role of *AtPHO1* in the Guard Cell Movements of *Arabidopsis thaliana*

Author : Céline Zimmerli

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive.
http://serval.unil.ch

Département de Biologie Moléculaire Végétale

The Role of *AtPHO1* in the Guard Cell Movements of *Arabidopsis thaliana*

Thèse de doctorat ès sciences de la vie (PhD)
présentée à la Faculté de Biologie et de Médecine de l'Université de Lausanne

par

Céline Zimmerli
Diplômée ingénieur agronome de l'Institut National Agronomique Paris-Grignon (Paris, France)

Jury

Prof. Christian Fankhauser. Président

Prof. Yves Poirier. Directeur de thèse

Prof. Enrico Martinoia. Expert

Dr. Nathalie Leonhardt. Expert

LAUSANNE

2012

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur	Prof. Christian **Fankhauser**
Directeur de thèse	Monsieur	Prof. Yves **Poirier**
Experts	Monsieur	Prof. Enrico **Martinoia**
	Monsieur	Dr Nathalie **Leonhardt**

le Conseil de Faculté autorise l'impression de la thèse de

Madame Céline Zimmerli

Ingénieur agronome de l'Institut National Agronomique, Paris-Grignon, France

intitulée

The Role of *AtPH01* in the Guard Cells Movements of *Arabidopsis thaliana*

Lausanne, le 31 août 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Christian Fankhauser

Summary

In plants, stomatal opening and closing are driven by ion fluxes that cause changes in guard cell turgor and volume, a process that is in turn regulated by complex environmental and hormonal signals such as light and the phytohormone abscisic acid (ABA). With this study, we present genetic evidence that stomatal movements in response to ABA are influenced by *PHO1* expression in guard cells of *Arabidopsis thaliana*. PHO1 is a phosphate exporter involved in phosphate loading into the root xylem vessels and, as a result, the *pho1* mutant is characterized by low shoot phosphate levels. In leaves, *PHO1* was found expressed at higher level in guard cells, and was quickly up-regulated following treatment with ABA. The *pho1* mutant was unaffected in ROS production following ABA treatment, and in stomatal movements in response to different light cues, high extracellular calcium, auxin, and fusicoccin. However, stomatal movements in response to ABA treatment were severely impaired, both in terms of induction of closure and inhibition of opening. Stomatal movements in response to hydrogen peroxide and reduced CO_2 was altered as well. Micro-grafting a *pho1* shoot scion onto wild-type root stock resulted in plants with normal shoot growth and Pi content, but failed to restore normal stomatal response to ABA treatment, showing that the impairment was not a simple pleiotropic consequence of phosphate deficiency. *PHO1* knockdown using RNAi specifically in guard cells of wild-type plants caused a reduced stomatal response to ABA. In agreement, specific expression of *PHO1* in guard cells of *pho1* plants complemented the mutant guard cell phenotype and re-established ABA sensitivity, although full functional complementation was co-dependent on shoot Pi sufficiency. Down-regulation of *PHO1* in guard cells did not alter the expression of ABA marker genes, indicating that *PHO1* does not affect the ABA signal transduction cascade at the transcriptional level. Together, these data

reveal an important role for phosphate and *PHO1* action in the stomatal response to ABA.

Résumé

L'ouverture et la fermeture des stomates des plantes sont des mouvements contrôlés par des flux d'ions causant des fluctuations de la turgescence des cellules de garde. Ce procédé est en retour régulé par des signaux environnementaux et hormonaux complexes, comme la lumière et l'hormone végétale acide abscissique (ABA). Nous présentons ici des preuves génétiques montrant que les mouvements stomatiques en réponse à l'ABA sont influencés par l'expression de *PHO1* dans les cellules de garde d'*Arabidopsis thaliana*. PHO1 est un exporteur de phosphate, impliqué dans l'efflux de phosphate des cellules corticales racinaires vers les vaisseaux de xylème. En conséquence, le mutant *pho1* est caractérisé par de faibles niveaux de phosphate dans les parties aériennes. Dans les feuilles, *PHO1* est exprimé préférentiellement dans les cellules de garde, comparé au mésophylle, et est rapidement induit par le traitement à l'ABA. Le mutant *pho1* n'est pas affecté dans la perception de l'ABA, dans la production de ROS en réponse à l'ABA, et dans la réponse des stomates aux traitements de lumière, à l'auxine, à la fusiccocine, et la forte concentration extracellulaire de calcium. En revanche, les mouvements de stomates en réponse aux traitements à l'ABA sont fortement affectés, dans l'induction de la fermeture des stomates comme dans l'inhibition de leur ouverture. De plus, les mouvements de stomates en réponse au péroxyde d'hydrogène et à la diminution du CO_2 sont aussi compromis. La création de micro-greffes composées d'une partie aérienne *pho1* greffés sur un système racinaire sauvage génère des plantes avec une croissance et une teneur en phosphate normale, mais ne permet pas de restaurer la réponse des stomates à l'ABA, ce qui démontre que le défaut de réponse à l'ABA n'est pas une simple conséquence pléiotropique de la carence en phosphate. La répression par RNAi de l'expression de *PHO1* dans les stomates de plantes sauvages provoque une réduction de la réponse des stomates à

l'ABA, mais n'affecte pas la réponse de gènes marqueurs à l'ABA, ce qui suggère que *PHO1* n'agit pas au niveau transcriptionnel. Parallèlement, l'expression de *PHO1* dans les cellules de gardes de mutants *pho1* complémente le phénotype stomatique mutant et rétablit la réponse à l'ABA, bien que la totale complémentation nécessite l'apport normal de phosphate aux parties aériennes. Ensemble, ces résultats révèlent l'influence importante de *PHO1* et du phosphate dans la réponse des stomates à l'ABA.

Abbreviations

ABA: abscisic acid

ATP: adenosine-5’-triphosphate

DNA: deoxyribonucleic acid

EMS: ethyl methane sulfonate

GFP: green fluorescent protein

GST: gene sequence tag

GUS: ß-glucuronidase

NMR: nuclear magnetic resonance

OPDA: 12-oxo-phytodienoic acid

PCR: polymerase chain reaction

Pi: inorganic phosphate

PSI: phosphate starvation inducible

RNA: ribonucleic acid

RNAi: ribonucleic acid interference

ROS: reactive oxygene species

RT-PCR: reverse transcription polymerase chain reaction

qRT-PCR: quantitative real-time polymerase chain reaction

SE: standard error

List of original publications and conférences

Zimmerli C, Ribot C, Vavasseur A, Bauer H, Hedrich R and Poirier Y (2012). *PHO1* expression in guard cells mediates the stomatal response to abscisic acid in *Arabidopsis*. *Plant J*, Accepted manuscript online: 21 May 2012. DOI: 10.1111/j.1365-313X.2012.05058.x.

Not associated with this manuscript:

Ribot C, Zimmerli C, Farmer EE, Reymond P, and Poirier Y (2008). Induction of the *Arabidopsis PHO1;H10* Gene by 12-Oxo-Phytodienoic Acid But Not Jasmonic Acid via a CORONATINE INSENSITIVE1-Dependent Pathway. *Plant Physiol*, 147(2):696–706.

Conférences:

Zimmerli C, Ribot C, Poirier Y (2011). *AtPHO1* Expression In Guard Cells Influence The Response Of Stomata To Abscissic Acid. 22nd International Conference on Arabidopsis Research, University of Wisconsin, Madison USA (Poster).

Zimmerli C, Poirier Y (2010). *AtPHO1*: a phosphate transport-related gene involved in guard cells movements. 1st Swiss Plant Science Web Summer School, Mürren Switzerland (Poster).

Contents

“With this model and the key to it, it will be possible to go on forever inventing plants and know that their existence is logical; that is to say, if they do not actually exist, they could, for they are not the shadow phantoms of vain imagination,but possess an inner necessity and truth.“

— Goethe, Rome July 31,1787

1 - Introduction

Guard cells integrate complex environmental signals into ion fluxes

The stomatal pore, a gateway between the plant and the atmosphere

Terrestrial plants need to adapt to the local and global ever-changing environments. In particular, they tightly control the gas exchanges between above ground organs and the surrounding atmosphere (Hetherington and Woodward, 2003): CO_2 has to penetrate the leaf to allow photosynthesis, all the while keeping water loss at a minimum to avoid desiccation. Plants are able to minimize dehydration thanks to the presence of the cuticle, an impermeable wax and cutin layer that covers 95% of the epidermal surface of their stems and leaves. The simultaneous regulation of transpirational water loss as well as the CO_2 uptake is then optimized by functionally specialized epidermal cells, known as guard cells, arranged in pairs surrounding a stomatal pore that connects the interior of the leaf to the atmosphere.

The aperture of the stomata, as well as the number of stomata on the epidermis, thus directly influence photosynthesis and water status of the plant, allowing tight regulation of gas exchanges depending on varying environmental conditions such as

light, CO_2 concentration, humidity or time of day. In addition, stomata also act as a barrier against pathogens or pollutants such as ozone (Torsethaugen *et al.*, 1999; Melotto *et al.*, 2006; Zeng *et al.*, 2010). These environmental signals act either in a synergistic or antagonistic manner on stomatal movement: light and reduced CO_2 induce the opening of the stomata to allow more CO_2 uptake for photosynthesis. Conversely, drought, the phytohormone abscisic acid (ABA), darkness, elevated CO_2 and ozone typically cause stomatal closure in an effort to reduce water loss, regulate photosynthesis, or protect the inside of leaves from ozone-induced oxidative damage (Kim *et al.*, 2010). It is believed that both the acquisition of stomata and of an impermeable cuticle constitute key steps in the adaptation of advanced terrestrial plants, allowing them to adapt quickly to a large range of different and fluctuating environments (Hetherington and Woodward, 2003).

Morphologically, guard cells are small dumb-bell, in Graminaea, or kidney-shaped cells, in Dicots, that have the particularity of being completely autonomous due to the loss of plasmodesmatal connections early in cell development (figure 1.1a and b). In most plants, stomata can be found on both the adaxial (upper) and abaxial (lower) leaf surface, but the majority is usually found on the abaxial epidermis. Stomata can also be found on stems, petiole or non-photosynthetic organs (Roelfsema and Hedrich, 2005). Stomatal proliferation and development is intimately linked to environmental parameters such as the levels of humidity, light, and carbon dioxide (Bergmann and Sack, 2007).

Stomatal movements depend on guard cell turgor

The regulation of the stomatal pore size by guard cells involves fluctuations in their hydrostatic pressure and volume status, a unique process in that it is dynamically

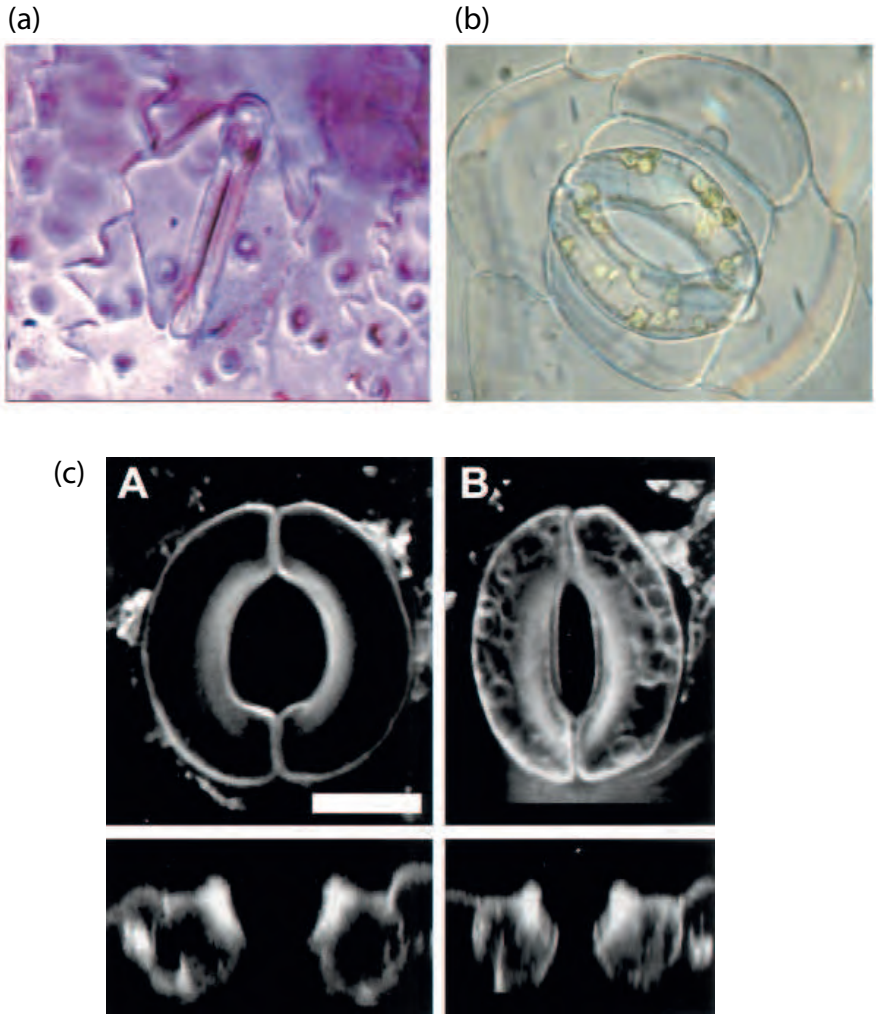


Figure 1.1: **Guard cells morphology and the opening/closing of the stomatal pore.** (a) Dumb-bell-shaped stomata of rice typical of the grasses and (b) the kidney-shaped stomata typical of other species such as *Arabidopsis thaliana* (Hetherington and Woodward, 2003). (c) Paradermal (upper) and transverse (lower) confocal images of opened (left) and closed (right) *Vicia faba* guard cells, visualised with a membrane-specific fluorescent dye. Scale bar = 20 μm (Shope *et al.*, 2003).

and reversibly adjustable, making it possible to rapidly fine tune the response to complex environmental and intrinsic hormonal signals (Nilson and Assmann, 2007; Schroeder *et al.*, 2001). The elevation of guard cell turgidity occurs via an influx and a re-mobilization of solutes, inorganic and organic ions and sugars, which leads to an increase in osmotic potential and a decrease in cell water potential. This mechanism relies on the activity of ion channels, vacuolar and membrane transporters, as well as on the reconversion of metabolites. Because of the high hydraulic conductivity of the plasma membrane, water is then drawn into the guard cell to equilibrate water potential with the apoplast. This influx of water elevates turgor pressure resulting in cell swelling (Roelfsema and Hedrich, 2005; Sirichandra *et al.*, 2009*b*). The asymmetrical structural reinforcement of the guard cell wall, due to a radial arrangement of cellulose fibrils, cause a differential longitudinal expansion as the cell inflates, forcing the two guard cells to bend apart instead of pushing more closely together, and thus widening the stomatal pore. Conversely, stomatal closure occurs when an efflux of solutes triggers a decrease in cell turgidity, and thus promotes cell deflation. During opening and closure, guard cells therefore undergo profound changes in volume and plasma membrane surface area, apparently through vacuole fragmentation (Martinoia *et al.*, 2012), and vesicles fusion (Shope *et al.*, 2003; figure 1.1c).

The ion fluxes occurring across the plasma membrane and the tonoplast of guard cells during stomatal opening and closure (figure 1.2) were elucidated early in the 1980s and 1990s, thanks to a large set of physiological studies. It was soon discovered that stomatal opening involves proton efflux into the apoplast through activation of proton pumps ATPases (H^+-ATPases) located in the plasma membrane. This H^+ efflux hyperpolarizes the membrane potential beyond the equilibrium potential of K^+, thus activating voltage dependent inward K^+ channels leading to the influx of osmotically

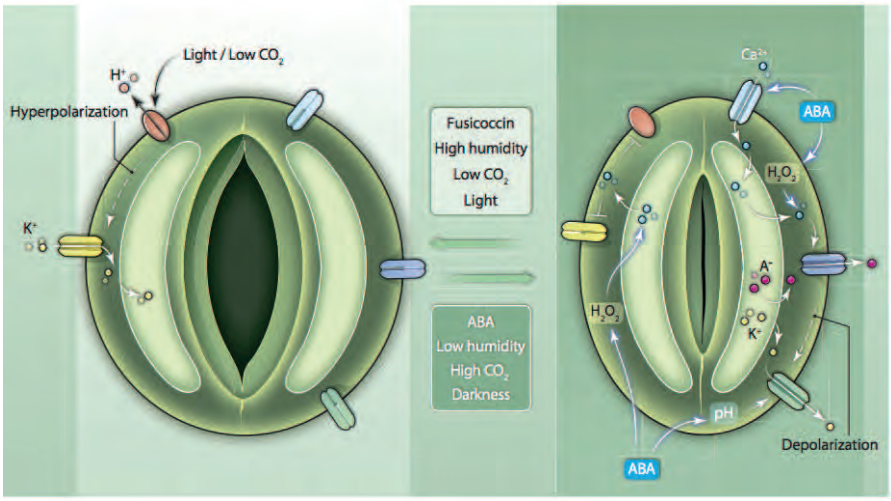


Figure 1.2: **Ion fluxes behind stomatal opening (left) and closing (right) in response to environmental and hormonal signals.** The vacuole is represented in pale green. Red, H^+-ATPase; yellow, K^+ inward-rectifying channel; light blue, Ca^{2+} permeable channel; dark blue, anion channels; light green, K^+ outward-rectifying channel (Joshi-Saha *et al.*, 2011).

active K^+ (Sirichandra *et al.*, 2009*b*). It is now believed that Cl^-, NO_3^- (Guo *et al.*, 2003) and sugars are also imported during stomatal opening, and that a large part of the anionic species accumulation comes from the breakdown of starch into $malate^{2-}$ (Kim *et al.*, 2010; Pandey *et al.*, 2007). During stomatal closure, the activity of H^+-ATPases is repressed, and anion channels are activated to trigger a passive efflux of anion into the apoplast, mainly in the form of Cl^-, NO_3^-, and $malate^{2-}$. The resulting membrane depolarization activates K^+ efflux through voltage-dependent outwardly-rectifying K^+ channels, leading to the decrease of osmotic potential that precedes cell deflation. Frequently, an elevation of cytosolic Ca^{2+} is observed during stomatal closure, via activation of Ca^{2+}-permeable channels at the plasma membrane, or remobilisation from internal compartments (Roelfsema and Hedrich, 2005; Nilson and Assmann, 2007; Pandey *et al.*, 2007; Sirichandra *et al.*, 2009*b*; Kim *et al.*, 2010).

Identification of guard cell ion channels and transporters in *Arabidopsis*

Because stomata integrate complex environmental signals into ion fluxes and an easily scored phenotype (i.e. stomatal aperture), guard cells have become an ideal genetic model to study membrane transport systems. In *Arabidopsis*, a lot of research efforts have thus been gathered in the search of the molecular identities that signal changes in guard cell turgor through ion transport and remobilisation (figure 1.3). Since guard cells lack plasmodesmata, ion fluxes in and out of the cells must occur at the plasma membrane via transporters and channels. Ion channels mediate ions fluxes down their electrochemical gradient, usually through pore-forming proteins (passive transport). Ion transporters on the other hand include carriers, symporters, antiporters, and also pumps that use energy (active transport), for example in the form of ATP or of the concentration gradient of another ion, to drive ion transport against their energy gradient (Pandey *et al.*, 2007).

Proton pump H^+-ATPases - The identification of the *AHA1* locus encoding a proton pump H^+-ATPases (Merlot *et al.*, 2007, 2002) was elucidated through forward genetic screens of mutants with increased transpiration as visualized by lower leaf temperature: the allelic mutations *openstomata2 ost2-1* and *ost2-2* in the *AHA1* locus render the AHA1 H^+-ATPases constitutively active, keeping therefore the stomata open even under drought stress. *AHA1* belongs to a gene family comprising 10 other members, all expressed in guard cells (Ueno *et al.*, 2005), suggesting some level of functional redundancy.

K^+ channels - Analysis of K^+ currents revealed two different types of K^+ channels in guard cell plasma membrane: outward rectifying, which are activated by depolarisation and are responsible for potassium extrusion; and inward-rectifying, which are

activated by hyperpolarisation and are responsible for potassium uptake (Schroeder *et al.*, 1987). Among the nine K^+ channels identified in *Arabidopsis*, if was found that *KAT1* and *KAT2* encode the majority of the guard cells voltage dependent K^+ inward-rectifying channels (Pilot *et al.*, 2001), while the GORK gene encoding for a K^+ outward-rectifying channels is apparently responsible for all potassium outward currents (Hosy *et al.*, 2003).

Anion and sugar transporters - Little is still known on how guard cells import anions, in particular in the case of Cl^-, although it is likely that guard cells possess a H^+-coupled Cl^- transporter (Pandey *et al.*, 2007). On the other hand, there are some evidence suggesting that AtNRT1.1 (CHL1) mediates NO_3^- uptake during stomatal opening under light (Guo *et al.*, 2003). The *chl1* mutant displays smaller stomatal aperture than wild-type under light when NO_3^- is supplied instead of Cl^-, indicating the involvement of CHL1 in the uptake of nitrate during stomatal opening. A recent study also demonstrated that AtABCB14, an ABC transporter that modulates stomatal closure on transition to elevated CO_2, is involved in malate import into the guard cells (Lee *et al.*, 2008). Finally, it is likely that *Arabidopsis* guard cells also import sucrose via the sucrose transporter AtSUC3, which is expressed in guard cells (Meyer *et al.*, 2004), and via the AtSTP1 H+/monosaccharide symporter (Stadler *et al.*, 2003).

Anion channels - Early studies described the presence of rapid-transient (R-type) and slow-activating (S-type) anion current (Schroeder and Keller, 1992) during stomatal closure, in response to ABA, elevated CO_2 and calcium transients. However little was known about the molecular identity behind the anion channels involved in guard cell anion efflux, until two recent independent genetic screens converged to identify a component of a S-type anion channel, SLAC1 (Negi *et al.*, 2008; Vahisalu *et al.*, 2008), followed later by the characterization of its homologue SLAH3 (Geiger *et al.*,

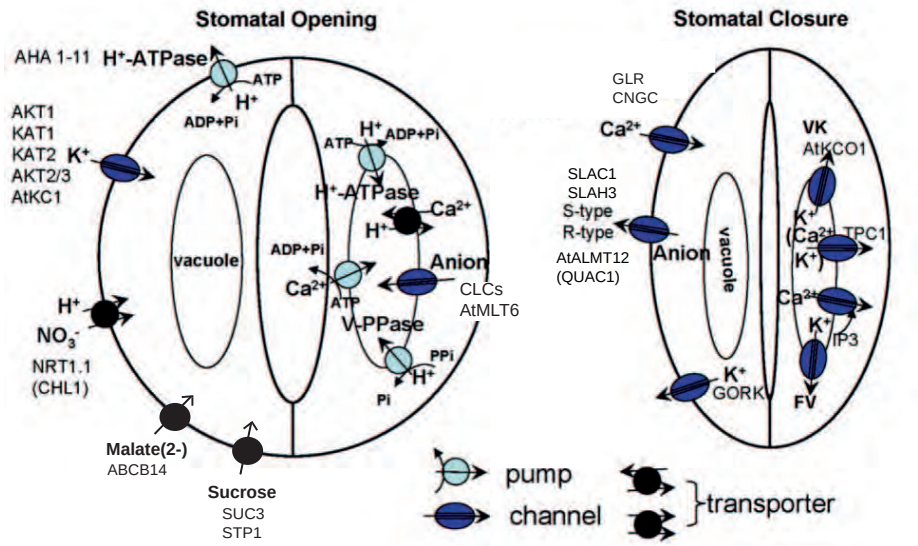


Figure 1.3: **Ion channels and transporters functioning in stomatal movements.** The left stomata shows transport proteins active during stomatal opening and the right stomata shows transport proteins active during stomatal closure (adapted from Pandey *et al.*, 2007).

2011). SLAC1 and SLAH3 are both expressed in guard cells and localize to the plasma membrane, and heterologous expression in Xenopus oocytes demonstrated their conductance to chloride and nitrate. Recently, AtALMT12 (also known as QUAC1, for quick anion channel 1) was also identified as a convincing guard cell R-type anion channel candidate permeable for malate (Meyer *et al.*, 2010; Barbier-Brygoo *et al.*, 2011; Roelfsema *et al.*, 2012).

Ca^{2+}-permeable channels - In *Arabidopsis*, several gene families have been predicted to encode Ca^{2+}-permeable channels (Pandey *et al.*, 2007; Roelfsema and Hedrich, 2010). The genes with high homology to the animal cyclic nucleotide gated channels (CNGC family) being good candidates, recent work on AtCNGC2 (cyclic nucleotide gated channel 2) showed that this channel is permeable for Ca^{2+} in guard cell pro-

toplasts (Ali *et al.*, 2007). A second family of genes are the ionotropic glutamate receptor-like (GLR) genes, which are believed to either conduct Ca^{2+} or activate a Ca^{2+} channel, based on the ability of glutamate to induce a Ca^{2+} influx in root cells. In this context, Cho *et al.* (2009) reported that the *Arabidopsis* glutamate receptor homologue AtGLR3.1 is preferentially expressed in guard cells compared to mesophyll cells, and that its over-expression affects Ca^{2+} oscillation-regulated stomatal movements. Although it is generally assumed that these channels reside in the plasma membrane, the cellular localization has not been confirmed, and localization in the vacuole or other organelles is possible.

Vacuolar transporters - About 90 % of the solutes released out of the guard cells comes in fact from the vacuole (Kim *et al.*, 2010; Schroeder *et al.*, 2001), emphasizing the importance of transport across the tonoplast during stomatal movements. Guard cell vacuoles store H^+, and guard cell patch clamp recordings suggest that vacuolar proton accumulation depend on the activity of vacuolar-type H^+-ATPases (V-ATPases) as well as H^+ translocating pyrophosphatases (V-PPases, Pandey *et al.*, 2007). At least 26 different V-ATPase subunit genes exist in *Arabidopsis*, and their importance in guard cells was backed up by experiments with the de-etiolated 3 (*det3*) mutant, lacking a subunit of the vacuolar H^+-ATPase (Allen *et al.*, 2000). Importantly, proton gradient also indirectly influence the transport of other solutes via H^+-coupled antiporter, or modulation of tonoplast potential to regulate voltage dependent channels.

Several cation channel activities have been identified at the guard cell vacuolar membrane, including fast vacuolar (FV), slow vacuolar (SV) and Ca^{2+}-activated K^+-selective vacuolar (VK) cation channels (Pandey *et al.*, 2007). These channels play an important role for K^+ and Ca^{2+} remobilization during stomatal movements (Ward and Schroeder, 1994). FV and SV channels are voltage regulated and mediate K^+ and Ca^{2+} cur-

rents, while VK channels are voltage-independent and specific to K^+. Recently, the SV channel has been identified and found to be encoded by the TPC1 (two-pore channel) gene (Peiter *et al.*, 2005), while KCO1/TPK1 was identified as a tonoplast voltage-independent VK channel involved in K^+ release, and activated by Ca^{2+} (Gobert *et al.*, 2007).

Regarding guard cell anion transport at the tonoplast, little was known until recent reports: Nagy *et al.* (2009) investigated the role of AtMRP5/AtABCC5, an inositol hexakisphosphate transporter located in the tonoplast and shown to be involved in stomatal movements; members of the AtCLC family seem to be involved in NO_3^- transport (AtCLCa, a tonoplast NO_3^-/H+ exchanger expressed in guard cells, as described by De Angeli *et al.*, 2006), and Cl^- transport (AtCLCc, targeted to the tonoplast and implicated in stomatal movements as described by Jossier *et al.*, 2010); finally AtALMT6 was recently reported as a guard cell vacuolar inward-rectifying malate channel (Meyer *et al.*, 2011; Martinoia *et al.*, 2012; Kollist *et al.*, 2011).

The regulation of stomatal movements by light, CO_2 and ABA

The activity of the transporters and channels mentioned above must be tightly regulated in order to allow stomata to quickly regulate their stomatal aperture in response to environmental and hormonal signals, and to support their central role in regulating photosynthetic CO_2 absorption and water loss. Some major signals include light, CO_2 concentration, and the drought hormone abscisic acid (ABA).

As mentioned before, light, a decrease in CO_2 concentration, and high humidity favor stomatal opening. Conversely, dark, an increase in CO_2 concentration, and water stress, all promote stomatal closure. These signaling pathways share both common and unique signaling steps.

Light signaling - Light is an central regulator of stomatal movement and is intimately linked to metabolism: by controlling tightly the stomatal aperture in response to light cues, plants are able to exploit the available light efficiently by modulating CO_2 fixation and photosynthesis, without losing unnecessary amounts of water (Shimazaki *et al.*, 2007).

Light triggers stomata to open, and distinct regulation mechanisms exist depending on the part of the light spectrum: red light acts primarily as an energy source: it induces stomatal opening by driving photosynthesis in mesophyll and guard cell chloroplasts, thus decreasing the intercellular CO_2 concentration (Roelfsema *et al.*, 2002). Stomatal opening under red light may then result from the direct response to this decrease in CO_2 concentration, as well as to the accumulation of sugars from the combination of photosynthesis, starch degradation, and sugar import from the apoplast. Alternatively, red light can act as a signal, through phytochrome B, controlling the expression of transcription factors involved in the regulation of stomatal opening (Wang *et al.*, 2010).

Blue light on the other hand acts primarily as a signal to trigger stomatal opening: blue light is captured by receptor kinases known as phototropins (PHOT1 and PHOT2 in *Arabidopsis*), which are phosphorylated and in turn activate, by direct or indirect phosphorylation, the plasma membrane H^+-ATPases (Assmann *et al.*, 1985; Kinoshita *et al.*, 2001) while repressing S-type anion channels (Marten *et al.*, 2007). Additionally, some data suggest that cryptochromes (CRY1 and CRY2) function additively with phototropins in mediating blue light-induced stomatal opening, and that Constitutively Photomorphogenic 1 (COP1), acts to repress stomatal opening in darkness (Mao *et al.*, 2005). Stomata are highly sensitive to blue light, this signal being twenty times more effective than red light in opening stomata, but interestingly, the

blue light response is only weak in the absence of red light, and is exacerbated in background of red light. Intercellular CO_2 concentration and guard cell chloroplasts could play a role in this synergistic action (Shimazaki *et al.*, 2007).

CO_2 signaling - In response to elevated CO_2 concentration, guard cells present a rapid physiological response as well as a sustained developmental mechanism (Hetherington and Woodward, 2003; Kim *et al.*, 2010): because less opening is required for efficient carbon intake, a rise in CO_2 concentration triggers rapidly stomatal closure, and in long term provokes a reduction in stomatal density, further reducing overall stomatal conductance. There is however surprisingly little knowledge on how CO_2 regulates stomatal aperture. In particular, it is matters of debate whether the CO_2 response resides entirely in guard cells, or is indirectly controlled by unknown signals emerging from the mesophyll cells, such as for example extracellular malate, which is known to activate guard cell anion channels. Yet, there is evidence for a direct functional role of guard cells in mediating at least some of the CO_2 response, meaning that both cell types could contribute to the signaling mechanisms (Mott, 2009).

Elevated CO_2 promotes stomatal closure by activation of anions channels (Negi *et al.*, 2008) and K^+ outward-rectifying channels, a response that seems co-dependent on Ca^{2+} signaling. Reports show that CO_2 induces cytosolic calcium elevations, a process which in turn could inhibit the activity of H^+-ATPases and K^+ inward-rectifying channels (Vavasseur and Raghavendra, 2005). In that sense, the CO_2 signaling pathway shows convergence with the ABA signaling pathway (see *ABA signaling,* below). However unlike ABA, elevated CO_2 does not appear to act through changes in cytosolic pH, but requires the action of carbonic anhydrases early in the signaling pathway , which catalyse CO_2 into bicarbonate ions and protons (Hu *et al.*, 2010). A recent report demonstrated that it is the elevated bicarbonate, more so than elevated CO_2,

that acts as intracellular signaling molecule to activate guard cell anion channels (Xue *et al.*, 2011).

ABA signaling - The phytohormone abscisic acid (ABA, figure 1.4a) was discovered in the 1960s. Numerous physiological, biochemical and genetic analyses have uncovered roles for ABA in numerous stress (e.g. water stress) and developmental processes (e.g. germination and seedling growth).

Importantly, ABA is the key actor of the regulation of stomatal movements in response to water stress. Under drought and high salinity, an immediate hydraulic signal is perceived by the plant (Christmann *et al.*, 2007) which triggers ABA levels to rapidly increase to physiologically active levels, primarily through *de novo* synthesis in the vasculature (Raghavendra *et al.*, 2010). ABA is then taken up (Kang *et al.*, 2010; Kuromori *et al.*, 2010) and perceived by guard cells, which respond by activating stomatal closing mechanisms, in an effort to minimize the loss of water through transpiration (figure 1.4b): ABA promotes stomatal closure by activating the R-type (rapid) and S-type (slow) anion channels that drive a release of anions (such as Cl^- and NO_3^-) and organic acids (such as $malate^{2-}$) to the outside of the cells, leading to membrane depolarization. ABA triggers also an influx and internal remobilization of Ca^{2+}, as well as a raise in internal pH; these signals stimulate the K^+ outward-rectifying channels, and all these ion fluxes cause the reduction in turgor pressure that precedes stomatal closure. In parallel, ABA inhibits H^+-ATPase pumps and K^+ inward- rectifying channels, thus preventing stomatal opening (Pandey *et al.*, 2007; Sirichandra *et al.*, 2009*b*).

The regulation of ABA signal transduction is very complex, and involves many secondary messengers including elevations of $[Ca^{2+}]_{cyt}$, production of reactive oxygen species, nitric oxide, phosphatidic acid (PA), phosphatidyl-inositol- 3-phosphate (PIP3),

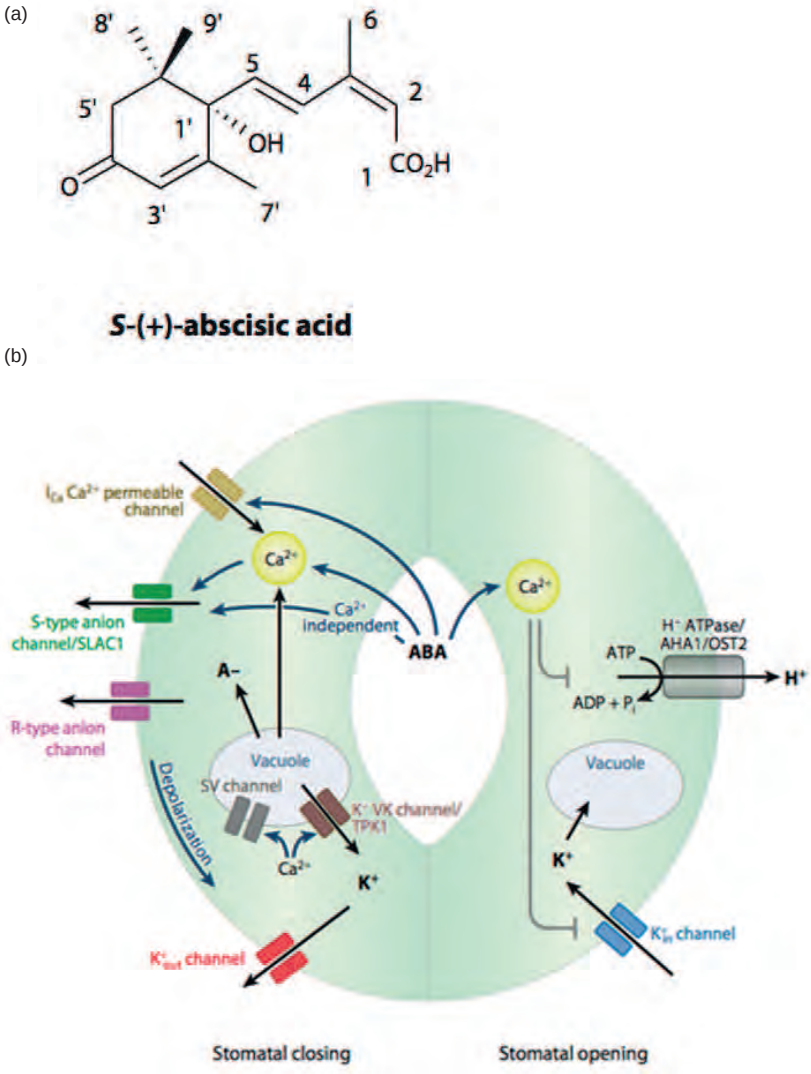


Figure 1.4: **Summary of ABA guard cell signaling and ion channel regulation.** (a) Chemical structure of ABA (Cutler *et al.*, 2010). (b) ABA-induced signal transduction across the plasma membrane and vacuolar membrane of guard cells. signaling events during stomatal closing are shown in the left guard cell, and major regulation steps for ABA-inhibition of stomatal opening mechanisms are shown in the right guard cell. Abbreviations: ABA, abscisic acid; ICa, inward Ca^{2+} current; S-type, slow-type; SLAC1, slow anion channel associated 1; R-type, rapid-type; SV, slow vacuolar; VK, vacuolar K^+ selective; TPK1, two pore K^+ channel 1; AHA1, *Arabidopsis* H^+ ATPase 1; OST2, open stomata 2 (Kim *et al.*, 2010).

inositol-3-phosphate (IP3), inositol-6-phosphate (IP6), sphingolipids, as well as cytosolic pH variations and regulation of gene expression (Sirichandra *et al.*, 2009*b*; Kim *et al.*, 2010).

Much progress has been recently made in the understanding of the earliest events involved in ABA signal transduction. Over the last decade, several ABA binding proteins had been proposed intermittently (Razem *et al.*, 2006; Shen *et al.*, 2006; Liu *et al.*, 2007; Pandey *et al.*, 2009), but their exact roles in ABA signaling remained somewhat controversial, in particular because of a complicated model where each putative ABA receptor had random cellular locations and complex relationships to one another (McCourt and Creelman, 2008).

In 2009, the core signaling pathway model of ABA was dramatically updated (Sheard and Zheng, 2009; Cutler *et al.*, 2010; Hubbard *et al.*, 2010; Joshi-Saha *et al.*, 2011; Kim *et al.*, 2010; Raghavendra *et al.*, 2010; Umezawa *et al.*, 2010; Weiner *et al.*, 2010) with the discovery of a soluble receptor family with properties matching the required physiological and molecular profiles: the PYR/PYL/RCARs (PYRABACTIN RESISTANCE / PYR1 LIKE / REGULATORY COMPONENT OF ABA RECEPTOR) . PYR/PYL/RCARs dimers were found to bind ABA, then dissociate to inhibit the activity of specific protein phosphatase enzymes, the type 2C plant PP2Cs (such as ABI1). PP2Cs are known negative regulators of the ABA response that act by sequestration of SnRK2s (SNF1-related protein kinase 2s, such as OST1) (Ma *et al.*, 2009; Park *et al.*, 2009; Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Fujii *et al.*, 2009; Santiago *et al.*, 2009; Nishimura *et al.*, 2009*b*,*a*; Vlad *et al.*, 2009; Umezawa *et al.*, 2009). By inhibiting PP2Cs, ABA therefore allows autoactivation of SnRK2s, which subsequently phosphorylates target proteins such as transcription factors (Furihata *et al.*, 2006; Kobayashi *et al.*, 2005), proteins responsible for secondary messenger pro-

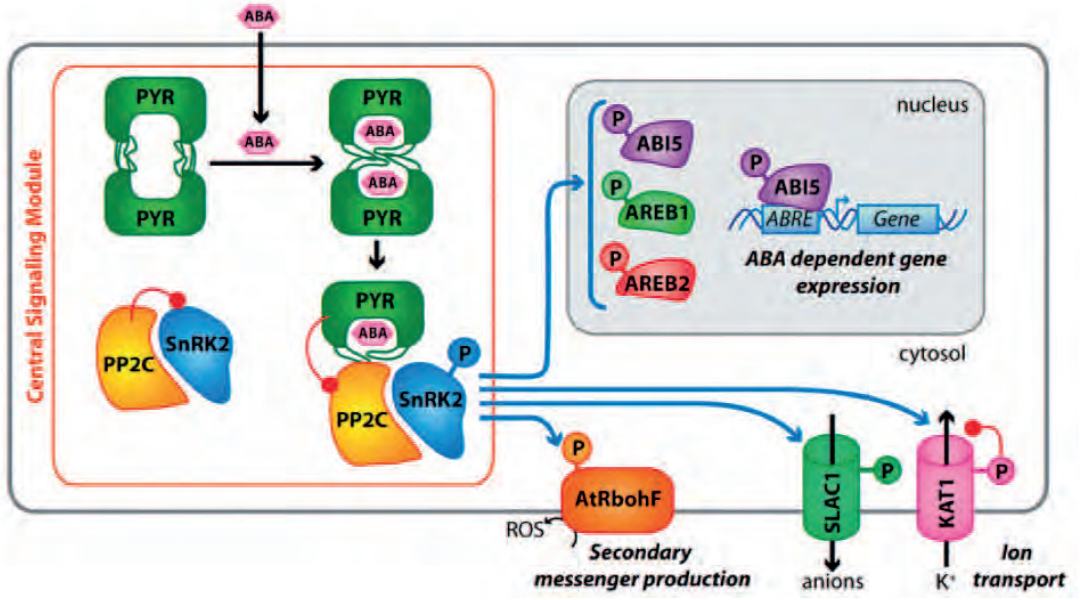


Figure 1.5: **The PYR/RCAR–PP2C–SnRK2 signal transduction model for the core ABA signaling pathway**. Red connections on left indicate an inhibitory interaction. (Hubbard *et al.*, 2010).

duction (Sirichandra *et al.*, 2009*a*), or directly ion channels such as KAT1 and SLAC1 (Geiger *et al.*, 2009; Lee *et al.*, 2009; Sato *et al.*, 2009; Vahisalu *et al.*, 2010) (figure 1.5).

In fact, PP2Cs can also modulate ABA signaling through interaction with a number of other proteins. For example, reports show that several calcineurin-B-like protein (CBL)-interacting protein kinases (CIPK) (Guo *et al.*, 2002), and calcium-dependent protein kinase CPKs (Geiger *et al.*, 2010, 2011) are regulated by PP2Cs. Calcium sensors such as CBL-CIPKs and CPKs are known regulators of ABA signaling, acting by regulation of downstream components such as ion channels (Mori *et al.*, 2006; Cheong *et al.*, 2007; Geiger *et al.*, 2011) or regulation of ABA-responsive transcription factors activity (Zhu *et al.*, 2007). Sofar, evidence suggest that CPKs are positive regulators of

ABA-induced stomatal closure, while CBL-CIPKs are negative regulators (Kim *et al.*, 2010). Importantly, these calcium sensors are part of the potential signal transduction targets of the $[Ca^{2+}]_{cyt}$ elevations signals triggered during both ABA and CO_2 response (Hubbard *et al.*, 2012). A recent study demonstrated that about 70% of the stomatal ABA response is calcium-dependent, and suggested a model where ABA acts in fact to enhance (”prime”) the calcium sensitivity of the targeted calcium sensors (Siegel *et al.*, 2009). In guard cells, $[Ca^{2+}]_{cyt}$ contributes to ABA-induced stomatal closing through activation of S-type anion channels (Mori *et al.*, 2006; Vahisalu *et al.*, 2008; Siegel *et al.*, 2009), activation of R-type anion channels (Meyer *et al.*, 2010), down-regulation of plasma membrane proton pumps (Kinoshita *et al.*, 1995), down-regulation of K^+ influx channels (Siegel *et al.*, 2009) and activation of vacuolar K^+ (VK) channels (Gobert *et al.*, 2007). The importance of calcium in the guard cell signal transduction network is crucial, and further research should provide new insights on its role in signal transduction.

As exemplified with the few signaling pathways described above, a large number of components and regulatory mechanisms in guard cell signal transduction have already been identified. This demonstrates how guard cells are a well-suited model for dissecting genes and proteins functions in signaling cascades. Great progress is constantly made towards understanding the intricate pathways that orchestrate signal perception, cell signaling and ion transports. In a global context where the issues of fresh water scarcity and continuous atmospheric CO_2 rise are principal environmental concerns, guard cell signaling research not only enriches our general understanding of plant cell signaling, but it can also highlight new approaches in the engineering of improved water-use efficiency and desiccation avoidance in crops (Schroeder *et al.*, 2001).

AtPHO1, a phosphate exporter and regulator of phosphate signaling

Phosphate transport and homeostasis

Phosphorus (P) is one of the six essential plant macronutrients. It participates in many aspects of plant biology: it is a critical component of nucleic acids and membrane phospholipids, but it also takes part in many cellular processes including energy transformation, cell signaling and regulation of enzymes through phosphorylation (Poirier and Bucher, 2002). Plants assimilate phosphorus from the soil in the form of orthophosphate (Pi, inorganic phosphate), through Pi transporters at the epidermal and cortical root cells. Pi is then secreted from xylem parenchyma cells, and loaded into the xylem vessels, which distributes and releases it to the shoot tissues (Poirier and Bucher, 2002). Because of the presence of a Casparian strip surrounding the root stele, and since xylem cells are dead cells (and thus reflects the extracellular space), the radial transport of phosphate from the root-soil interface to the vascular cylinder requires a minimum of two transport events across cell plasma membranes: the first for the uptake outside of the endodermal cells, and the second for the efflux of phosphate out of the stelar cells and into the xylem (Hamburger *et al.*, 2002).

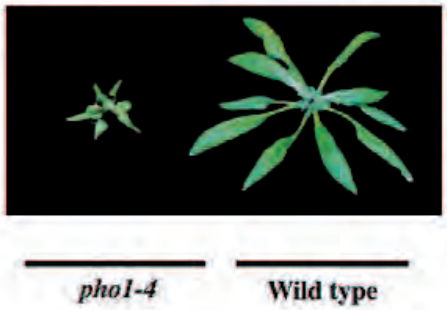
Plants therefore require multiple Pi transport systems, for uptake, export and relocation from subcellular compartments. In that respect, several families of Pi transporters have already been identified in *Arabidopsis,* for example with the characterization of genes belonging to the *PHT1*, *PHT2*, *PHT3*, and *PHT4* families, which encode proteins involved in the acquisition of Pi across the plasma membrane, chloroplast, mitochondria, and Golgi, respectively (Mudge *et al.*, 2002; Versaw and Harrison, 2002; Guo *et al.*, 2008; Chen *et al.*, 2008).

Although the total amount of phosphorus in the soil may be high, the availability of Pi in the soil is often limited, because of its tendency to create insoluble complexes by binding with organic and inorganic compounds. In the event of an inadequate Pi supply, plants need to maintain constant cellular Pi concentrations in order to sustain growth and survival, and adaptive responses have thus been developed to enhance acquisition of external Pi, while conserving and remobilizing internal Pi (Lin *et al.*, 2009). As examples, the root architecture is altered, more phosphate transporters are produced, vacuolar Pi is mobilized, and phosphatases are secreted to extract Pi from soil organic compounds (Plaxton and Tran, 2011). These biochemical, morphological and developmental responses are accompanied by numerous changes at the gene expression levels. These responses are in turn orchestrated by a complex Pi-deficiency signaling cascade (Rouached *et al.*, 2010). Several important transcription factors have now been identified, and great progress has been made with the elucidation of the systemic signals that control the expression of the regulators of Pi-responsive genes (Lin *et al.*, 2009; Rouached *et al.*, 2010, 2011).

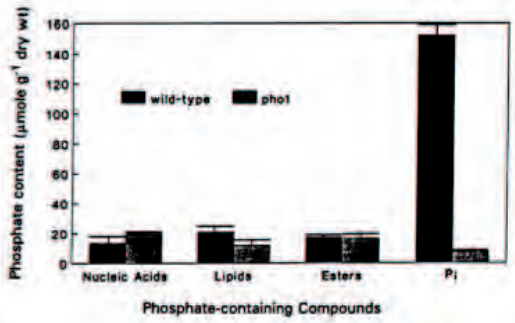
AtPHO1 has a role in root-to-shoot phosphate transport

In an effort to investigate the regulatory loci of phosphate homeostasis, an EMS mutant screen for altered inorganic phosphate levels in the leaves was carried out in *Arabidopsis thaliana* (Poirier *et al.*, 1991). Among the selected mutants, *pho1* was identified suffering from a severe reduction in shoot Pi content. The *pho1* mutant accumulates approximately 24-44% as much total phosphate and 5% as much inorganic phosphate as a wild-type (Poirier *et al.*, 1991), but presents normal Pi concentration in the roots (Delhaize and Randall, 1995). Experiments using radioactive labeled phosphate revealed that *pho1* displays normal Pi uptake into the roots over a wide

(a)



(b)



(c)

Plant Line	Phosphate	
	Root uptake	Transfer to shoot
	nmol g^{-1} h^{-1}	%
Pho1	1559 ± 144	0.9 ± 0.1
Wild type	1593 ± 130	35 ± 3

Figure 1.6: **The mutant *pho1* of *Arabidopsis thaliana***. (A) Morphological phenotype of a *pho1* plant compared with a wild-type plant grown in soil. (B) Content of various phosphate-containing compounds in wild-type and *phol* mutant plants. (C) Root uptake and transfer to the shoot of phosphate and sulfate (Poirier *et al.*, 1991).

range of external phosphate concentration, and normal phosphate movement through the xylem, but only 3-10% of the wild type Pi translocation from roots to shoots in media containing no more than 200μM Pi. Together, these results suggested that *PHO1* is involved in root-to-shoot phosphate transfer. The mutant *pho1* displays typical symptoms of Pi starvation: a severe reduction in rosette size, an accumulation of anthocyanins, a higher starch content in the leaves (Ciereszko *et al.*, 2001), a delay in flowering, and a poor production of seeds (figure 1.6).

The *PHO1* gene (*At3g23430*, 5.8 kb containing 14 introns) was subsequently identified by map-based cloning (Hamburger *et al.*, 2002). Sequence analysis suggested that *PHO1* encodes a 782 amino acids protein containing at least 6 transmembrane domains in its 40kDa C-terminal region, and a 40 kDa N-terminal hydrophilic portion, with no homology to any characterized solute transporters, including H+/Pi cotransporters (figure 1.7a and b).

By Northern blot analysis, *PHO1* was found to be expressed primarily in roots, but weak expression was also detected in rosette leaves, stems, cauline leaves, and flowers with developing siliques. Promoter-GUS fusion revealed predominant expression in the stelar cells of the roots and the hypocotyl, including the pericycle and xylem parenchyma cells, which seemed consistent with the role of loading phosphate into the root xylem (figure 1.7c).

AtPHO1 mediates specific Pi export from cells

In an effort to further study the role of PHO1, over-expressing lines were generated. The transformation of wild-type *Arabidopsis* using a T-DNA vector containing the

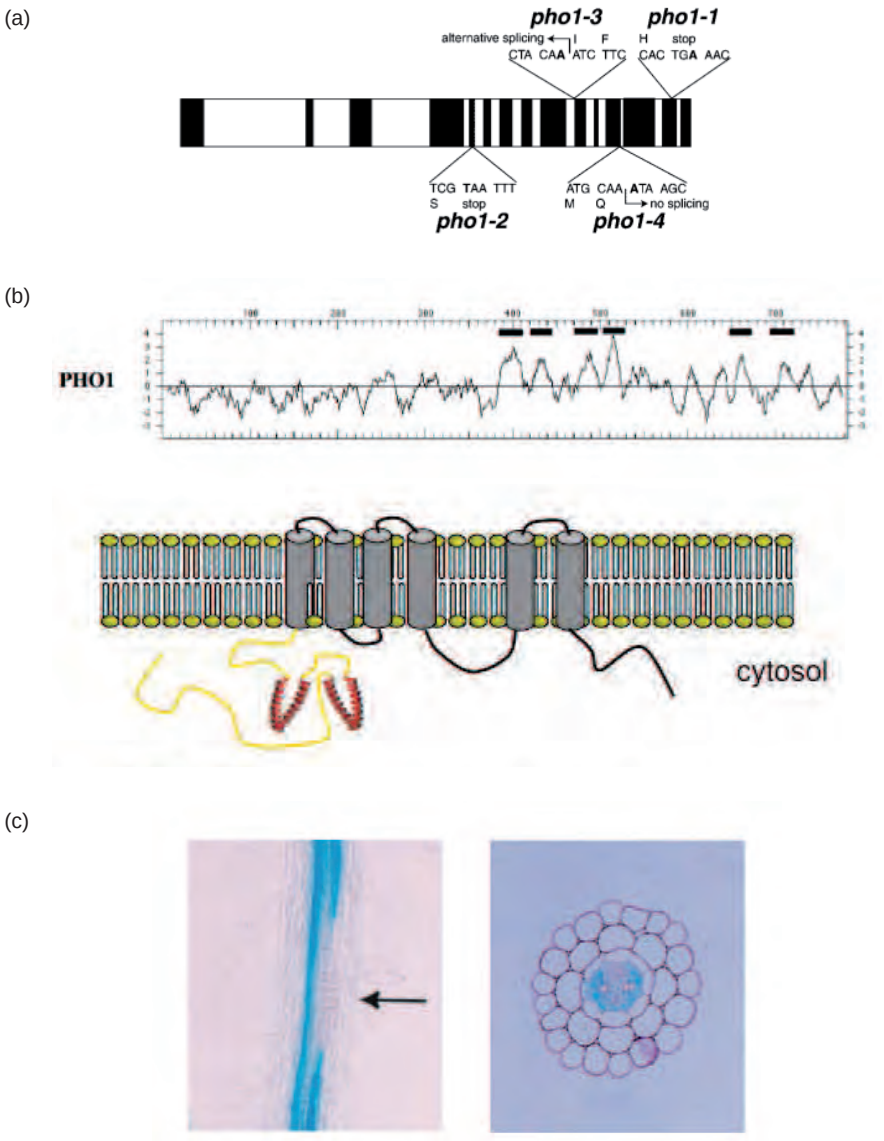


Figure 1.7: **Structure of *AtPHO1* and localization of expression in the root.** (A) Intron (white box) and exon (black box) structure of the *AtPHO1* gene and of the four *pho1* alleles. (B) Hydropathy profile of AtPHO1 (transmembrane segments are indicated by closed rectangles) and representation of the predicted topology. (C) Root expression pattern of a 2.1-kb fragment of *AtPHO1* promoter region using GUS reporter gene (Hamburger *et al.*, 2002).

PHO1 gene under the control of its own promoter yielded a number of independent over-expressing lines that were identified and characterized (L3 and SL5, Stefanovic *et al.*, 2011). These lines presented small, dark-green and slightly curled leaves, and strikingly higher *PHO1* expression levels than WT, especially in the shoot vascular system. *PHO1* ectopic over-expression in leaves triggered an accumulation of Pi in the leaves, with two to threefold higher Pi content in the L3 and SL5 lines compared to WT, a result that is in agreement with the role of PHO1 in root-to-shoot phosphate transfer. In vivo ^{31}P-NMR as well as measurements of Pi fluxes out of leaves (figure 1.8a) revealed that *PHO1* over-expression in leaves leads to a dramatic increase of Pi export into the apoplast, with a consequent loss of Pi vacuolar pool.

Using an inducible promoter, Arpat *et al.* (2012) further demonstrated that induction of *PHO1* in leaves from soil-grown plants, in leaves and roots of plants grown in liquid culture, or in leaf mesophyll protoplasts, all led to a specific release of Pi into the extracellular medium, thus revealing the crucial role for PHO1 in Pi efflux from cells (figure 1.8b). In contrast with H^+/Pi co-transporters, PHO1-mediated Pi export was independent of H^+ gradient across the membrane, and was enhanced by high extracellular Pi (Arpat *et al.*, 2012). Together, these data strengthened the model of PHO1 function in the roots, in which PHO1 acts as a Pi exporter from root cylinder cells into the xylem vessels (figure 1.9).

At the moment, the mechanisms and molecular players that govern the regulation of PHO1 activity are still investigated. Significant advances were made with the identification of a set of proteins interacting with PHO1, using the complete N-terminal hydrophilic region of PHO1 as a bait in a yeast two-hybrid screen, with consequent confirmation of the interaction using full length PHO1 and the full length version of the interacting partners in a yeast split-ubiquitin system. Among the putative inter-

actors of PHO1 was a CBL-interacting protein kinase (CIPK). With their interacting partners, the calcineurin B-like (CBL) proteins, CBL/CIPKs are known components of the Ca^{2+}-dependent signaling network that regulate responses to various abiotic stresses in plants, hormone reactions and ion transport processes (Batistic and Kudla, 2009). Current research efforts aim at characterizing how a set of CBL/CIPKs, and possibly other calcium dependent kinases, can phosphorylate PHO1 and regulate its Pi-export activity.

AtPHO1 localizes to the Golgi and *trans*-Golgi network

In order to investigate PHO1 subcellular localization in *Arabidopsis* root cells, Arpat *et al.* (2012) generated transgenic lines complementing the *pho1* mutant with a PHO1-GFP fusion construct. As predicted, GFP was seen in pericycle cells surrounding xylem poles, but surprisingly, subcellular localization did not reveal any GFP signal at the plasma membrane. Rather, GFP expression was observed in punctate structures, that co-localized with the Golgi, trans-Golgi network (TGN) and uncharacterized vesicles when expressed in onion epidermal cells or in tobacco mesophyll cells (figure 1.8c). Pi export activity was witnessed in these PHO1-GFP transformed cells, which confirmed the importance of not only PHO1, but also of the Golgi and associated endosomes for the mediation of phosphate release to the outside of the cell. Interestingly, the fluorescence signal was partially relocated to the plasma membrane under high extracellular phosphate, possibly to ensure greater phosphate export. This suggested that action site of PHO1 depends largely on Pi homeostasis (Arpat *et al.*, 2012).

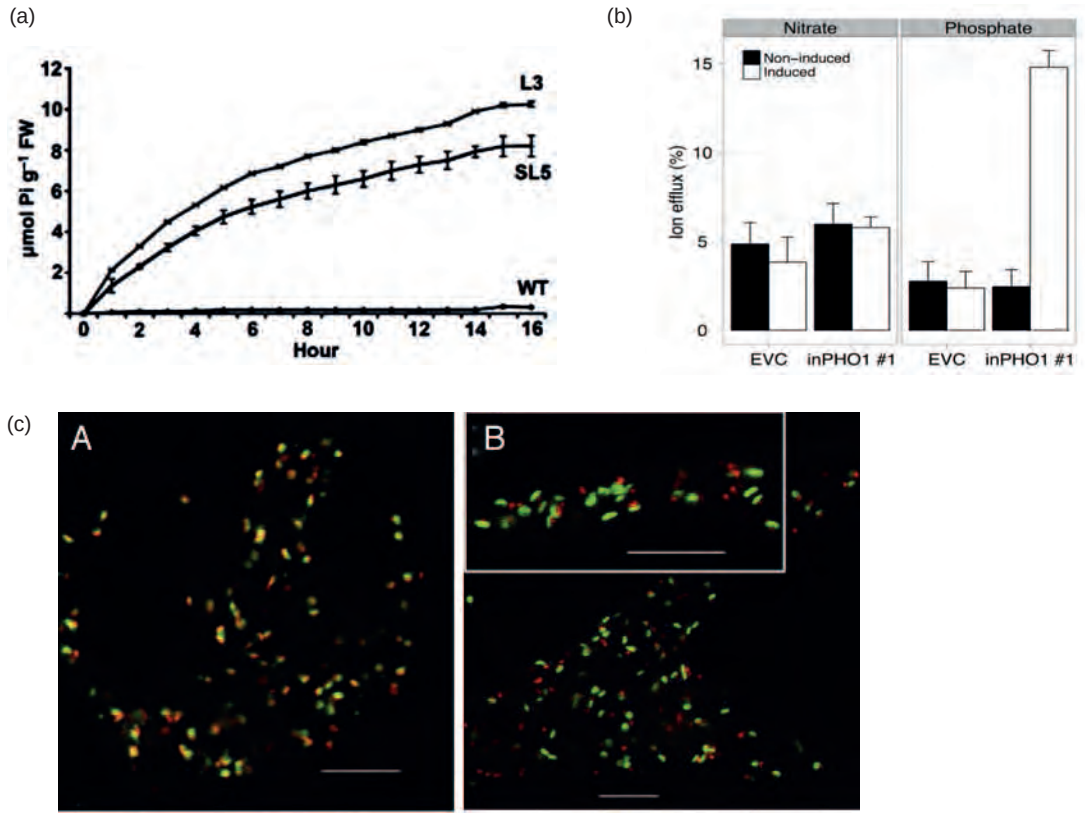


Figure 1.8: **PHO1 mediates phosphate efflux out of cells into the xylem vessels, and localizes to the Goli and the TGN.** (a) Export of Pi from leaves in *PHO1* over-expressing plants (L3 and SL5 transgenic lines) compared to wild-type, as revealed by increasing phosphate concentration in the bathing solution **(Stefanovic *et al.*, 2011)**. (b) PHO1-dependent specific phosphate efflux from mesophyll protoplasts (Arpat *et al.*, 2012). inPHO1#1: transgenic line expressing PHO1 under the control of an inducible promoter; EVC: control line. (c) Sub-cellular localization of PHO1-GFP and co-expression with different markers in onion cells (adapted from Arpat *et al.*, 2012). Onion epidermis was co-bombarded with PHO1-GFP (green, A-B), and Golgi markers Got1p-mCherry (A) or TGN marker VTI12-mCherry (B) (red). Bars = 10 µm.

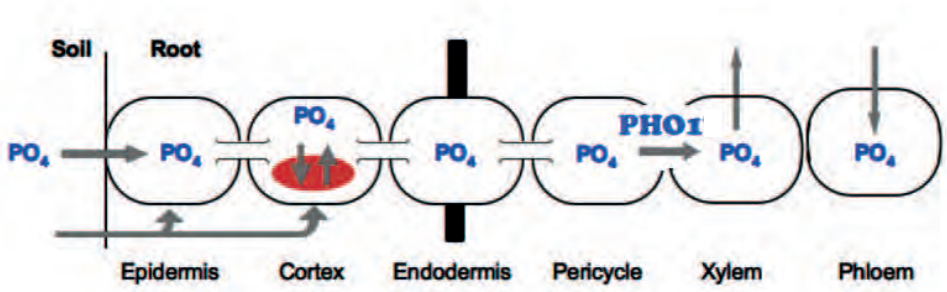


Figure 1.9: **Schematic representation of the putative role of PHO1 in phosphate export from root cylinder cells into the xylem vessels.** The transverse section of a plant root is shown, with grey arrows indicating the apoplastic and symplastic acquisition routes of Pi.

AtPHO1 regulates phosphate deficiency signaling

During the same transformation experiment mentioned above (transformation of wild-type *Arabidopsis* with *PHO1* under the control of its own promoter), a number of independent under-expressing lines were also identified and characterized (B1 and B3, Rouached *et al.*, 2011). The under-expressing lines of *PHO1*, just like the mutant *pho1*, were defective in the transfer of Pi from root to shoot, but despite their low shoot Pi, presented a surprising normal shoot growth with no signs of Pi deficiency even at the gene expression level.

Comparative microarray analysis between the mutant *pho1* and the underexpressor lines suggested that PHO1 is involved in the transmission of a root-to-shoot signal, possibly a small signaling molecule, that suppresses phosphate starvation inducible (PSI) genes in the shoot. In the underexpressing lines, this signal could be sufficient to repress starvation response, resulting in plants that do not respond to their internal Pi depletion.

This analysis revealed therefore that beside Pi transport into the xylem, *PHO1* plays

a critical role in the signal transduction of phosphate deficiency response. This dual function of PHO1 protein, both root-to-shoot phosphate transporter and long distance regulator of Pi deficiency signaling, echoes with other numerous examples of proteins in bacteria and yeast that participate both as a nutrient transporter and either a nutrient sensor or a component of the signal transduction pathway (Rouached *et al.*, 2011).

The *AtPHO1* gene family suggest roles beyond root phosphate transport

PHO1 was the first characterized gene in a gene family composed of eleven members in *Arabidopsis*. The ten homologues to PHO1 were found and designated as PHO1;H1 to PHO1;H10. Protein sequence alignment identified two conserved domains, with significant similarities to a number of proteins found in *Arabidopsis* as well as in non-plant eukaryotes: an SPX (for SYG1, PHO81 of yeast and XPR1 of human) and an EXS domain (for yeast ERD1, human XPR1, and yeast SYG1), respectively in the hydrophilic and the hydrophobic regions of PHO1 (Wang *et al.*, 2004). The SPX domain is found in several plant proteins, including several which were recently shown to be involved in Pi transport and signaling (Secco *et al.*, 2012). The activity of the EXS domain however is still unknown.

Expression analysis of the *PHO1* gene family revealed a broad expression pattern, not restricted to vascular tissues, and only *PHO1;H1*, the closest homologue to the original *PHO1*, was able to complement the mutant *pho1*, although they were found to be differentially regulated (Stefanovic *et al.*, 2007). These results raised the important question of the biological role of the other homologues, and the possibility for PHO1 proteins to have roles that are beyond phosphate transport.

In fact, Kang and Ni (2006) demonstrated that *PHO1;H4* is involved in cryptochrome

signaling and hypocotyl growth under blue light, although its exact function remains unclear (Kang and Ni, 2006; Zhou and Ni, 2010). Additionally, the homologue *PHO1;H10* was found to have a unique expression pattern with strong induction upon numerous biotic and abiotic stresses, such as wounding, dehydration, cold, salt and pathogen attack, and phytohormones treatments including OPDA and ABA (Ribot *et al.*, 2008*a*,*b*). Altogether, these studies support the involvement of *PHO1* genes in signal transduction pathways that are not confined to phosphate homeostasis.

AtPHO1, phosphate and ABA signaling

Recently, Ribot (2006) identified and thoroughly described some new phenotypes for the mutant *pho1*. These traits were particularly well known to be linked with abscisic acid (ABA) signaling: *pho1* exhibits a pronounced seed dormancy, a hypersensitivity to ABA in terms of germination and root growth, and displays higher rate of water loss during dehydration assays. The rate of water loss was comparable to known ABA-mutant, and was independent of the phosphate deficiency occurring in the mutant *pho1*, as demonstrated through the use of wild type roots micrografts (Ribot, 2006). In agreement, some simple observations of *pho1* epidermis suggested that *pho1* stomata did not close in response to ABA, suggesting a potential role for PHO1 in guard cell ABA signaling.

However to our knowledge, little is known about the link between phosphate and abscisic acid. The role of other phytohormones (e.g auxin, cytokinins) in plant response to phosphate starvation is quite well documented (Yuan and Liu, 2008); instead, the role of ABA in phosphate homeostasis is poorly described, although a raising number of studies report evidence that ABA might regulates some branches of the Pi deficiency response (Trull *et al.*, 1997; Ribot *et al.*, 2008*a*; Rubio *et al.*, 2009; Yang and

Finnegan, 2010). Conversely, the potential role of phosphate in ABA signaling has not been addressed in the literature. Only a few studies report specific accumulation of phosphorus in guard cells (Outlaw *et al.*, 1984; Heath *et al.*, 1997), suggesting that phosphate might be of biological significance for the guard cell ABA response.

While most previous research effort on PHO1 had focused on its role in the roots, the results described by Ribot (2006) incited to further study the potential novel role for PHO1 and phosphate in guard cell signaling. Moreover, studying PHO1 in guard cells represents a new approach to understand its general role, action and regulation mechanisms in plant cells, while bringing valuable knowledge in both the fields of phosphate transport and guard cell signaling; this approach is particularly relevant considering the great amount knowledge gathered by the scientific community on guard cells and ABA signaling, which makes stomatal research very attractive for the study of ion transport regulation.

2 - Aim of this thesis

The aim of the present thesis was to investigate the expression and the importance of *AtPHO1* in guard cell movements, using several complementary approaches, with a focus on the stomatal response to ABA during induction of stomatal closure and repression of stomatal opening under light.

In the first part, we analyzed the patterns of expression of *PHO1* in guard cells in comparison to mesophyll cells. The observation of *pho1.4* transgenic lines expressing the *PHO1-GUS* reporter gene fusion under the control of the *PHO1* promoter revealed that aside from *PHO1* known expression in root vascular tissue, specific expression was seen in leaf stomata, with increased expression upon treatment with ABA. This observation was in accordance with published microarrays (Leonhardt *et al.*, 2004; Yang *et al.*, 2008), and results from qRT-PCR on guard cell protoplasts confirmed the preferential expression of *PHO1* in guard cells protoplasts compared to mesophyll cells protoplasts, with induction of gene expression following treatment with ABA.

In the second part, we explored the stomatal movement response of the mutant *pho1*. We found that the stomata in *pho1* are severely impaired in their response to ABA, both for the induction of stomatal closure as well as for the repression of stomatal opening under light. We showed that *pho1* remains able to close stomata in transition to dark as well as under high calcium treatment. We demonstrated that *pho1* stomata

open like a wild type under red and blue light and other stomatal opening-inducing stimuli. We concluded that while unresponsive to ABA, *pho1* stomata maintain the ability to open and close under other treatments. We demonstrated that the mutant *pho1* is able to sense ABA, and produces reactive oxygen species (ROS) in response, but that the induction of stomatal closure by hydrogen peroxide is not functional, showing that the impairment in the signal transduction occurs downstream of ROS production. In addition, we observed that *pho1* exhibits a reduced response to changes in CO_2 concentration, suggesting that PHO1 might be involved in a signaling step common to both ABA and CO_2. Finally, using micrografting techniques, we report that in Pi-sufficient *pho1* the stomata remain unresponsive to ABA and hydrogen peroxide, an evidence that this impairment is not a simple pleiotropic consequence of phosphate deficiency.

In a third part, we investigated the effect of guard-cell-specific regulation of *PHO1* gene expression on the stomatal movement response to ABA. We found that a knock-down of *PHO1* expression in guard cells altered the stomatal movements in response to ABA, but did not alter the transcriptional response of known ABA-responsive genes. This observation suggests that PHO1 does not act through modifications of the transcriptional response to ABA. Conversely, guard-cell-specific expression of *PHO1* in the *pho1* mutant background restored stomatal responsiveness to ABA, although full complementation was co-dependent on the restoration of shoot phosphate sufficiency through grafting. Therefore, both PHO1 and Pi deficiency have an effect on guard cell movements response to ABA.

On the basis of these findings, we conclude that PHO1 is a novel important player in the response of guard cells to ABA, and we discuss the potential mechanisms behind the implication of *PHO1* and phosphate in the guard cell ABA signaling cascade.

PHO1 is involved in the release of phosphate into the vascular cylinder (Poirier *et al.*, 1991), and more generally in the export of phosphate from cells (Stefanovic *et al.*, 2011; Arpat *et al.*, 2012). Therefore, in stomata, we suggest the hypothesis of *PHO1* acting in the regulation of phosphate transport in guard cells, possibly through phosphate efflux to the apoplast, or regulation of phosphate fluxes between intracellular compartments. Finally, we discuss different scenarios where phosphate fluxes mediated by PHO1 could influence the ABA-induced osmotic pressure changes in guard cells.

3 - Expression analysis of *AtPHO1* in guard cells and in response to ABA

Céline Zimmerli* and Yves Poirier

*The production of transgenic *A. thaliana* plants expressing the *GUS* reporter gene under the control of the *PHO1* promoter was done by Dirk Hamburger (Hamburger *et al.*, 2002); the transgenic *pho1.4* plants expressing *PHO1* fused to *GUS* under the control of the *PHO1* promoter were produced by Aleksandra Stefanovic; microarray experiments were published by Yang *et al.* (2008); all other experiments were designed, performed and data analyzed by Céline Zimmerli under the supervision of Yves Poirier.

Introduction

While the expression profile of *AtPHO1* has been extensively studied using various approaches including Northern blot, ß-glucuronidase (GUS) reporter gene, and semi-quantitative RT-PCR (Hamburger *et al.*, 2002; Stefanovic *et al.*, 2007; Ribot *et al.*, 2008*a*), most studies described only its predominant expression in roots. In an early study, RNA gel blot analysis revealed that consistent with its role in loading phosphate into the root xylem, *PHO1* was expressed mostly in roots, with a slight up-regulation under low Pi concentration. However, weak expression also was detected in rosette leaves, stems, cauline leaves, and flowers with developing siliques (Hamburger *et al.*, 2002), suggesting that *PHO1* might have roles in other cell types. Using transgenic plants expressing the ß-glucuronidase (GUS) gene under the control of fragments encompassing 0.55, 1.1, 1.6, and 2.1 kb of the promoter region of *PHO1*, Hamburger *et al.* (2002) reported expression of the *PHO1* promoter in the stelar cells of the root and the lower part of the hypocotyl, e.g. pericycle cells, xylem parenchyma cells and passage cells. However, despite the low *PHO1* expression detected in leaves by RNA gel blot analysis, no GUS staining was detected in any above ground tissues in these transgenic lines.

Stefanovic *et al.* (2007) further confirmed preferential expression of *PHO1* in roots, and demonstrated that the increase of *PHO1* expression under Pi deficiency was independent of *PHR1* and was not influenced by phosphite. Ribot *et al.* (2008*a*) explored further the regulation of *PHO1* expression. They reported that, in whole seedlings, *PHO1* was up-regulated by sucrose, but repressed by long-term treatment with several phytohormones such as the cytokinin kinetin, auxin 2,4-D, and ABA. These results highlighted the fact that *PHO1* expression regulation is complex and involves

the interaction between multiple signaling pathways.

Interestingly, expression profile analysis of the *PHO1* homologues (Wang *et al.*, 2004), through RT-PCR and promoter:GUS reporter, revealed that while the majority of genes were expressed in the plant vasculature, some genes were more broadly expressed and included expression in flowers, pollen or root cortical cells. The homologue *PHO1;H10* was found to be strongly induced by numerous stresses, including dehydration and the local response to wounding, and was upregulated by OPDA (12-oxo-phytodienoic acid) and ABA (Ribot *et al.*, 2008*b*). Together, these data indicated that the role of the *PHO1* gene family may not limited to the roots, nor to Pi movement to or from vascular tissues.

In an effort to identify important regulators of guard cell signaling, several recent microarray analysis presenting overviews of ABA-induced and repressed genes in guard-cell and mesophyll-cell protoplasts were recently published (Leonhardt *et al.*, 2004; Yang *et al.*, 2008). Data from these hybridization experiments suggested that *PHO1* is expressed in guard cells, at higher levels compared to mesophyll cells, and is induced under short-term ABA treatment, which is in apparent contradiction with results from Hamburger *et al.* (2002) and Ribot *et al.* (2008*a*). In this context, it was of interest to confirm these microarray data and investigate the potential expression of *PHO1* in stomata, which could have been overlooked during previous reports.

In this chapter, using transgenic lines expressing GUS and PHO1-GUS fusions under the control of the *PHO1* promoter, we reveal *PHO1* promoter activity and localization of PHO1-GUS fusions in stomata of hypocotyl and leaf blade of *Arabidopsis thaliana*. Increased GUS staining suggested that promoter activity was enhanced after a 3-hour ABA treatment. Using qRT-PCR on purified guard cell and mesophyll protoplasts, we detected expression of *PHO1* in guard cells, with higher transcript levels than in mes-

ophyll cells. ABA treatments on guard cell protoplasts confirmed rapid induction of *PHO1*, under a range of ABA concentrations. Consistent with these results, analysis of the *PHO1* promoter region revealed the presence of ABA responsive elements as well as motifs corresponding to DOF-binding sites, some suggested cis-elements regulating guard cell specific expression. Together, these results show that, in addition to its known expression in the roots, *PHO1* is expressed in guard cells, suggesting a potential biological role for PHO1 in stomata.

Results

Expression of the *PHO1* genes in guard cells as revealed by microarray data

As mentioned above, microarray analyses presenting overviews of ABA-induced and repressed genes in guard-cell and mesophyll-cell protoplasts were published in recent years (Leonhardt *et al.*, 2004; Yang *et al.*, 2008). The hybridization experiment performed by Yang *et al.* (2008) included expression data of *PHO1* and several *PHO1* homologues (figure 3.1). The homologues *PHO1;H1*, *-H2*, *-H3*, *-H4*, *-H5*, *-H7* and *PHO1;H9* displayed weak expression in both cell types and chemical treatment. However, *PHO1* stood out with a much higher expression level in guard cells than all other homologues; *PHO1* also appeared up-regulated in guard cells under a 4-hour treatment with 100 µM ABA. In contrast, *PHO1* expression in mesophyll cells appeared below detectable levels. The homologue *PHO1;H10* was expressed at low levels in both guard cells and mesophyll, but was strongly up-regulated by ABA in both cell types, which is consistent with previous published results, showing strong induction of *PHO1;H10* in the leaves in response to various biotic/abiotic stresses and ABA treatments (Ribot *et al.*, 2008*b*,*a*).

Interestingly, this microarray suggests that *PHO1* expression in the leaf blade is restricted to guard cells. Under ABA treatment, *PHO1* presents comparable expression levels as some known genes encoding guard cells ion channel such as the inwardly rectifying K^+ channel *KAT1* (248888_at: 1602.6 AU, Arbitrary Unit), the outwardly rectifying K^+ channel *GORK* (249619_at: 4518.4 AU) or the putative slow anion channel *SLAC1* (259514_at: 4129.5 AU), while remaining five times less expressed than the *OST2* gene encoding the proton ATPase *AHA1* (266939_at: 20083.6 AU).

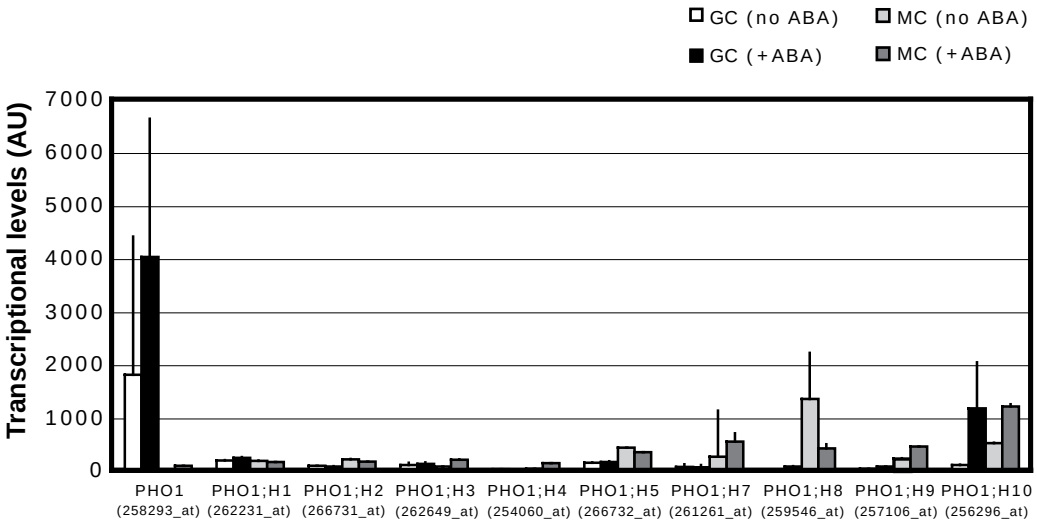


Figure 3.1: **Transcriptional profiles of genes from the *PHO1* gene family, in guard cells (GC) or mesophyll cells (MC), with or without ABA treatments.** Datas are from the microarray experiment described by Yang *et al.* (2008), registered under the accession number E-MEXP-1443 available on the ArrayExpress database (www.ebi.ac.uk/arrayexpress).

Gus staining reveals *PHO1* promoter activity in guard cells

The results from the microarray experiment of Yang *et al.* (2008) incited us to investigate *PHO1* promoter activity more closely in the aerial part of the plant, under ABA treatment, using the ß-glucuronidase (*GUS*) reporter gene. We used two different type of transgenic lines, the first being transgenic *Arabidopsis thaliana* lines having the GUS reporter gene under the control of a 2.1kb DNA fragment upstream of *PHO1* start codon (*pPHO1::GUS;* Hamburger *et al.*, 2002), the second being transgenic *pho1.4* lines in which the *PHO1-GUS* fusion gene was expressed under the same *PHO1* promoter (*pho1.4 pPHO1::PHO1-GUS*; Stefanovic A., unpublished).

Two- to three-week old seedlings of the *Arabidopsis* lines carrying *pPHO1::GUS* were grown vertically in plates and immersed in 100 µM ABA for 4 h prior to GUS staining.

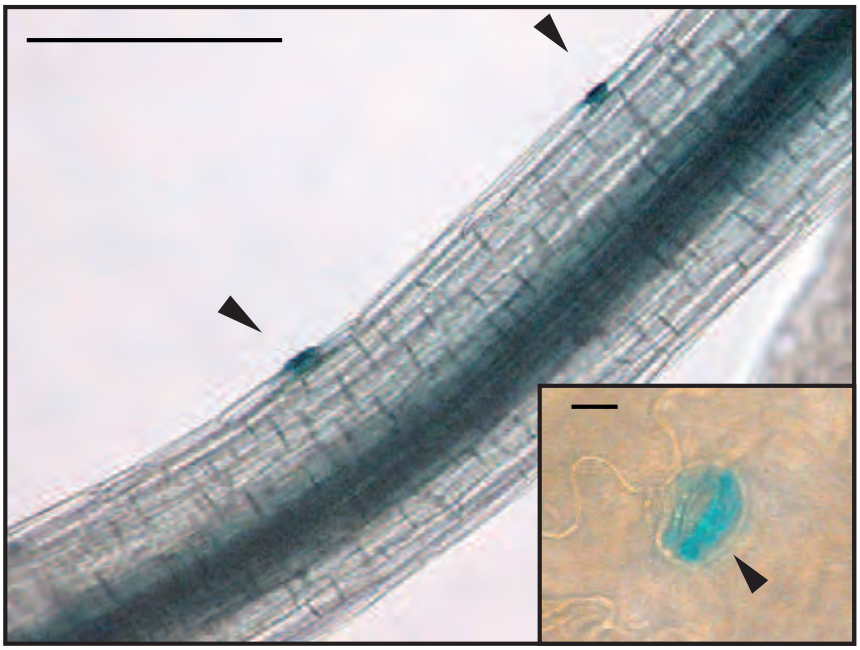


Figure 3.2: **The *PHO1* promoter region drives GUS activity mainly in the vasculature, but also in hypocotyl guard cells under ABA treatment.** Localization of *GUS* reporter gene activity in seedling hypocotyls (bar = 100 μm) and cotyledons epidermis (bottom right picture, bar = 10 μm). Expression was under the control of 2.1 kb of the *AtPHO1* promoter region. Seedlings were submerged in 100 μM ABA for 4 h before GUS staining. Arrows indicate guard cells. GUS staining in vascular tissue can also be seen, indicative of known *AtPHO1* expression patterns (Hamburger *et al.*, 2002).

Consistent with previous reports (Hamburger *et al.*, 2002), we observed GUS activity in stellar cells of the root and hypocotyl. However, GUS activity in hypocotyl stomata was also consistently observed following ABA treatment in several independent transgenic lines (figure 3.2). GUS activity in stomata of leaves and cotyledons was also observed on occasion, although not as frequently and at lower intensity compared to the hypocotyl (figure 3.2, bottom right picture).

Promoter activity and *PHO1* localization were further investigated using the *pho1.4 pPHO1::PHO1-GUS* lines. In an effort to use more physiologically meaningful ABA concentrations, two week-old rosettes were cut at the hypocotyl and floated in water with or without 10 µM ABA for 3 h prior to GUS staining. For both treatments, we observed GUS staining in the vasculature of the hypocotyl, in the petiole as well as in hydathodes. Staining in the entire leaf blade vasculature was seen on occasion (not shown). Interestingly, GUS staining was also seen in the guard cells of the leaf blade (figure 3.3a). Moreover, the intensity and frequency of the guard cell staining was enhanced under ABA treatment (figure 3.3a).

qRT-PCR on guard cells protoplasts confirm *PHO1* expression in guard cells with induction under ABA

To assess expression levels of *PHO1* in guard cells, we performed quantitative real-time PCR using RNA extracted from guard cell and mesophyll cell protoplast preparations of wild-type (WT) *Columbia* plants. We attested the relative purity of each protoplast preparation by measuring the expression of the known guard-cell-specific gene *KAT1* (Schachtman *et al.*, 1992) and the mesophyll-cell-specific gene *At4G26530* (Yang *et al.*, 2008) . In guard cells, *PHO1* transcripts were approximately six times more abundant compared to mesophyll cell protoplast preparations (figure 3.4a). *PHO1*

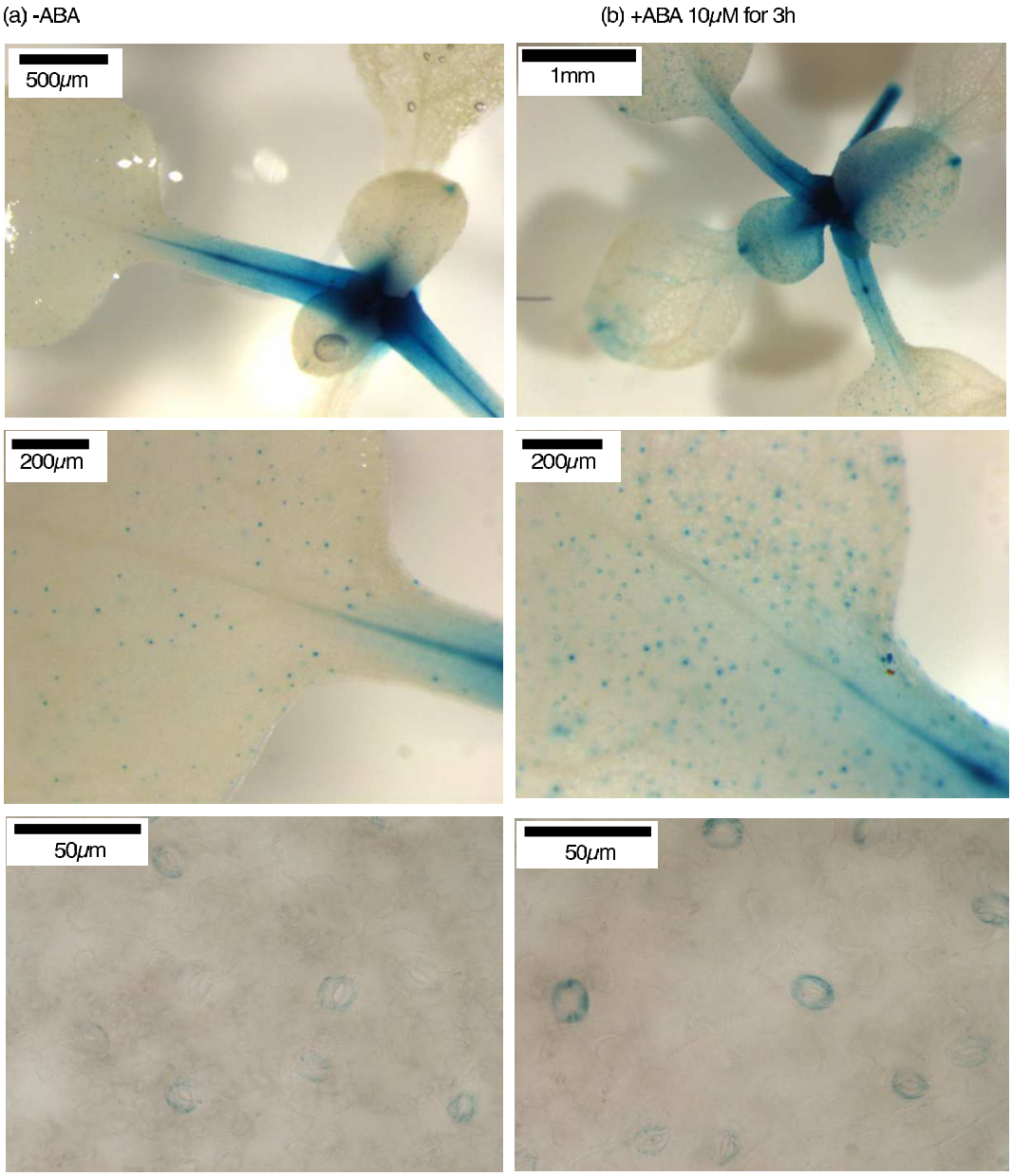


Figure 3.3: **The *PHO1::GUS* translational fusion protein activity in *pho1.4* leaves localizes to guard cells and petiole vasculature.** Expression was under the control of 2.1 kb of the *PHO1* promoter region. Rosettes were cut at the hypocotyl and floated in water with or without 10 μM ABA for 3 h before GUS staining. In the leaf blade, GUS staining is visible in guard cells, and the intensity is increased under ABA treatment. Staining in vascular tissue of the leaf petiole can also be seen.

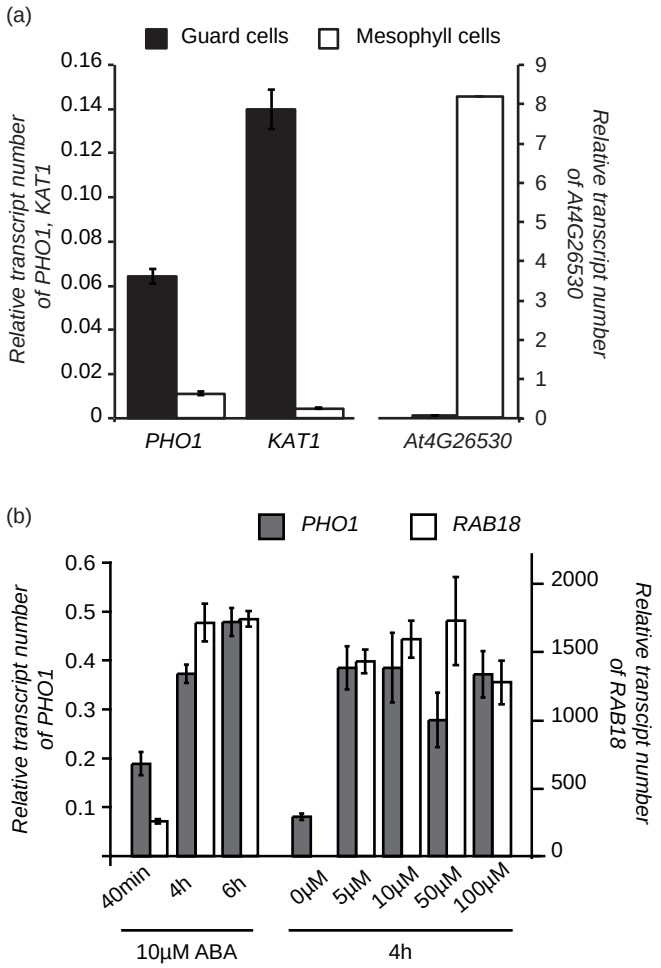


Figure 3.4: ***AtPHO1* is preferentially expressed in guard cells compared to mesophyll cells, and expression is induced by ABA.** (a) Variation in *AtPHO1* transcript level between wild-type guard cell and mesophyll cell protoplasts preparations. The guard cell-specific gene *KAT1* and the mesophyll cell-specific gene *AT4G26530* were also measured as controls. n = 3 biological replicates; average ± SE. (b) Expression levels of *PHO1* in wild-type guard cell protoplasts following treatment with 10 µM ABA for 40 min to 6 h, and treatment with 0 to 100 µM ABA for 4 h. Expression levels of the known ABA-induced gene *RAB18* are also represented. n = 3 biological replicates; average ± SE. Relative expression levels refers to transcript abundance of target genes, normalized against expression of the reference gene *AT1G13320* (Czechowski *et al.*, 2005).

expression in guard cell protoplasts was in the same value range as the known K^+ channel, with a transcript number up to nearly half that of *KAT1*.

The expression of *PHO1* in guard cell protoplasts subjected to ABA treatment was then analyzed. The expression level of *PHO1* increased between 40 min to 6 h after addition of 10 µM ABA (figure 3.4b, left side). Also, *PHO1* expression was similarly induced by 4h treatments with varying concentrations of ABA ranging from 5 µM to 100 µM (figure 3.4b, right side).

Analysis of the *PHO1* promoter sequence

In *Arabidopsis*, little information is available on promoter *cis*-elements regulating guard cell-specific expression, but several studies suggested a role for [T/A]AAAG DOF-binding sites (Plesch *et al.*, 2000, 2001; Galbiati *et al.*, 2008; Cominelli *et al.*, 2011). Clusters of [A/T]AAAG motifs have been found in the regulatory regions of most of the genes that were upregulated in guard cells (Galbiati *et al.*, 2008), and by mutagenesis of these sites in the promoter of *AtMYB60*, Cominelli *et al.* (2011) confirmed the importance of DOF motifs of the same cluster in driving guard cell expression. Analysis of the *PHO1* promoter sequence revealed a large number of [A/T]AAAG motifs in the 2.1kb upstream of the start codon (figure 3.5), with the presence of a cluster of three [A/T]AAAG motifs, located on the same strand within a region of 100 bp, between -860 and -930 bp. Analysis of the *PHO1* promoter sequence also revealed the presence of a few ACGT core motifs of the G-box-containing ABREs (ABA responsive elements; figure 3.5). ABRE motifs are classical *cis*-acting elements in ABA-responsive gene expression (Wang *et al.*, 2011). Although statistical analysis of the promoter sequences would be needed to assess whether these motifs are particularly enriched in *PHO1* promoter, the presence of guard cell specific and ABA

Figure 3.5: **Promoter region of *PHO1* with Dof target sites and ABRE motifs.** The 2.1 kb sequence upstream of the ATG start codon is represented. Nucleotides are numbered on the left with the translational start site designated as +1. The ATG is in bold. The Dof target sites, (+)5'-TAAAG-3', (-) 5'-CTTTA-3', (+) 5'-AAAAG-3' or (-) 5'-TTTTA-3' are boxed and labelled. The abscisic acid-response element (ABRE), (-) 5'-CACGT-3' motifs are also indicated.

response motifs is consistent with the expression pattern of *PHO1* in stomata, as revealed by GUS staining and qRT-PCR data.

Discussion

In agreement with its role in transferring phosphate into the root vasculature, *PHO1* is primarily expressed in root stelar cells (Hamburger *et al.*, 2002). In this chapter, with microarray data analysis, ß-glucuronidase (GUS) reporter gene analysis, and quantitative RT-PCR, we gathered evidence that beside its known expression in the vasculature, *PHO1* is also expressed in guard cells of the leaf blade. *PHO1* expression in guard cells was at much higher levels than in the mesophyll cells, and was in the same range as some well known guard cell ion channels. Together, these results unravel a possible biological significance of *PHO1* stomatal expression for guard cell functioning.

Ribot *et al.* (2008*a*) observed that a long-term (two-day) treatment with 10 μM ABA repressed *PHO1* in whole seedlings, the majority of the repression occurring most likely in the root vascular cylinder, the tissue with the highest *PHO1* expression. Their result corroborated the idea that ABA might be involved in the regulation of Pi homeostasis (Trull *et al.*, 1997; Ribot *et al.*, 2008*a*; Rubio *et al.*, 2009; Yang and Finnegan, 2010). In this study we examined more precisely the transcriptional response of *PHO1* to short-term (up to 6 h) ABA in guard cells. We show that in stomata, *PHO1* expression increases rapidly following ABA application. This rapid up-regulation of *PHO1* in guard cells under ABA treatment suggest a possible involvement of *PHO1* in guard cell ABA signal transduction.

While weak expression in the shoots had been detected before through RNA blots (Hamburger *et al.*, 2002), evidence of *PHO1* expression outside the roots were lacking. Our results show that *PHO1* expression in leaf guard cells as well as leaf vasculature might account for the weak expression in the shoots detected by Hamburger

et al. (2002). Interestingly, prior use of *Arabidopsis* lines carrying *pPHO1::GUS* revealed *PHO1* promoter activity in the root and hypocotyl vascular cylinder, but failed to detect relevant promoter activity in the leaves (Hamburger *et al.*, 2002), suggesting a possible low sensitivity of the method, or the requirement of other elements than the 2.1-kb region upstream of the *PHO1* gene for its expression in above ground tissues. In accordance with Hamburger *et al.* (2002), we show that in the *pPHO1::GUS* lines, *PHO1* promoter activity is seldom detected in shoots, apart from staining in hypocotyl guard cells and occasionnaly in a few leaf stomata. In contrast, the use of *pho1.4* transgenic lines carrying the *pPHO1::PHO1-GUS* construction (PHO1-GUS fusion) revealed surprisingly consistent expression in stomata and leaf vasculature. The result differences between the two transgenic lines suggest that the full expression patterns of *PHO1* might involve other regulatory elements outside the promoter region. Intronic regulatory elements, such as enhancers, may be involved in such a regulation, as was previously demonstrated for other plant genes (Schauer *et al.*, 2009).

However, even though possible intronic regulatory elements must be considered, the 2.1-kb region sequence of upstream of PHO1 start codon did indicate the presence of several motifs typical of cis-acting regulatory elements involved in guard cell-specific expression and ABA responsiveness. The *PHO1* promoter displayed several [A/T]AAAG motifs including a cluster of three located between -860 and -930 bp. [A/T]AAAG motifs are required for binding of DOF-type transcription factors, a class of zinc finger transcription factors. [A/T]AAAG motifs have been described as an essential cis-active element for guard cell-specific expression of the potato potassium channel *KST1* gene (Plesch *et al.*, 2001), and of the transcription factor *AtMYB60* (Cominelli *et al.*, 2011). Although the presence of a DOF-binding site alone is not sufficient to explain guard

cell-specific expression patterns (Yang *et al.*, 2008; Cominelli *et al.*, 2011), it appears that it is the presence of clusters of at least three [A/T]AAAG motifs (defined as located on the same strand within a region of 100 bp), that is important to confer guard cell-specificity to a promoter region (Galbiati *et al.*, 2008). Within a cluster, DOF-binding sites exert additive roles in mediating gene expression in stomata (Cominelli *et al.*, 2011). The *PHO1* promoter region also displayed a few ACGT core motifs of ABA-responsive elements (ABREs). The ACGT core is a sequence known to be recognized by plant bZIP proteins, and it has been demonstrated that multiple ABREs can establish a minimal ABA-responsive complex (ABRC), that can confer ABA responsiveness to a minimal promoter (Gomez-Porras *et al.*, 2007).

Finally, it is well known that ion channels/transporters are involved in a wide range of physiological processes in plants, including plant nutrition, osmoregulation and cell signaling (Barbier-Brygoo *et al.*, 2011). In root cells, transporters and channels are required, among other roles, to take up ions from the soil and to release them in the root vascular cylinder for subsequent distribution to the shoots. In guard cells, ion transporters and channels are involved in cell signaling and in the uptake and efflux of osmolytes that regulate the osmotic pressure controlling stomatal aperture. It is therefore not unusual to find members of the same channels/transporters families expressed in these different cell types, and eventually with different functions. As an example of expression in both root and guard cells, the plant Shaker family of K^+ channels displays members with roles in root K+ accumulation from soil (AKT1; Hirsch *et al.*, 1998), in root release of K^+ into the xylem (SKOR, expressed in the vascular cylinder of the root; Gaymard *et al.*, 1998), but also in guard cell K^+ efflux (GORK; Hosy *et al.*, 2003) as well as in guard cell K^+ influx (AKT1, AKT2, KAT1 and KAT2; Pilot *et al.*, 2001; Kwak *et al.*, 2001; Szyroki *et al.*, 2001). The unraveling of

the expression of *PHO1* in guard cells suggests that PHO1 might have similar diverse roles in *Arabidopsis*, and prompts for further investigations to provide insights on PHO1 function in stomatal biology.

Experimental procedures

Plant material and growth conditions *Arabidopsis thaliana* wild type plants were from the Columbia ecotype (wild-type Col). For *in vitro* plant cultures, seeds were surface sterilized and sown on plates containing 1/2 Murashige Skoog medium pH 5.6 with Gamborg vitamins (Duchefa), 1% w/v sucrose, and 0.8 % w/v agar, then placed under short-day growth conditions (20°C, 10 h light/ 18 h dark) for two to three weeks. Chamber-grown plants were directly sown in 7 cm diameter pots containing potting compost and vernalized for 2 days at 4°C, before being placed for 2 weeks at 18°C, 60% relative humidity, 10 h light/ 14 h dark, 100 $\mu Em^{-2}s^{-1}$.

Gus staining Transgenic *A. thaliana* (Columbia) plants expressing the ß-glucuronidase (*GUS*) reporter gene under the control of the *PHO1* promoter region (2.1 kb upstream of the transcribed sequence, see Hamburger *et al.*, 2002) were grown on agar plates for two to three weeks. One hour after the beginning of the light cycle, seedlings were immersed for 4 h in a 1/2 MS solution containing 100 µM ABA. GUS staining was then conducted as explained below.

The transgenic *pho1.4* plants expressing *PHO1* fused to the ß-glucuronidase (*GUS*) was done by cloning the *PHO1* promoter (2.1 kb upstream of the transcription start) along with the complete *PHO1* ORF into the pMDC139 vector (Curtis and Grossniklaus, 2003). Three hours after the beginning of the light cycle, two-week-old rosettes were cut at the hypocotyl and floated in water or 10 µM ABA for 3h prior to GUS staining. For both constructions, GUS staining was conducted as follows: seedlings were incubated in 1mL of X-gluc solution (0.5 mM X-Gluc/DMF, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 0.01 % v/v Triton X-100, 50 mM sodium phosphate) for 48 h at 37 °C. Stained tissue was fixed in 5% v/v formalin for 10 min, followed by a 10 min in-

cubation in 5% v/v acetic acid. To remove chlorophyll, stained and fixed tissue was incubated in serial dilutions of 30 %, 50 %, 70 %, and 100 % EtOH.

Guard cell and mesophyll cell protoplast preparation Guard cell and mesophyll cell protoplasts were isolated according to Pandey *et al.* (2002). For guard cell protoplasts, mature leaves from 12-24 five-week-old plants (main vein discarded) were blended in cold distilled water for 2 min using a small Waring blender on high speed. Processed tissue was passed through a 100 µm nylon mesh to isolate epidermal fragments and rinsed with water until clear. Epidermal fragments were then submerged in a digestion mixture containing 0.7-1.4 % Cellulysin Cellulase *Trichoderma viride* (Calbiochem), 0.1 % PVP40 (Sigma), 0.25 % BSA (Sigma), 13.75 mL basic medium (104 g/L D-sorbitol (Sigma), 0.5 mM ascorbic acid (Sigma), 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM MES, pH 5.5 with KOH) and 11.25 mL distilled water. Digestions were incubated for 3-4 hours at 27 °C incubated in a shaking water bath in the dark. The extent of cellular digestion was monitored microscopically until completion, upon which digests were passed through a 50 µm nylon mesh and rinsed with basic medium. Isolated epidermal cells were transferred to a second digestion mixture containing 1.5 % Cellulase RS onozuka (Yakult pharmaceuticals, Japan), 0.03 % Pectolyase Y23 (Interchim, France), 0.25 % BSA (Sigma) and 25 mL basic medium. These secondary digestions were incubated slowly shaking for 2 hours at 17 °C and the formation of guard cell protoplasts was monitored microscopically until completion. Digests were passed through a 10 µm nylon mesh and isolated protoplasts were collected in Falcon tubes. Triplicate washes were performed where protoplasts were pelleted at 350 g for 15 min in a swing rotor centrifuge and resuspended in basic medium. The purity of guard cell protoplasts in the final pellet was then verified using a hemacytometer. For mesophyll

cell protoplasts, mature leaves from five-week-old plants (main vein discarded) were cut into 1mm strips and submerged directly into a digestion mixture containing 1 % Cellulase R-10 (Serva), 0.4 % Macerozyme R-10 (Serva), 5mM MES, 0.2 % BSA, 0.1 % PVP40 and 25 mL medium (118.9 g/L D-sorbitol, 1 mM $CaCl_2$). Digestions were incubated slowly shaking for two hours in the dark at room temperature. Digests were passed through a 25 µm nylon mesh and the resulting medium containing protoplasts were collected in Falcon tubes. Triplicate washes were performed where protoplasts were pelleted at 200 g for 15 min in a swing rotor centrifuge and resuspended in medium. The purity of mesophyll cell protoplasts in the final pellet was then verified using a hemacytometer.

ABA treatments on guard cell protoplasts Guard cell protoplasts were re-suspended in 50 mL of basic medium to which 50 uL of 0, 5, 10, 50 or 100 mM ABA was added. Replicated treatments were set up allowing analysis immediately following the addition of ABA and after 4 and 6 h incubations in the dark at room temperature. Treated protoplasts were collected by duplicate 15 min centrifugations in a swing rotor centrifuge at 350 g.

RNA extraction and real-time quantitative RT-PCR Pelleted protoplasts were collected in 1.5 mL tubes and concentrated by centrifugation. All but 100 µL of supernatant was removed from the pellet. Total RNA was extracted using 1 mL TRIZOL reagent (Invitrogen) following manufacturer's instructions. Glycogen (Roche) was used as an RNA carrier. Total RNA was treated with DNase (Qiagen,) to remove residual genomic DNA, and was subsequently purified using a RNAeasy Minelute Cleanup Kit (Qiagen). Reverse transcription was performed using M-MLV reverse transcriptase (Promega). qRT-PCR analysis was performed using SyberGreen mix

and the reference dye ROX (ABgene) in a Stratagene MPx3000 instrument. The following thermal cycle was observed: an initial incubation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. At the end of the 40 cycles was a dissociation step of 95 °C for 15 sec, 55 °C for 15 sec and 95 °C for 15 sec, in order to verify the absence of nonspecific products. All amplification plots were analyzed using MxPro QPCR software (Stratagene) with an Rn threshold of 0.1 to obtain Ct values. Transcript abundance was estimated as per the standard curve method (Rutledge and Cote, 2003) observing default settings. Transcript abundance of target genes was normalized against expression of the reference gene *AT1G13320* (described by Czechowski *et al.*, 2005). For analysis of *PHO1* expression in guard cells versus mesophyll cells, *PHO1* was amplified using the oligonucleotides 5'-acc gta ccg tta ccg ttc ctt ga-3' and 5'ctt cgt ttt gca ctt tgg agc gt-3', and using 5'-tgc tga aga cag gca ctg gag ag-3' and 5'-tgc tgc att gcc cat tca gga cc-3' for the reference gene *AT1G13320*. For analysis of gene expression in response to ABA treatment of guard cells, *PHO1* was amplified using the oligonucleotides 5'-aca cca ttc cag gca tcc tcc tc-3' and 5'-acg gtg agc aaa caa tct tcc gc-3', whilst *RAB18* was amplified using 5'-tgg gag gaa tgc ttc acc gct c-3' and 5'-cca tcg ctt gag ctt gac cag ac-3'. To assess the purity of guard and mesophyll cell preparations, transcript abundance of guard-cell-specific genes *KAT1* and the mesophyll-cell-specific gene *At4G26530* was determined using primers 5'-atg ctc atg ctg acg atg gac ga-3' and 5'-tcc act ctt ccc atc cca tgc t-3', and 5'-agg cct tga acg acc acc atg tc-3' and 5'-acc tgc agg tgg gac tgt gcg-3', respectively. In latter reactions, the oligonucleotides 5'-taa cgt ggc caa aat gat gc-3' and 5'-gtt ctc cac aac cgc ttg gt-3' were chosen for the reference gene *AT1G13320*. The mesophyll-cell-specific gene *At4G26530* was chosen according to microarray data described by Yang *et al.* (2008).

4 - Stomatal movement analysis of the mutant *pho1*

Céline Zimmerli* and Yves Poirier

*The experiments were designed, performed and data analyzed by Céline Zimmerli under the supervision of Yves Poirier.

Introduction

Guard cells are able to sense and integrate a myriad of complex environmental and hormonal signals. In response, they regulate the aperture of the stomata by driving ion fluxes across their plasma membrane, therefore causing changes in guard cell turgor and volume. This rapid closing and opening of the stomatal pore allows tight regulation of gas exchanges depending on varying environmental conditions such as light, CO_2 concentration, humidity or time of day (Hetherington and Woodward, 2003; Nilson and Assmann, 2007). Environmental signals can act either in a synergistic or antagonistic manner on stomatal movement: red/blue light and reduced CO_2 induce the opening of the stomata to allow more CO_2 uptake for photosynthesis, while the drought hormone abscisic acid (ABA), darkness, and elevated CO_2 typically cause stomatal closure in order to reduce water loss and regulate photosynthesis. These various signals involve common as well as distinct signaling events and molecular players (for details, see *Chapter 1 - Introduction*).

In *Arabidopsis,* PHO1 is involved in the export of phosphate into the root xylem vessels and, as a result, the *pho1* mutant is characterized by low shoot phosphate levels. In the previous chapter (see *Chapter 3 - Expression analysis of AtPHO1 in guard cells and in response to ABA*), we presented evidence that beside its expression in the root vasculature, *PHO1* was expressed in guard cells and up-regulated under ABA treatment. These results indicated that PHO1 might have a role in guard cell ABA signaling. Incidentally, previous results from Ribot (2006) showed that the mutant *pho1* displays phenotypes linked to ABA: *pho1* exhibits a pronounced seed dormancy, a hypersensitivity to ABA in terms of germination and root growth, and has a higher rate of water loss during dehydration assays, comparable to *aba* mutants (ABA-deficient

mutants). Through micro-grafting on wild-type roots, Ribot (2006) demonstrated that the higher rate of water loss could be seen even in Pi-repleted *pho1,* suggesting that the impairment was independent of phosphate deficiency. In addition, observation of wild-type and *pho1* epidermis suggested that the stomata of *pho1* did not close in response to ABA treatment. However, the extent of the impairment was not quantified, nor was it further explored. It was thus of interest to investigate in greater detail the potential role of *PHO1* in guard cell ABA signaling.

In this chapter, we explore the stomatal response of the mutant *pho1* in response to various stimuli, in an effort to narrow down the precise pathways that might be affected by the mutation. With stomatal aperture measurements on epidermal peels, we show that *pho1* is impaired in the stomatal movement response to ABA, during both the induction of stomatal closure and the repression of stomatal opening under light, and this even at high concentrations of ABA. Interestingly, *pho*1 response to changes in CO_2 concentration appeared altered as well. We demonstrate that the impairment occurs downstream of ABA-induced ROS production, but that it does not affect the stomatal response to light transitions, chemicals such as auxin and fusicoccin, and high extracellular calcium. In order to rule out a possible effect of phosphate deficiency on the stomatal response of *pho1*, we used micro-grafting techniques to generate Pi-repleted *pho1.* Alleviation of *pho1* phosphate deficiency did not improve *pho1* stomatal response to ABA. Together, these results demonstrate that the *pho1* mutation does not affect the overall ability of stomata to open and close, but rather interferes specifically, and independently of the plant phosphate status, with the guard cell response to ABA. The signaling event involving PHO1 is likely to be common to both ABA and CO_2 signaling.

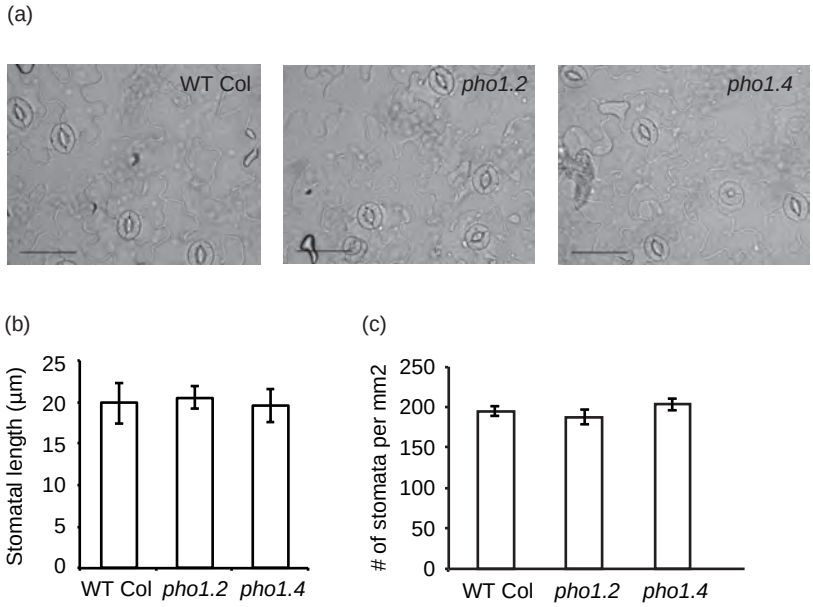


Figure 4.1: **The *pho1* mutant presents normal guard cell morphology**. (a) Representative images of WT, *pho1.2* and *pho1.4* epidermal peels (bar = 20 µm). (b) Stomatal length in WT, *pho1.2* and *pho1.4*. n= 30 stomata over five sampled epidermis; average ± SE. (c) Stomatal density in the epidermis of WT, *pho1.2* and *pho1.4*. n= 10 sampled epidermis; average ± SE. The differences between WT and the *pho1* mutants were not significant, as confirmed by Student t-test.

Results

The *pho1* mutant is impaired in both ABA-induction of stomatal closure and repression of stomatal opening

To assess *PHO1* function in guard cells, we analyzed stomatal movements in the *pho1* mutant in response to different stimuli. Epidermal peels were prepared by detaching the epidermis from the mesophyll. Visually, no differences were observed in the morphology, the size, or the density of the WT stomata and those of the allelic mutants *pho1.2* and *pho1.4* (figure 4.1).

We analyzed the stomatal movements in the allelic mutants *pho1.1, pho1.2, pho1.3* and *pho1.4* (Poirier *et al.*, 1991; Delhaize and Randall, 1995; Hamburger *et al.*, 2002) following treatment with ABA. Epidermis preparations of WT, *pho1.1, pho1.2, pho1.3* and *pho1.4* were subjected to light in order to induce stomatal opening, then were treated with 10 µM ABA for two hours to induce stomatal closing. In average over three independent experiments, stomatal apertures in *pho1.1, pho1.2, pho1.3* and *pho1.4* were slightly higher than WT before treatment with ABA (figure 4.2; t = 0). However, later experiments revealed that this finding was not consistently observed (figure 4.3; 0 µM ABA). More importantly, following a 1-h and 2-h of ABA treatment, stomatal apertures in *pho1.1, pho1.2, pho1.3* and *pho1.4* were consistently wider than observed in WT (figure 4.2; t = 1 h, 2 h), suggesting that ABA is unable to fully induce stomatal closure in the mutants *pho1*.

In order to assess the response of *pho1* under varying ABA concentrations, epidermis preparations of WT, *pho1.2* and *pho1.4* were subjected to light in order to induce stomatal opening, then were treated with varying concentrations of ABA for two hours to induce stomatal closing. In this set of independent experiment, stomatal apertures in *pho1.2* and *pho1.4* were comparable to WT in the absence of ABA (figure 4.3a; 0 µM ABA). Following ABA treatments, induction of stomatal closure was successfully seen in WT stomata, with final stomatal aperture getting smaller as the ABA concentration was increased. However, stomatal apertures in *pho1.2* and *pho1.4* were consistently wider than observed in WT (figure 4.3a), meaning that ABA failed to induce proper stomatal closure in *pho1*, even under high concentration of ABA (50 µM).

To further investigate stomatal movements in *pho1* in response to ABA, we analyzed ABA-inhibition of stomatal opening in *pho1.2*. WT and *pho1.2* epidermal peels were prepared and incubated in darkness to ensure stomata were closed. Interestingly,

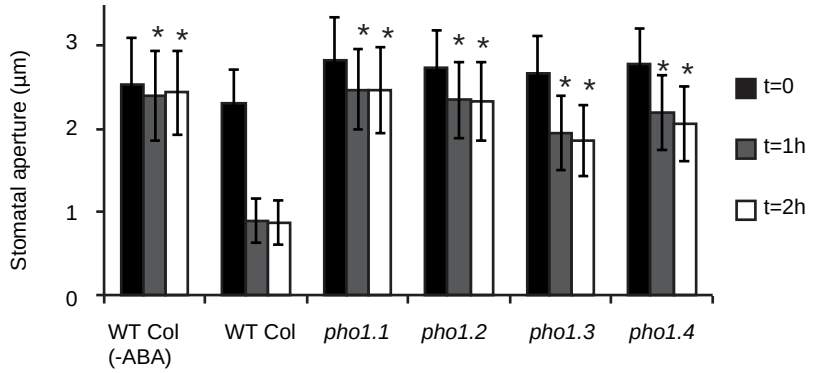


Figure 4.2: **The *pho1* allelic mutants present consistent impairment of stomatal closure in response to ABA.** Induction of stomatal closure, as represented by decreasing apertures, in WT and in the allelic mutants *pho1.1*, *pho1.2*, *pho1.3* and *pho1.4* (Poirier *et al.*, 1991; Delhaize and Randall, 1995; Hamburger *et al.*, 2002), in response to 10 µM ABA, after 0, 1, and 2 h. Stomatal opening of WT without ABA (WT Col -ABA) was also monitored. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values per time point ($P < 0.05$).

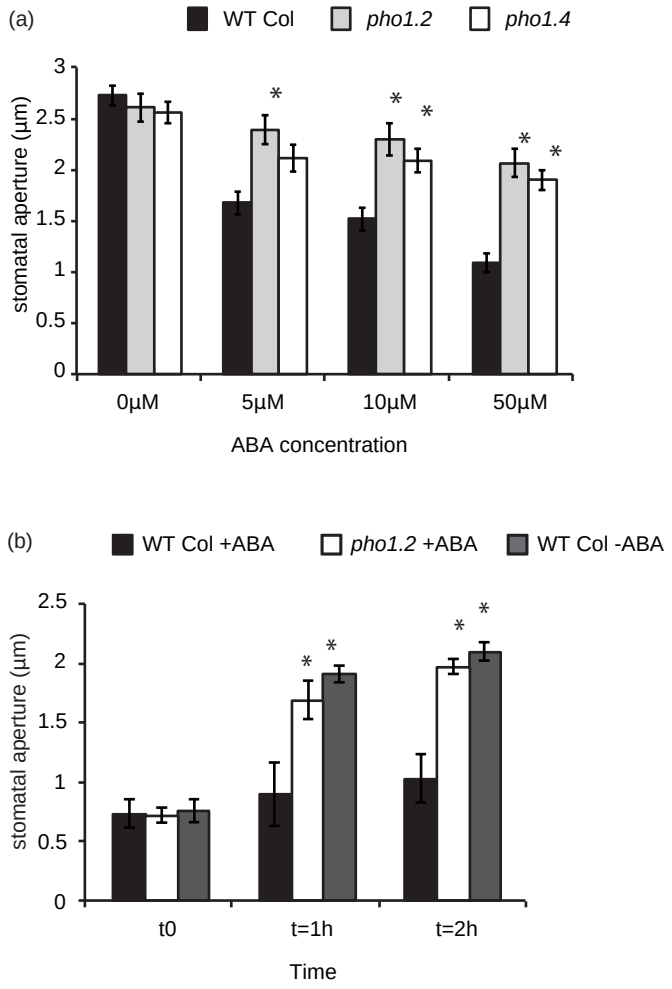


Figure 4.3: **The guard cells of *pho1* are non-responsive to ABA both during induction of stomatal closure and inhibition of stomatal opening.** (a) Stomatal closure in WT, *pho1.2*, and *pho1.4*, as represented by decreasing stomatal apertures, following 2-h treatments with ABA concentrations varying from 0 to 50 µM. n = 3 independent experiments; average ± SE. (b) Stomatal opening under light in WT and *pho1.2*, as represented by increasing stomatal apertures, following 0, 1, and 2-h treatments with 10 µM ABA. Stomatal opening of WT without ABA (WT Col -ABA) was also monitored. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

prior to treatment, stomata in *pho1.2* were fully closed and stomata aperture widths were comparable to WT (figure 4.3b; t0), indicating that the inability of ABA treatment to induce stomatal closure in *pho1* is not due to a mechanical incapacity of *pho1* stomata to close. Wild type and *pho1.2* epidermal peels were placed under light and treated with 10 µM ABA for two hours. ABA treatment was seen to effectively inhibit stomatal opening of WT. In contrast, stomata in *pho1.2* were unresponsive to ABA treatment and opened fully (figure 4.3b), displaying final aperture widths similar to untreated WT stomata. This result suggests that ABA is also unable to repress stomatal opening in *pho1*.

pho1 perceives ABA and produces ROS but does not close under H_2O_2 treatment

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are known mediators of stomatal movements and are produced in response to ABA (Wang and Song, 2008). Therefore, it was of interest to examine whether guard cells in *pho1* were able to perceive ABA and produce ROS. The H_2O_2-sensitive fluorescent probe 2´,7´-dichlorofluorescein diacetate (DCFDA) was used to assess relative ROS levels in WT and *pho1.2* guard cells prior to and following ABA treatment. Wild type and *pho1.2* epidermal peels were incubated in 50 µM DFCDA followed by 50 µM ABA. DCFDA fluorescence in guard cells of both *pho1.2* and WT increased following ABA treatment (figure 4.4a and b), suggesting that ROS production is increased in *pho1* in response to ABA. This provides evidence that an impaired stomatal ABA response in *pho1* occurs downstream of ABA perception and ROS production. In agreement with this hypothesis, H_2O_2 treatment also failed to induce stomatal closure in *pho1.2* (figure 4.4c).

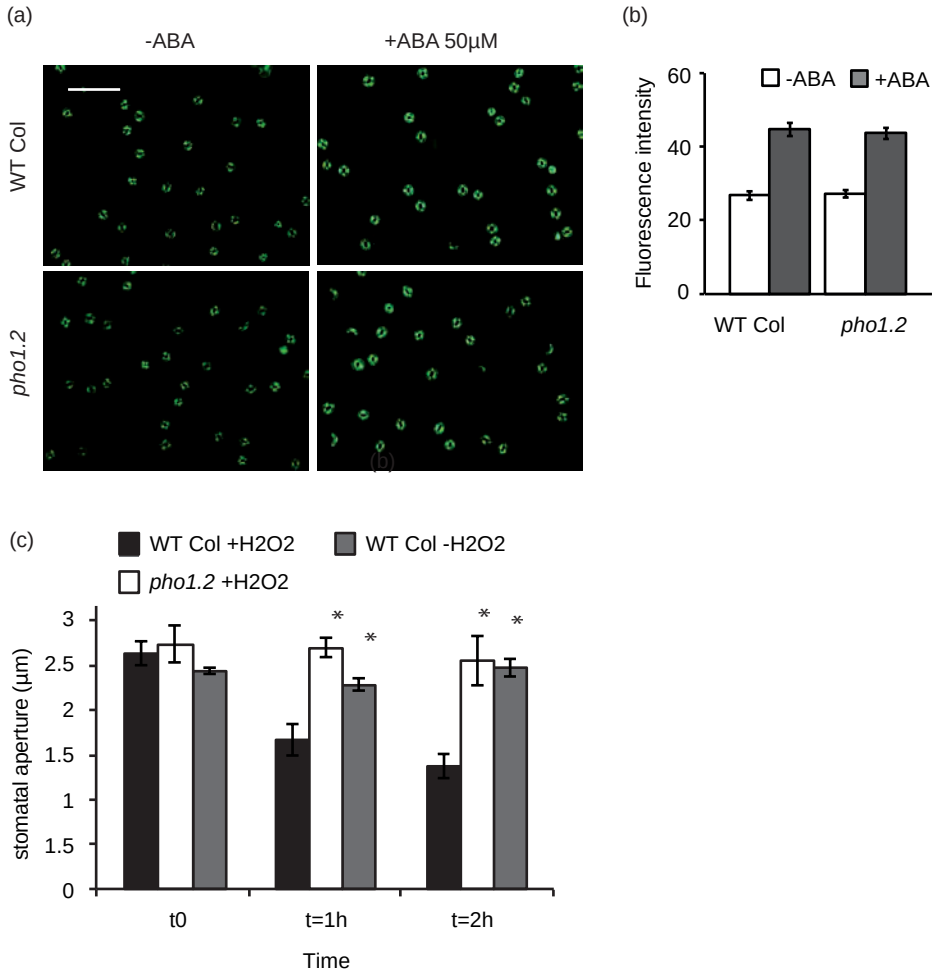


Figure 4.4: **The mutant *pho1* produces ROS in response to ABA and does not close under H_2O_2 treatment.** (a) ROS production in leaf epidermal stomata of WT and *pho1.2* following application of 50 µM ABA, as revealed by fluorescence levels of the fluorescent dye DCFDA. (-ABA) pictures represent epidermis before addition of ABA; (+ABA) represent epidermis 3 min after addition of ABA. Bar = 100 µm. (b) Quantification of the average fluorescence intensity (n = 45 stomata; average ± SE). (c) Stomatal closure in WT and *pho1.2* following 0, 1, and 2-h treatments with 100 µM H_2O_2. Stomatal aperture of WT without H_2O_2 treatment (WT Col -H_2O_2) was also monitored. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

Stomata of *pho1* are able to open and close in response to other treatments

To assess whether impaired stomatal movements in *pho1* were specific to the ABA pathway, we tested whether stomata in *pho1.2* were responsive to other stimuli.

We first experimented with treatments inducing stomatal opening, including light signals such as red and blue light, and also chemical signals such as auxin and fusicoccin. Wild-type and *pho1.2* epidermal peels were maintained in darkness to promote stomatal closure, followed first by a 1-h exposure to strong red light (50 $\mu Em^{-2}s^{-1}$) and then a 2-h blue light (10 $\mu Em^{-2}s^{-1}$) superimposition. Interestingly, similar stomatal aperture widths were observed in both WT and *pho1.2* under red light (figure 4.5a; RL 1 h) and red+blue light treatments (figure 4.5a; RL+BL 2 h). In contrast, the stomata of the phototropin double mutant *phot1;phot2* did not respond to blue light, as previously described (Kinoshita *et al.*, 2001).

Furthermore, we examined stomatal opening in darkness following treatment with auxin or fusicoccin (figure 4.5b). Wild-type and *pho1.2* epidermal peels were maintained in darkness to promote stomatal closure (figure 4.5b; t0), then auxin (IAA 1 mM) or fusicoccin (1 μM) were applied. Again, WT and *pho1.2* presented similar stomatal opening responses to auxin and fusicoccin (figure 4.5b).

We then tested treatments that induce stomatal closure, such as transition from light to dark, and high extracellular calcium treatment. Wild-type and *pho1.2* epidermal peels were subjected to light in order to induce stomatal opening (figure 4.6a; t0), then light was turned off and stomatal aperture was monitored after 30 and 60 minutes in the dark (figure 4.6a; t = 30 min and t = 60 min). We observed similar stomatal apertures in both WT and *pho1.2* at the selected time points. Finally, application of high extracellular calcium concentration (5 mM $CaCl_2$) to pre-opened stomata triggered

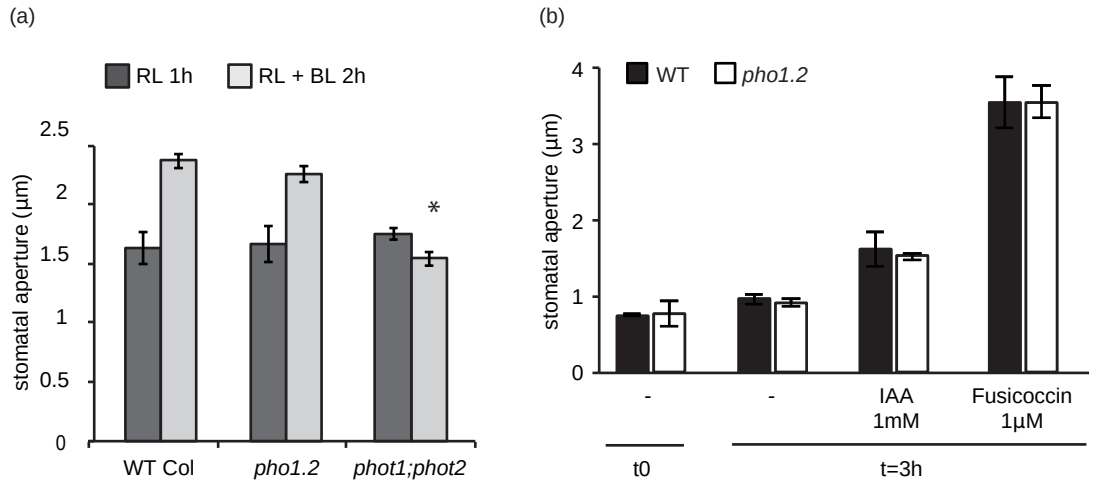


Figure 4.5: **The mutant *pho1* maintains normal stomatal movement under blue light, auxin, and fusicoccin treatments.** (a) Stomatal opening under blue light in WT and *pho1.2*. Stomatal apertures were measured following 1 h exposure to red light (RL 1 h), and again after a 2-h superimposition of blue light (RL+BL 2 h). The double mutant *phot1,phot2* was used as a control. (b) Stomatal opening in darkness in WT and *pho1.2* following treatment with auxin 1mM (IAA) and fusicoccin 1µM. Pre-treatment (t0) measurements were of stomata maintained in darkness. n = 3 independent replicates; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

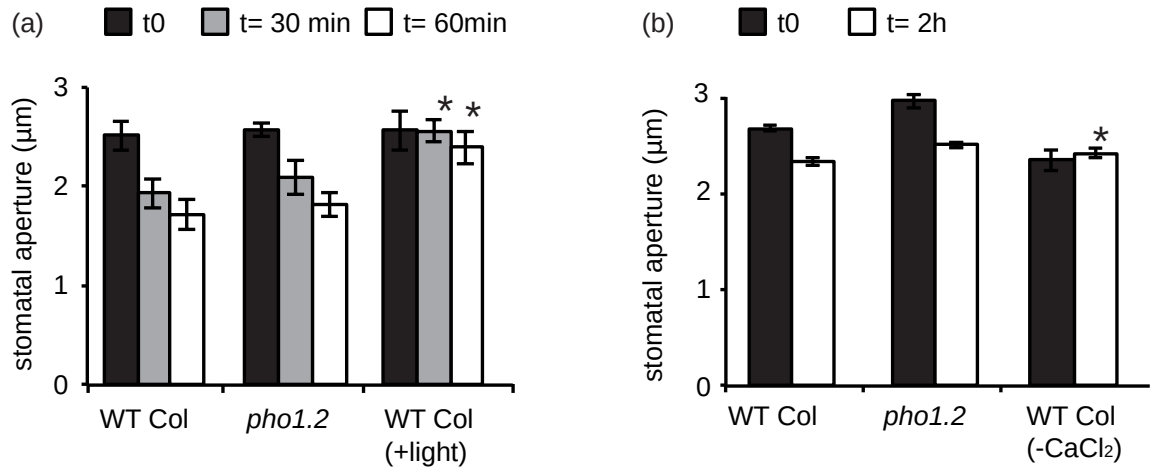


Figure 4.6: **The stomata of *pho1* are able to close under transition to dark and high calcium loads.** (a) Stomatal closure in WT and *pho1.2* following 30 and 60 minutes of darkness. Stomatal aperture of WT kept under light (WT Col +light) was also monitored. n = 3 independent experiments; average ± SE. (b) Stomatal closure in WT and *pho1.2* following treatment with 5 mM $CaCl_2$. Stomatal aperture of WT without $CaCl_2$ treatment (WT Col - $CaCl_2$) was also monitored. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

similar stomatal closure response in both WT and *pho1.2* (figure 4.6b).

The stomatal response of *pho1* to changes in CO_2 concentration is also affected

We further investigated the response of *pho1* to changes in CO_2 concentration. WT, *pho1.2* and *pho1.4* epidermal peels were prepared and kept in darkness to ensure that stomata were fully closed (figure 4.7; t = 0). The epidermal peels were then incubated in the dark for two more hours in incubation buffer satured with CO_2-free air, or were kept in the control buffer saturated with atmospheric air. When kept under normal conditions, stomata of WT, *pho1.2* and *pho1.4* stayed closed (figure 4.7; t = 2

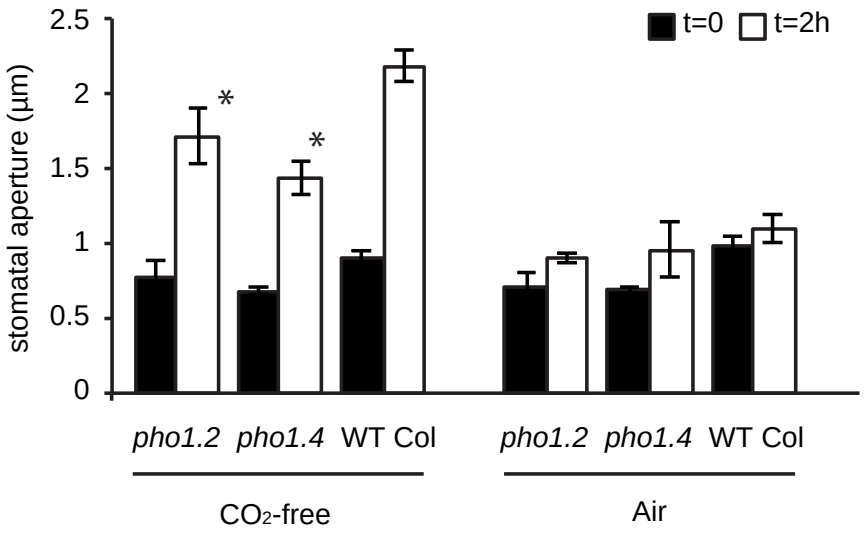


Figure 4.7: **The mutants *pho1* respond less than WT to a decrease in CO_2 concentration.** Stomatal opening in the dark in WT, *pho1.2* and *pho1.4,* in response to a 2-h incubation with CO_2-free air or atmospheric air. Epidermal peels were kept in the dark (t = 0) before treatment. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate final stomatal aperture that are statistically different from the wild-type value ($P < 0.05$).

h "Air"). In contrast, the CO_2-free treatment successfully induced stomata to open in WT. Interestingly, while the stomata of *pho1.2* and *pho1.4* opened in response to the CO_2 decrease, the average final stomatal aperture after two hours was significantly lower in the mutants than in the WT, showing that they did not open to the same extent as the WT. This result suggests that the *pho1* mutation also affects the stomatal response to changes in CO_2 concentrations (figure 4.7; t = 2 h "CO_2-free").

Figure 4.8: **Micro-grafts composed of a *pho1* shoot scion onto wild type rootstock result in plants with normal shoot growth and Pi content.** Representative growth of 4-week-old WT, *pho1.2*, and chimeric plants composed of either WT or *pho1.2* scion micro-grafted onto WT rootstock (WT/WT and *pho1.2*/WT, respectively). Shoot growth of *pho1.2* scion with WT rootstock resembled growth of WT and the WT/WT micro-graft control, indicating restored Pi content in the *pho1.2* scion.

Stomata in *pho1* scion micro-grafted onto WT rootstock remain poorly responsive to ABA, despite Pi sufficiency.

Phosphorus starvation induces a myriad of transcriptional, biochemical, and physiological effects, including adaptive changes in shoot growth, carbohydrate metabolism and ion composition (Hammond and White, 2008). Because such pleiotropic effects could potentially impair the stomatal response to ABA, we were interested in determining whether the impaired ABA induced stomatal movements observed in *pho1* were dependent on the specific *PHO1* function in guard cells or on the phosphate deficiency alone. The approach was to generate Pi-sufficient chimeric plants composed of a *pho1* scion micro-grafted to a WT rootstock (*pho1*/WT). As previously described, WT rootstock restored root-to-shoot phosphate transfer to *pho1* scions, resulting in WT level of shoot Pi content and normal shoot growth (Stefanovic *et al.*, 2007) (figure 4.8). We also generated control WT plants composed of a WT scion micro-grafted to a WT rootstock (WT/WT). These chimeric plants were then tested for their stomatal response to ABA.

Epidermis preparations of WT/WT, *pho1.1*/WT, *pho1.2*/WT, *pho1.3* / WT and *pho1.4*/WT

micro-grafts were subjected to light in order to induce stomatal opening, then treated with 10 µM ABA for two hours to induce stomatal closing (figure 4.9a). Stomatal aperture was recorded after one hour and two hours of ABA treatment. The stomata of plants composed of a WT scion micro-grafted to a WT rootstock (WT/WT) closed normally under ABA treatment, suggesting that micro-grafting does not interfere with normal ABA-induction of stomatal closure. However, stomatal movements in plants composed of a *pho1* scion micro-grafted to a WT rootstock (*pho1.1*/WT, *pho1.2*/WT, *pho1.3* / WT and *pho1.4*/WT) remained poorly responsive to ABA, as ABA failed to induce full stomatal closure (figure 4.9a). Consistent with this result, treatment with 100 µM H_2O_2 also failed to induce stomatal closure in *pho1.2*/WT (figure 4.9b).

We further tested ABA-repression of stomatal opening in micro-grafted WT/WT and *pho1.2*/WT plants (figure 4.10). Epidermal peels of micro-grafted WT/WT and *pho1.2*/WT plants were kept in darkness to ensure that stomata were closed, then were treated with light and 10 µM ABA. Stomatal aperture was monitored after one hour and two hours of ABA treatment. Again, while ABA effectively inhibited the stomatal opening of WT/WT, we consistently measured wider stomatal aperture in *pho1.2*/WT after one hour and two hours under light and ABA treatment. This result shows that ABA still failed to repress stomatal opening in Pi-repleted *pho1*. Together, the results of the stomatal aperture assays on micro-grafted *pho1*/WT demonstrate that the impaired stomatal response of *pho1* can not be alleviated through phosphate sufficiency alone.

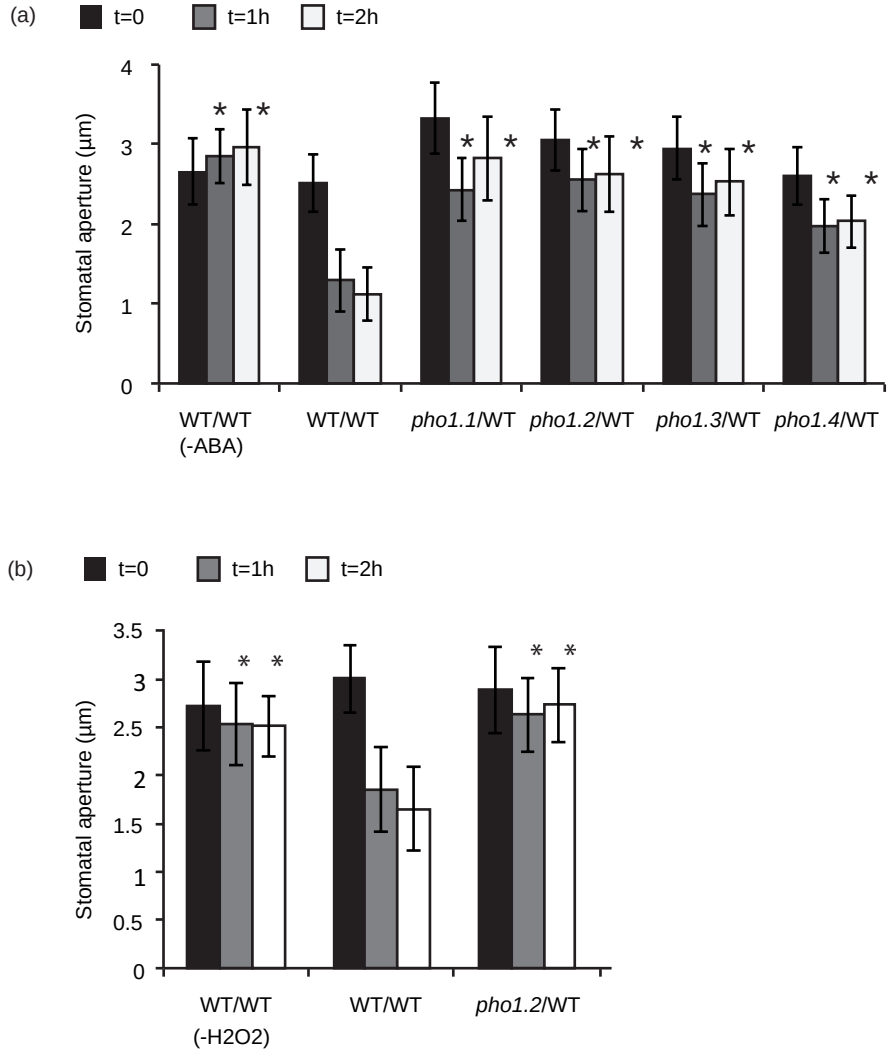


Figure 4.9: **Phosphate sufficiency through micro-grafting does not restore ABA and H_2O_2-induction of stomatal closure in *pho1*.** (a) ABA-induction of stomatal closure in micro-grafted WT/WT, *pho1.1*/WT, *pho1.2*/WT, *pho1.3* / WT and *pho1.4*/WT in response to 10 µM ABA, after 0, 1, and 2 h. n = 3 independent experiments; average ± SE. Stomatal opening of WT/WT without ABA (-ABA) was also monitored. (b) Stomatal closure in micro-grafted WT/WT and *pho1.2*/WT following 0, 1, and 2-h treatments with 100 µM H_2O_2. Stomatal aperture of WT/WT without H_2O_2 treatment (WT Col -H_2O_2) was also monitored. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

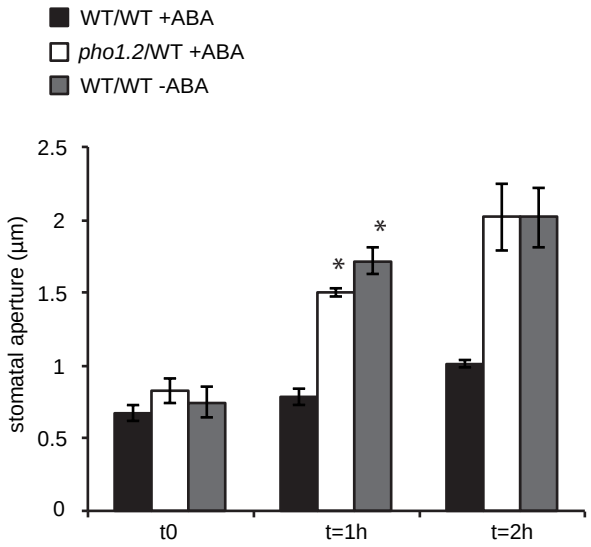


Figure 4.10: **Phosphate sufficiency through micro-grafting does not restore ABA-repression of stomatal opening in *pho1*.** ABA-repression of stomatal opening under light in micro-grafted WT/WT and *pho1.2*/WT, in response to 10 µM ABA, after 0, 1, and 2 h (n = 3 independent experiments, average ± SE). Stomatal closure and opening of WT/WT without ABA was also monitored as a control (WT/WT -ABA). Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding wild-type control values ($P < 0.05$).

Discussion

As was mentioned in the introduction, previous results suggested a potential role of *PHO1* in guard cell signaling: the ABA-related phenotypes of *pho1* described by Ribot (2006), and the specific expression of *PHO1* in guard cells as presented in *Chapter 3 - Expression analysis of AtPHO1 in guard cells and in response to ABA*. In this context, the aim of this chapter was to explore the stomatal response of the mutant *pho1* in response to ABA and to other various stimuli, in order to characterize a potential stomatal phenotype in *pho1*.

Consistent with the work of Ribot (2006), the *pho1* mutant displayed a striking impairment of the stomatal response to ABA during stomatal aperture assays (figure 4.2 and 4.3). Both the induction of stomatal closure and repression of stomatal opening under light were impaired, with the *pho1* mutant presenting higher stomatal aperture than WT following ABA treatment, even under high concentrations of ABA.

We addressed whether the impaired ABA-induced stomatal movements observed in *pho1* were dependent on a specific function of *PHO1* in guard cell ABA signaling, or on some pleiotropic effects induced by the *pho1* mutation. We first ruled out the presence of any structural or mechanical impairment that could potentially prevent normal stomatal movements in *pho1*, since observation of epidermal peels revealed normal stomatal morphology, stomatal size and density (figure 4.1), and that *pho1* stomata maintained the ability to open and close like a wild type following other treatments such as light cues, high extracellular calcium, auxin and fusicoccin treatments (figure 4.5 and 4.6). Secondly, we were interested in addressing more specifically the possible effect of *pho1* shoot phosphate deficiency on the stomatal response to ABA. Indeed, long-term Pi starvation affects photosynthesis and carbon assimilation, triggering ac-

cumulation of starch and sucrose in the leaves (Hammond and White, 2008), which in turn could potentially impair the stomatal response to ABA. Using micro-grafting experiments between *pho1* scions and WT root stocks, we demonstrated that the impaired stomatal response to ABA in *pho1* plants occurred even in Pi-sufficient leaves (figure 4.9 and 4.10). Together, these results suggested that the *pho1* mutation does not interfere with the overall ability of stomata to open and close, and that *pho1* phosphate deficiency can not explain alone *pho1* stomatal phenotype. Rather, it suggested that the *pho1* mutation might affect a specific signal transduction event occurring during ABA-induced stomatal movements.

Interestingly, the mutant *pho1* presented a wild-type aperture under light, in the dark, and under red and blue light treatments (figure 4.3, 4.5a and 4.6a). Red light triggers stomatal opening through activation of photosynthesis (Shimazaki *et al.*, 2007), as well as through phytochrome B signaling (Wang *et al.*, 2010). Blue light signals activation of the plasma membrane H^+ -ATPase and inhibition of S-type anion channels. The resulting membrane hyperpolarization activates uptake of K^+ via the voltage-gated inward-rectifying K^+ channels (Shimazaki *et al.*, 2007; Roelfsema *et al.*, 2012), and therefore promotes stomatal opening. In darkness, stomatal opening is repressed via *COP1,* a negative regulator of stomatal opening that functions downstream of both cryptochromes and phototropins (Mao *et al.*, 2005). Our results reveal that *pho1*'s stomata are functional in these pathways and can fully open and close in response to light cues. Moreover, the stomata of *pho1* presented a WT response following treatments with the phytohormone auxin and the fungal toxin fusicoccin (figure 4.5b), which are known inducers of stomatal opening that act through the activation of the H^+-ATPases (Kinoshita and Shimazaki, 2001; Acharya and Assmann, 2009). These results further suggested that *PHO1* is not involved in the activation of

H^+-ATPases pumps and inward-rectifying K^+ channels, and more generally, that the *pho1* mutation does not affect all stomatal movements responses.

An early component of ABA signaling in guard cells is the ABA-stimulated production of reactive oxygen species (ROS) by the NADPH oxidases AtrbohD and AtrbohF (Kwak *et al.*, 2003). AtrbohF activity is believed to be regulated by OST1 (Sirichandra *et al.*, 2009*a*), upon relief of inhibition of this kinase by PP2C phosphatases, which are themselves suppressed upon ABA perception (Ma *et al.*, 2009; Park *et al.*, 2009). Using a reactive oxygen species (ROS)-sensitive fluorescent dye, we showed that *pho1* stomata are able to perceive ABA and produce ROS in response (figure 4.4), suggesting that the *pho1* mutation does not affect the permeability of the cells to ABA, nor does it interfere with ABA perception or with the subsequent early ABA signaling events. In addition, we observed that the mutant stomata do not close under hydrogen peroxide treatment (figure 4.4c), further suggesting that the mutation is likely to affect a signaling step downstream of ABA perception and ROS production.

We then investigated the response of *pho1* to changes in CO_2 concentration. CO_2 can induce stomatal opening or closing depending on its concentration: low CO_2 induces stomatal opening, while elevated CO_2 induces stomata to close. The mutants *pho1.2* and *pho1.4* presented a lower aperture in response to reduced-CO_2 in the media, suggesting that *pho1* stomatal response to changes in CO_2 concentration is perturbed as well (figure 4.7).

Interestingly, *pho1* stomata presented a wild-type closure response under high extracellular Ca^{2+} loads, suggesting that this treatment is able to trigger the appropriate signal transduction mechanisms necessary for stomatal closure. It is known that external application of high calcium concentration (1 to 10 mM) triggers stomatal closing via the induction of $[Ca^{2+}]_{cyt}$ oscillations (Allen *et al.*, 2000). There are in fact

many $[Ca^{2+}]_{cyt}$-dependent mechanisms, and reports suggest a current model where $[Ca^{2+}]_{cyt}$ functions as a 'hub' within the guard-cell signaling network (Hetherington and Woodward, 2003). For example, ABA (Allen *et al.*, 2000; Siegel *et al.*, 2009), ROS (McAinsh *et al.*, 1996; Pei *et al.*, 2000), elevated- and reduced-CO_2 (Young *et al.*, 2006) are all known to induce stomatal movements through the modulation of repetitive $[Ca^{2+}]_{cyt}$ transient patterns, responsible for a large part of the stomatal response (Siegel *et al.*, 2009; Hubbard *et al.*, 2012). Calcium signals participate for example in stomatal closure via activation of S-type anion channels (Mori *et al.*, 2006; Vahisalu *et al.*, 2008; Siegel *et al.*, 2009), activation of R-type anion channels (Meyer *et al.*, 2010), down-regulation of plasma membrane proton pumps (Kinoshita *et al.*, 1995), and down-regulation of K^+ influx channels (Siegel *et al.*, 2009). Oscillations in $[Ca^{2+}]_{cyt}$ result from the interaction of three processes: extracellular calcium influx, intracellular calcium release, and sequestration into intracellular stores or across the plasma membrane (Allen *et al.*, 2000). How the *pho1* mutation could affect such calcium relocation during ABA, ROS and CO_2 signaling is unknown. The monitoring of $[Ca^{2+}]_{cyt}$ transient patterns in *pho1* vs wild-type, in response to ABA, ROS, CO_2 and high extracellular Ca^{2+}, could provide further insight on the possible effect of the mutation on $[Ca^{2+}]_{cyt}$ oscillations, as well as on how high extracellular calcium manages to trigger normal stomatal closure response in *pho1*. Further characterization of *pho1* stomatal response to calcium-related stimuli, such as artificially imposed calcium transients or lower concentrations of extracellular calcium, could also help understanding which aspects of calcium signaling are affected or not by the *pho1* mutation.

Together, the results of this chapter characterize stomatal phenotypes in the mutant *pho1*, which displays impairment in ABA-, ROS- and CO_2 -induced stomatal move-

ments. This suggests that *PHO1* is likely involved in a signaling step common to both ABA and CO_2 signaling, and downstream of ROS production. ABA and CO_2 signaling share indeed common components, with a convergence point occurring early between the two signaling pathways (Xue *et al.*, 2011). Beside the Ca^{2+} elevations mentioned above, ABA, ROS and elevated CO_2 promote stomatal closure by activating anion channels that drive a release of anions and organic acids to the outside of the cells, leading to membrane depolarization and stimulation of K^+ outward-rectifying channels (Sirichandra *et al.*, 2009*b*; Negi *et al.*, 2008; Kim *et al.*, 2010). In parallel, these signals inhibit H^+-ATPase pumps and K^+ inward- rectifying channels (Zhang *et al.*, 2001, 2004), thus preventing stomatal opening. Further investigation, for example using electrophysiology measurements during guard cell response, could help characterize the signaling events compromised by the *pho1* mutation. How *PHO1* might play a role in these signaling events will be discussed further in *Chapter 6 - Conclusion and perspectives*.

Experimental procedures

Plant material *Arabidopsis thaliana* wild type and mutants plants are all from the *Columbia* ecotype (wild type Col). The mutants *pho1.1*, *pho1.2*, *pho1.3* and *pho1.4* have previously been described by Poirier *et al.* (1991), Delhaize and Randall (1995) and Hamburger *et al.* (2002). The mutant *phot1;phot2* mutant line was kindly supplied by Dr. K. Shimazaki (Kyushu University, Japan).

Micro-grafting experiments Plant grafting using collars was performed as per the protocol previously described by Turnbull *et al.* (2002). Seedlings were grown for 5 days at 27°C under 8h light/16h dark cycles on 1/2 Murashige Skoog medium pH 5.6 with Gamborg vitamins (Duchefa) containing 1% w/v Sucrose (Acros organics) and 0.8% w/v Agar (Applichem). Root to shoot grafting was performed using sterile silicone tubing 0.3 mm in diameter. Following grafting, plants were incubated at 27°C for a further 4 to 6 days. Grafts unions were examined for the absence of adventitious roots, and successful grafts were transferred to soil for 4-5 weeks under short-day regiment (18°C, 10 h light/ 14 h dark). Prior to phenotype analyses, graft unions were re-examined to ensure that no adventitious roots had grown from the shoot scion.

Stomatal aperture bioassays Seed was sown directly onto potting compost contained in 7cm diameter pots and vernalized for 2 days at 4°C. Plants were grown for 4 to 6 weeks under short-day growth conditions (18°C, 60% relative humidity, 10 h light/ 14 h dark, 100 µEm-2s-1). The youngest fully expanded leaves from 4-6 weeks old plants were excised in darkness one hour before the beginning of the light cycle, and epidermal peels were prepared immediately as follows: the abaxial epidermis of the leaf was fixed to a microscope slide using liquid medical adhesive B (VM 355-1, Ul-

rich Swiss), and the resulting epidermal peels were floated in petri dishes containing 50 mL of incubation buffer (KCl 30 mM MES-KOH 10 mM pH 6.5) for 30 min in darkness before being subjected to light (100 $\mu Em^{-2}s^{-1}$, 25°C) and chemical treatments.

For stomatal closure experiments, the epidermal peels were exposed to light for two hours prior to any further treatments in order to trigger stomatal opening. Either ABA, $CaCl_2$ or H_2O_2 was then added to the incubation buffer up to the indicated concentrations. Abscisic acid stock was prepared in ethanol.

Analysis of stomatal opening under blue light was conducted as follows: epidermal strips were prepared before the beginning of the light cycle, floated in incubation buffer and kept in darkness for two hours to promote stomatal closure. Strong red light (50 $\mu Em^{-2}s^{-1}$) was applied for one hour using a red Plexiglas filter (GS Rot 501, Röhm schweiz GmbH). Initial stomatal apertures were recorded before blue light (10 $\mu Em^{-2}s^{-1}$), from a second light source with a blue Plexiglas filter (GS Blau 612, Röhm schweiz GmbH), was superimposed onto the red light. Blue/red light treatment was continued for two hours after which final stomatal apertures were measured.

For the measurement of stomatal apertures in the dark and under IAA or fusicoccin treatments, epidermal peels were prepared as above. After initial stomatal apertures were recorded, epidermal strips were floated in darkness for an additional three hours in incubation buffer containing 1 mM Indole-3-acetic acid or 1µM fusicoccin (Sigma) before final stomatal apertures were measured. IAA stock solution (100 mM) was prepared in water and fusicoccin stock solution (1 mM) was prepared in ethanol.

Analysis of stomatal opening in CO_2-free conditions was conducted as follow: epidermal peels were floated in darkness in incubation buffer for two hours to ensure that stomata were fully closed. After initial stomatal apertures were recorded, the epidermal peels were incubated for two more hours in incubation buffer satured with

CO_2-free air (Synthetic mixture of 80% N_2 20% O_2, Carbagas) or in the control buffer saturated with atmospheric air before final stomatal apertures were measured.

All stomatal apertures were observed under an optical microscope before digital images were taken and subsequently used to measure aperture width in IMAGEJ (National Institutes of Health). Approximately 40 stomatal apertures were measured for each independent experiment, time point, treatment and genotype. The significance of aperture fold change differences (stomatal aperture at t = 2 h/ stomatal aperture at t = 0) between genotypes was assessed by non-coupled one-tailed Student's t-test analysis. Values of $P<0.05$ were considered statistically significant.

Detection of ROS production in guard cells ROS production in response to ABA treatments in guard cells was observed using 2,7- ddichlorofluorescein diacetate (DCFDA, Sigma) according to Zhang *et al.* (2009). Epidermal peels were prepared, floated in incubation buffer (KCl 30 mM MES-KOH 10 mM pH 6.5) and exposed to light for 2 h to induce stomatal opening. Epidermal peels were then placed in incubation buffer containing 50 µM DCFDA for 10 min, followed by 5min of washes. Digital images were taken (-ABA) before epidermal peels were exposed to ABA 50 µM for 3 min and digital images taken again (+ABA). Observation of fluorescence was performed at an excitation at 430-510 nm and emission at 475-575nm. Fluorescence intensity was quantified as mean pixel intensity using IMAGEJ.

5 - Guard-cell specific approach to study the role of *PHO1* in the stomatal response to ABA

Céline Zimmerli*, Hubert Bauer, Rainer Hedrich and Yves Poirier

*The experiments were designed, performed and data analyzed by Céline Zimmerli under the supervision of Yves Poirier. ABA treatments and enriched guard cells preparations of the of the transgenic lines p*GC1*::*PHO1*RNAi were performed in collaboration with Hubert Bauer, who performed the qRT-PCR experiments for ABA-responsive genes under the supervision of Rainer Hedrich. All other data were produced by Céline Zimmerli under the supervision of Yves Poirier.

Introduction

In the precedent chapters (*Chapter 3- Expression analysis of AtPHO1 in guard cells and in response to ABA,* and *Chapter 4 - Stomatal movement analysis of the mutant pho1)* we provided evidence that beside the known expression and role of *PHO1* in the root vascular cylinder (Poirier *et al.*, 1991; Hamburger *et al.*, 2002), *PHO1* is also expressed in leaf guard cells, and the mutant *pho1* is strongly impaired in ABA-induced stomatal movements. However, as mentioned before, *PHO1* is primarily responsible for the appropriate transfer of phosphate from roots to shoots. As a result, the *pho1* mutation drastically affects shoot Pi levels, triggering acute phosphate deficiency symptoms that potentially have numerous pleiotropic effect in the leaves. Phosphate starvation in plants initiates a myriad of transcriptional, biochemical, and physiological responses, and is known in particular to induce a rapid change in shoot carbohydrate metabolism (Hammond and White, 2008), which in turn could potentially affect stomatal movements. The use of micro-grafting techniques, i.e. the creation of Pi-repleted *pho1* by grafting a *pho1* shoot scion to a WT root stock, is an elegant approach to circumvent the potential effects of phosphate deficiency in *pho1* shoots (Stefanovic *et al.*, 2007; and page 70 of this manuscript). However, micro-grafting is a very fastidious technique requiring high precision, with a relatively low success rate that doesn't allow the production of a large number of plants. For example, the use of micro-grafted plants is not convenient to generate the amount of plants required for guard cell protoplasts purification for transcriptional analysis. It is also likely that the wounds induced by micro-grafting seedlings trigger a variety of stresses that could further along influence the overall stomatal response of the fully-grown micro-grafted plants. Finally, *PHO1* is also expressed at low levels in the mesophyll cells and the leaf vasculature, and it is therefore difficult in a whole *pho1* shoot to differentiate the

effect of the mutation in the guard cells from the one in other tissues.

A different approach is to use available methods for targeted gene expression in guard cells. Manipulating gene function specifically in guard cells offers advantages over manipulation at the whole plant level, for example by allowing to study the effect of gene knockdown in stomata without affecting gene function in the rest of the plant. In *Arabidopsis*, the *KAT1* promoter was widely used for driving guard cell specific gene expression, but expression was not sufficiently strong for high-level expression or repression, and in some cases expression was also seen in root vascular tissues and inflorescences. Using microarray analyses presenting ABA-induced and repressed genes in guard-cell and mesophyll-cell protoplasts, Yang *et al.* (2008) identified a promoter, p*GC1* (*At1g22690*), that drives strong and specific reporter gene expression in guard cells, with a relatively constant expression under most abiotic stresses. They demonstrated that p*GC1* can effectively be used for strong guard cell anti-sense gene repression, making it a powerful tool for manipulating gene function specifically in guard cells.

It was therefore of interest to investigate the effect of targeted guard cell *PHO1* expression using two complementary approaches, the anti-sense repression of *PHO1* in guard cells, and the targeted expression of *PHO1* in *pho1* guard cells. Guard cell specific knock-down of *PHO1* allows to investigate the role of *PHO1* in guard cell signal transduction, independently of plant phosphate deficiency. Conversely, the guard cell-specific complementation of *pho1* permits to investigate whether the stomatal phenotype of *pho1* can be rescued by the sole expression of *PHO1* in a phosphate-deficient background.

In this chapter, using the strong guard cell promoter p*GC1*, we investigate the effect of guard cell-specific down-regulation of *PHO1*, and of the guard cell-specific comple-

mentation of *pho1*, on the stomatal response to ABA. We show that RNAi-mediated repression of *PHO1* in guard cells reduces the stomatal movement response to ABA, during both induction of stomatal closure and repression of stomatal opening, without altering the transcriptional response of known ABA-responsive genes. Conversely, driving guard cell-specific expression of *PHO1* in the *pho1* mutant background partially restored stomatal movement responsiveness to ABA, although full complementation was co-dependent on shoot phosphate sufficiency. Finally, we show that modulation of *PHO1* expression in guard cells affects guard cells Pi content. Therefore, we conclude that both *PHO1* and phosphate sufficiency are required for normal stomatal movements in response to ABA, and that *PHO1* likely acts through the regulation of guard cell phosphate homeostasis.

Results

Down-regulation of *PHO1* in guard cells reduces stomatal movement responsiveness to ABA

In order to assess the importance of *PHO1* expression specifically in guard cells, we generated transgenic *A. thaliana* lines with guard-cell-specific *PHO1* expression knockdown mediated by RNAi. The strong guard-cell-specific promoter *pGC1*, described by Yang *et al.* (2008), was used to express inverted repeats of a *PHO1* gene-specific tag (*PHO1* gst) (figure 5.1a) in *Columbia* plants. We isolated two independent transgenic lines, referred to as p*GC1*::*PHO1*RNAi #6-6 and #10-3, with corresponding non-transgenic segregates (NTS) as controls. On the whole-plant level, there was no obvious morphological difference between p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their respective NTS, WT #6-6 and #10-3 (figure 5.1b).

In order to assess the effect of the RNAi construct, we performed qRT-PCR measurements on guard cells and mesophyll cell protoplasts of the p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their respective NTS lines. Quantification of *PHO1* transcripts revealed that guard-cell-specific *PHO1* expression was reduced in lines expressing p*GC1*::*PHO1*RNAi compared to the NTS lines, with a 70% and 78% reduction in *PHO1* transcript level observed for #6-6 and #10-3, respectively (figure 5.2a). Differential expression of the mesophyll cell marker gene *AT4G26530* and the guard cell marker gene *MYB60* attested the purity of the guard cell and mesophyll cell protoplast preparations. In addition, we assessed the specificity of the RNA interference by measuring the transcript levels of the *Arabidopsis PHO1* homologues expressed in guard cells (figure 5.2b): the presence of the p*GC1*::*PHO1*RNAi construct did not affect the expression levels of *PHO1;H1, PHO1;H2, PHO1;H3, PHO1;H5* and *PHO1;H10* in guard

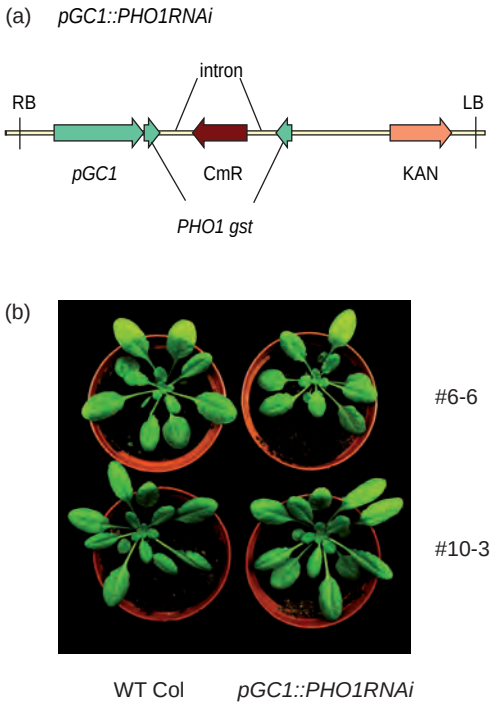


Figure 5.1: **Transgenic *Arabidopsis* lines expressing the guard-cell specific *PHO1* RNAi construction p*GC1*::*PHO1*RNAi.** (a) T-DNA region containing expression construct p*GC1*::*PHO1*RNAi consisting of a spacer region flanked by *PHO1*'s gene sequence tag (GST) sense and antisense sequences. Expression is driven by the guard cell-specific promoter *pGC1*. LB: left border, RB: right border, *KAN*: plant resistance gene to kanamycin. (b) Representative growth of two independent 6-week-old transgenic lines with *PHO1* expression knocked down in guard cells through RNAi (p*GC1*::*PHO1*RNAi #6-6 and #10-3) and their corresponding NTS (WT Col #6-6 and #10-3).

cells protoplasts. Together, these results show that the RNAi construction effectively and specifically down-regulate *PHO1* in guard cells.

Following confirmation of guard-cell-specific *PHO1* expression knockdown, p*GC1*::*PHO1*RNAi #6-6 and #10-3 were assessed for their stomatal responsiveness to ABA. ABA-induced repression of stomatal opening was seen affected by p*GC1*::*PHO1*RNAi expression. In darkness, p*GC1*::*PHO1*RNAi #6-6 and #10-3 and WT #6-6 and #10-3 possessed a similar average stomatal aperture (figure 5.3a, t0). However, following a 2-h light exposure in the presence of 10 µM ABA, repression of stomatal opening seen in WT #6-6 and #10-3 was not observed to the same extent in p*GC1*::*PHO1*RNAi #6-6 and #10-3 transgenic lines. Instead, p*GC1*::*PHO1*RNAi #6-6 and #10-3 consistently displayed wider stomatal apertures than their respective NTS (figure 5.3a, t = 2 h). ABA-induced stomatal closure was also compromised in p*GC1*::*PHO1*RNAi #6-6 and #10-3. Whilst average stomatal aperture was comparable between RNAi and WT lines in the dark prior to treatment (figure 5.3b, t0), after 2 h light exposure in the presence of 10 µM ABA, average aperture width was higher in p*GC1*::*PHO1*RNAi #6-6 and #10-3 compared to their respective NTS lines #6-6 and #10-3 (figure 5.3b, t = 2 h). Combined together, these results suggest that down-regulation of *PHO1* expression specifically in guard cells alters the ABA-mediated stomatal movement response.

Expression profiling of ABA-induced marker genes shows normal transcriptional response to ABA when *PHO1* is downregulated in guard cells

We investigated the effect of *PHO1* expression in guard cells on the transcriptional response of some ABA-responsive genes. Since phosphate deficiency in the mutant *pho1* induces major transcriptional changes, we chose to compare the transcript level responses to ABA in the phosphate-sufficient transgenic lines p*GC1*::*PHO1*RNAi #6-

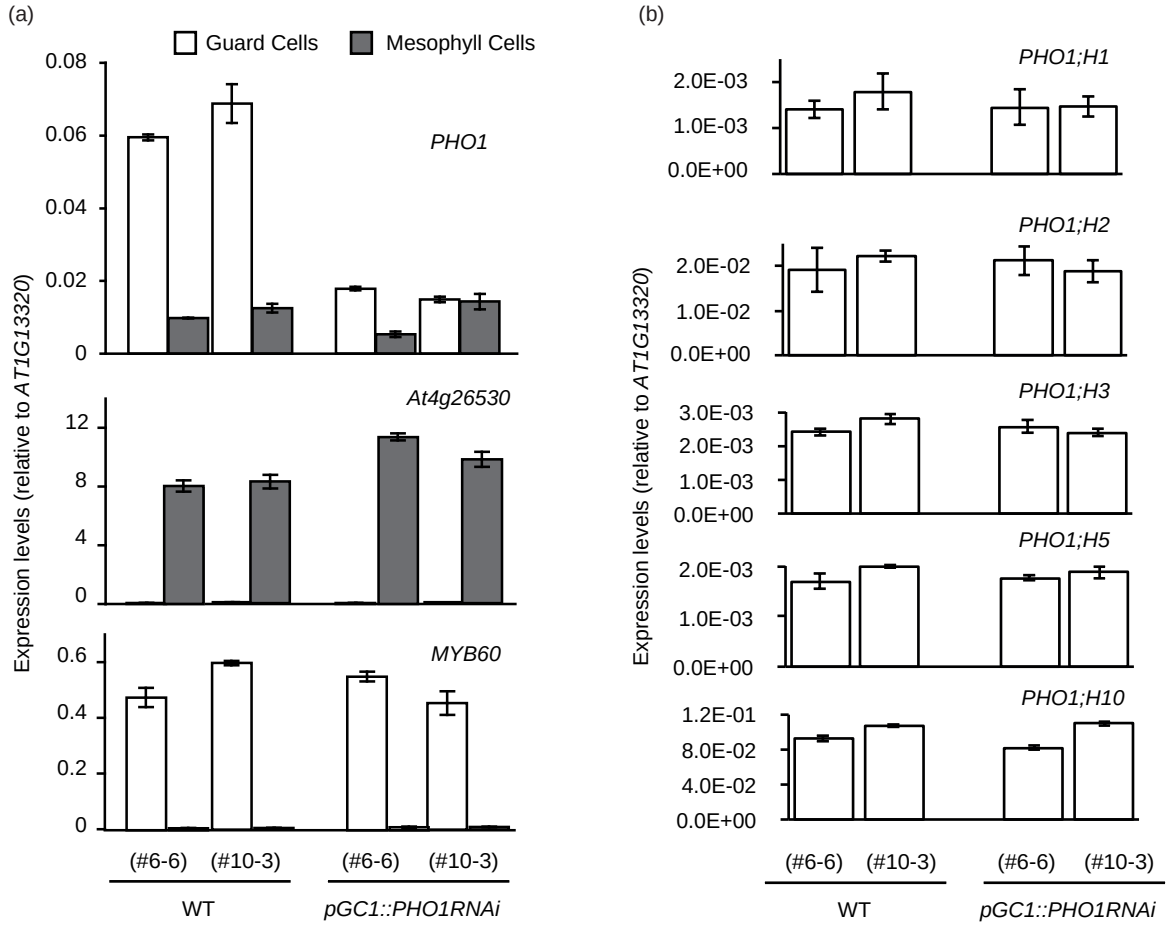


Figure 5.2: **Expression of *PHO1* is effectively reduced in the guard cells of the p*GC1*::*PHO1*RNAi transgenic lines, without affecting expression of other *PHO1* homologues.** (a) Variation in *PHO1* transcript level between guard cell and mesophyll cell preparations of transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding NTS (WT #6-6 and #10-3, respectively), normalized against expression levels of the reference gene *AT1G13320*. Transcripts of the mesophyll cell marker *AT4G26530*, and the guard cell marker *MYB60* were also quantified as controls. n = 3 biological replicates; average ± SE. (b) Transcript levels of *PHO1;H1*, *PHO1;H2*, *PHO1;H3*, *PHO1;H5* and *PHO1;H10* in guard cell protoplasts of transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding NTS (WT #6-6 and #10-3), normalized against expression levels of the reference gene *AT1G13320*. n = 3 biological replicates; average ± SE.

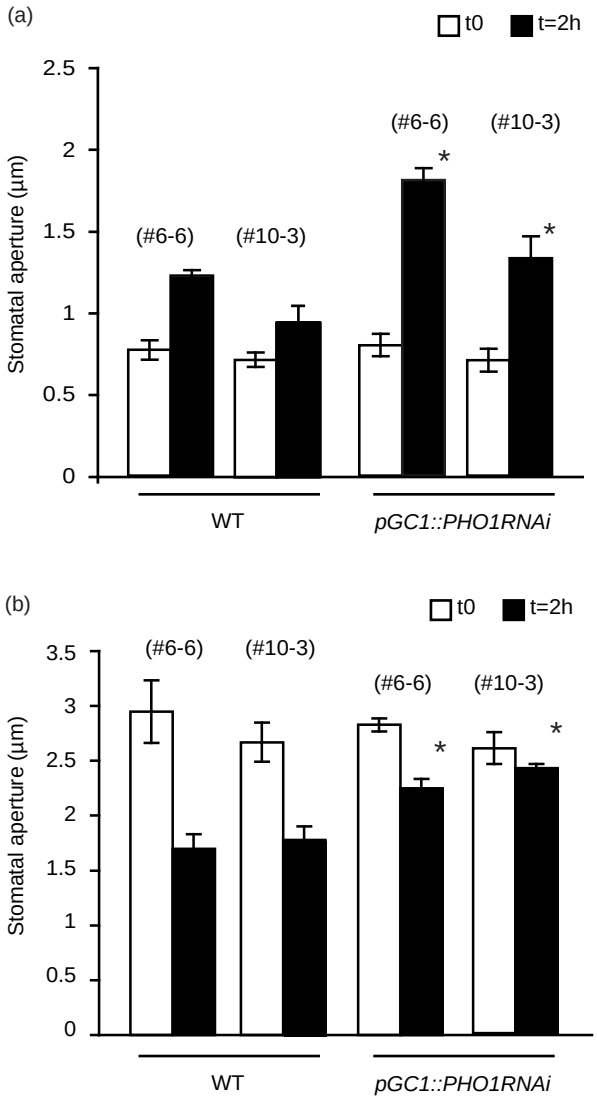


Figure 5.3: **Guard cell-specific expression knockdown of *PHO1* through RNAi reduces stomatal response to ABA.** (a) ABA repression of stomatal opening, as represented by and (b) induction of stomatal closure, in WT and the guard cells RNAi lines p*GC1*::*PHO1*RNAi, following treatment with 10 µM ABA, after 0 and 2 h. n = 3 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (WT) control values ($P < 0.05$).

6 and #10-3 and their corresponding NTS. One hour after the beginning of the light cycle, 6-week-old plants were sprayed with 50 μM ABA or control buffer and incubated under light for 4 h. RNA was then extracted from enriched guard cells preparations. We measured the following ABA-marker genes by qRT-PCR: *ABI1, ABI2*, *ABAR, HAI1, RD20*, *KIN2*, *LTP4*, *LEA6* (ABA-induction markers) and *MYB60* (ABA-repression marker). Overall, the expression levels changes induced by the ABA treatment were similar between the p*GC1*::*PHO1*RNAi lines when compared to their respective NTS (figure 5.4).

Guard-cell-specific complementation of *pho1.2* restores ABA-induced repression of stomatal opening, but long term phosphate sufficiency is required for stomatal closure

In parallel to guard-cell-specific *PHO1* expression knockdown, we assessed whether a WT stomatal phenotype was possible in *pho1.2* through guard-cell-specific complementation. For this, *pho1.2* was transformed with the full-length genomic sequence of *PHO1* (*PHO1g*) with expression governed by the guard-cell-specific promoter p*GC1* (figure 5.5a). Two independent transgenic lines were isolated, referred to as p*GC1*::*PHO1g* #10-1 and #12-1, along with their respective NTS as controls. Similar to what was seen with guard-cell-specific *PHO1* expression knockdown, at the whole-plant level, p*GC1*::*PHO1g* #10-1 and #12-1 displayed a comparable morphology to their NTS counterparts, *pho1.2* #10-1 and #12-1 (figure 5.5b).

qRT-PCR was used to verify *PHO1*g expression in guard cell preparations of p*GC1*::*PHO1g* #10-1 and #12-1 (figure 5.6). Almost no *PHO1*g transcript was detected in mesophyll cell preparations of the same transgenic lines, indicating guard-cell-specific *PHO1* complementation. Again, differential expression of marker genes *AT4G26530* and

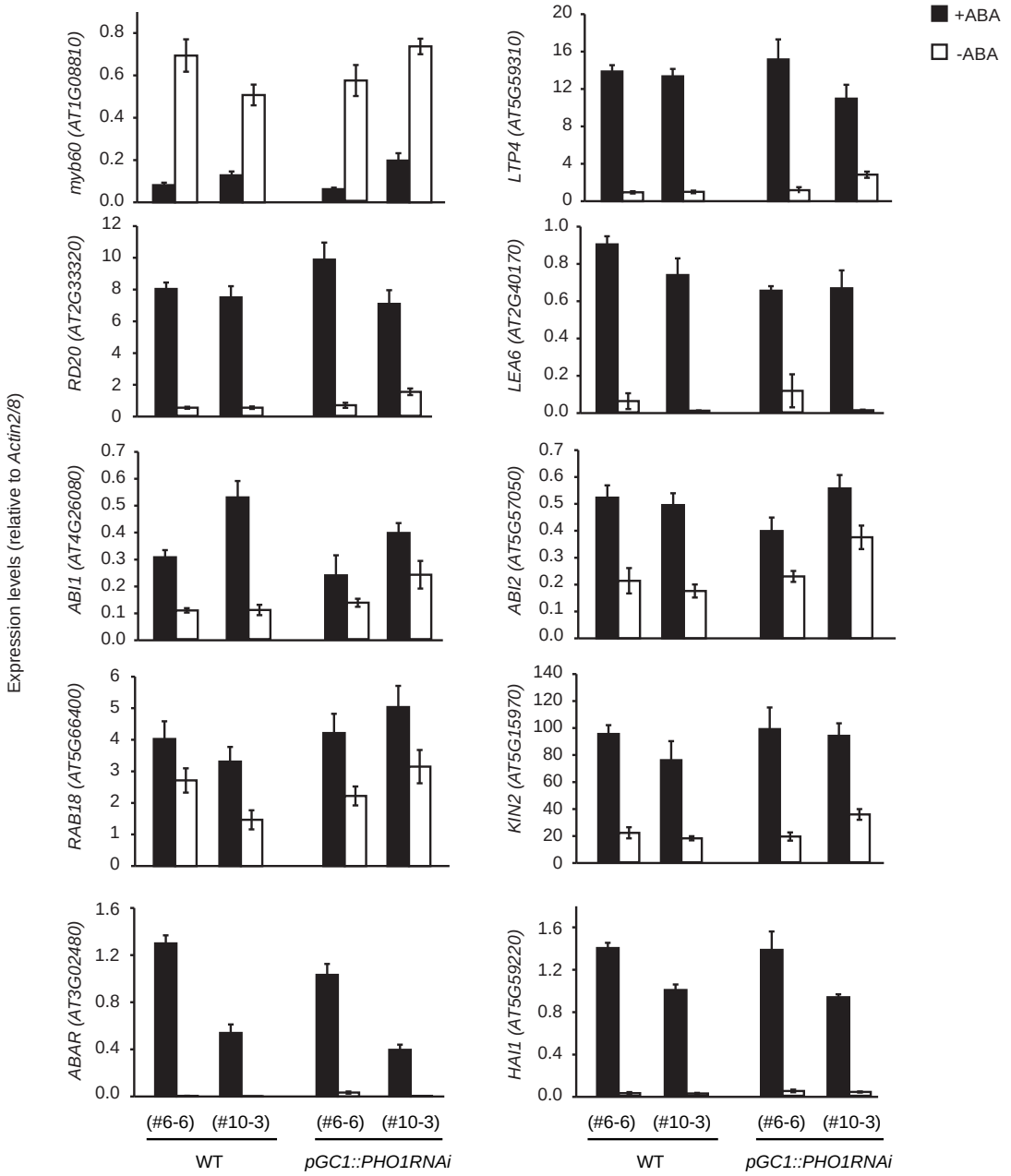


Figure 5.4: ***PHO1* down-expression in guard cells does not affect the transcriptional response of marker genes to ABA.** Transcript levels of known ABA-responsive marker genes (*ABI1, ABI2*, *ABAR, HAI1, RD20*, *KIN2*, *LTP4*, *LEA6*) in enriched guard cells preparations of the transgenic lines p*GC1*::*PHO1*RNAi #6-6 and #10-3 and their corresponding NTS (WT #6-6 and #10-3), following a 4-h treatment with or without 50 μM ABA, normalized against the mean expression levels of the reference genes Actin 2 and 8. n = 4 biological replicates; average ± SE.

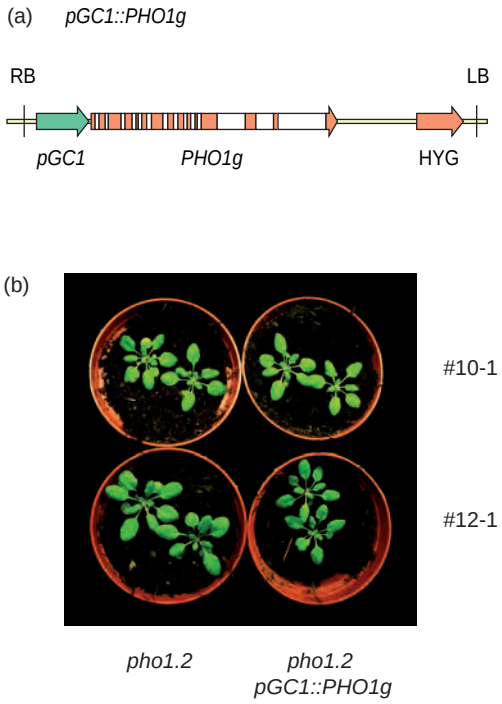


Figure 5.5: **Transgenic *pho1.2* lines expressing the guard-cell specific complementation construction p*GC1*::*PHO1*g.** (a) T-DNA region containing expression construct, p*GC1*::*PHO1*g, consisting of *PHO1* genomic sequence (*PHO1g*) under the control of the guard cell promoter p*GC1*. LB: left border, RB: right border, *HYG*: plant resistance gene to hygromycin. (b) Representative growth of two independent 6-week-old *pho1.2* transgenic lines with *PHO1* complementation in guard cells (*pho1.2* p*GC1*::*PHO1g* #10-1 and #12-1) and their corresponding NTS (*pho1.2* #10-1 and #12-1).

MYB60 attested the purity of the guard cell and mesophyll cell protoplast preparations.

We determined the stomatal responsiveness to ABA in the p*GC1*::*PHO1*g lines compared to their NTS controls with stomatal aperture assays as described above. ABA-induced repression of stomatal opening was markedly improved in guard cell-complemented p*GC1*::*PHO1*g #10-1 and #12-1 lines (figure 5.7a). Similar to the earlier observations in *pho1.2* (see *Chapter 4 - Stomatal movement analysis of the mutant pho1*), wider stomatal apertures were apparent in *pho1.2* NTS controls following a 2-h light exposure in the presence 10 µM ABA (figure 5.7a, t = 2 h). However, under the same conditions, stomatal apertures in the p*GC1*::*PHO1*g lines were narrower (figure 5.7a, t = 2 h), indicating that the stomata were more responsive to ABA-repression of stomatal opening in these lines.

Surprisingly, ABA-induced stomatal closure was not restored following guard cell-specific *PHO1* complementation. Stomatal aperture width was maintained in the complemented p*GC1*::*PHO1*g lines and their corresponding *pho1.2* NTS following 2-h light treatment in the presence of 10 µM ABA (figure 5.7b, t = 2 h), indicating that stomata failed to close in response to ABA treatment in all lines. It is thus apparent that guard cell-specific *PHO1* complementation is sufficient to restore ABA-induced repression of stomatal opening, but is not sufficient to restore ABA-induced stomatal closure.

We postulated then that the inability of guard cell-specific *PHO1* complementation to restore ABA-induced stomatal closure was due to phosphate unavailability to the guard cells or to the pleiotropic consequences of shoot phosphate deficiency. To assess this, phosphate was provided first through incubation of the epidermal peels in potassium phosphate buffer (30 mM KH_2PO4, 10 mM MES, pH 6.5) instead of

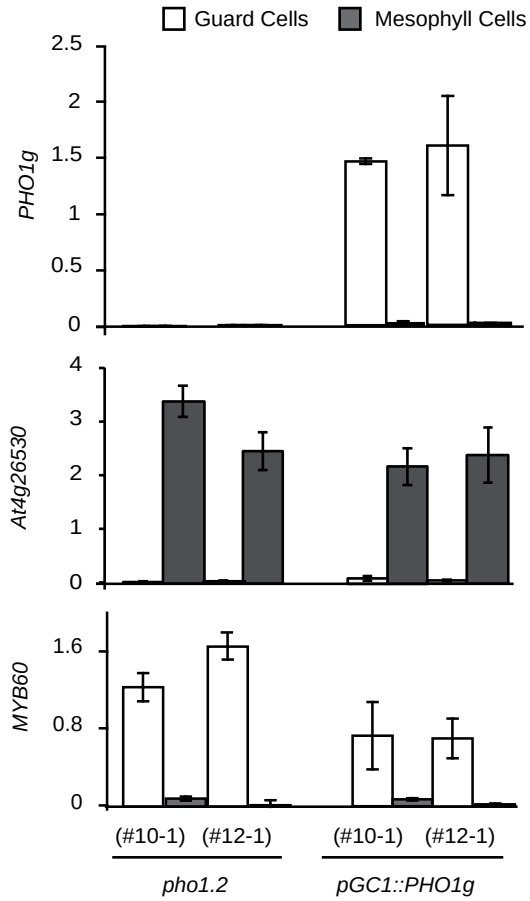


Figure 5.6: **The transgenic *pho1.2* complemented lines express effectively *PHO1g* in guard cells.** Guard cell and mesophyll cell transcript levels of the transgene *PHO1g*, in the guard cell complemented lines *pho1.2* p*GC1*::*PHO1g* #10-1 and #12-1, and their corresponding NTS (*pho1.2* #10-1 and #12-1), normalized against expression levels of the reference gene *AT1G13320*. Primers were chosen to hybridize to *PHO1*'s second last exon and to a sequence specific to pMDC32. Transcript levels of the mesophyll cell marker *AT4G26530* and the guard cell marker *MYB60* were also quantified as controls. n = 3 biological replicates; average ± SE.

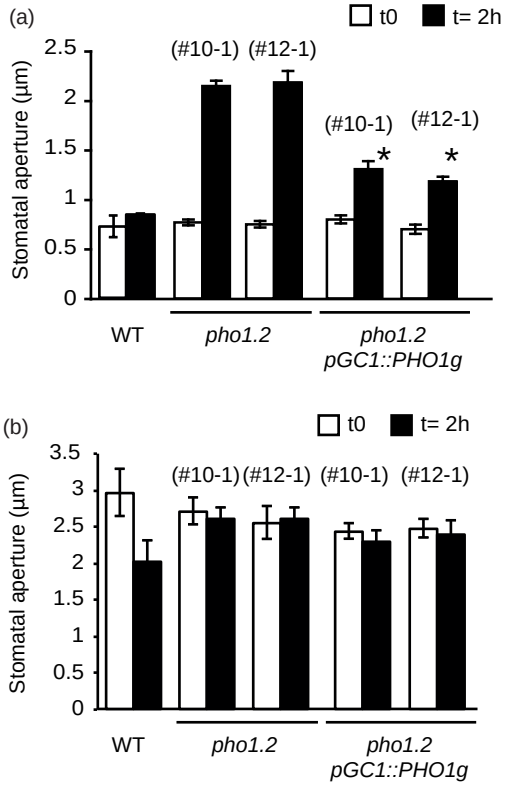


Figure 5.7: **Guard cell-specific complementation of *pho1.2* partially restores stomata responsiveness to ABA** (a) ABA repression of stomatal opening and (b) induction of stomatal closure, in the guard cell-complemented lines *pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1, and their corresponding NTS (*pho1.2* #10-1 and #12-1), in response to 10 µM ABA, after 0 and 2 h. $n > 3$ independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (*pho1.2*) control values ($P < 0.05$).

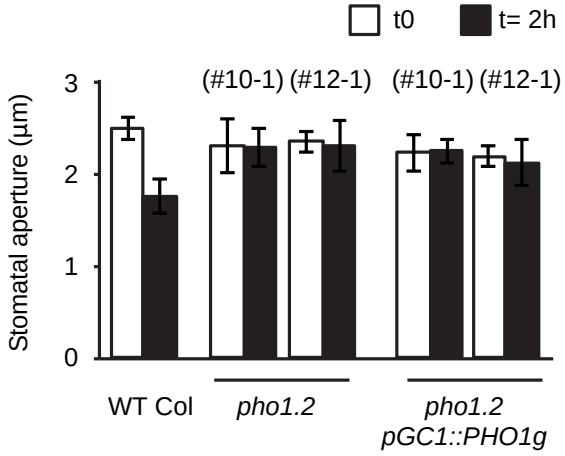


Figure 5.8: **Supplying phosphate to the epidermal peels is not sufficient to restore stomatal closure response to ABA in the guard cell complemented lines.** Induction of stomatal closure under light in WT, the *pho1.2* guard cell-complemented lines *pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1, and their corresponding NTS (*pho1.2* #10-1 and #12-1), after 0 and 2 h of treatment with 10 µM ABA in potassium phosphate buffer composed of 30 mM KH_2PO_4, 10 mM MES, pH 6.5. n = 3 independent experiments; average ± SE. Stomatal aperture fold changes in the *pho1.2* p*GC1*::*PHO1*g lines was not statistically different from the corresponding NTS (*pho1.2*) control values (P >0.05).

potassium chloride. After a 2-h incubation under light, 10 µM ABA was added to the buffer. While stomata of WT plants responded to the ABA treatment, the use of potassium phosphate did not improve the stomatal closure response of both the guard cell-specific PHO1-complemented *pho1.2* and their respective NTS (figure 5.8).

To alleviate effects of long-term shoot Pi deficiency, shoot phosphate sufficiency was then restored in each transgenic line by creating chimeric plants composed of *a* p*GC1*::*PHO1*g *pho1.2* scion micro-grafted onto WT rootstock (p*GC1*::*PHO1*g #10-1 and #12-1/WT). Chimeric plants composed of NTS scion micro-grafted onto WT rootstock (*pho1.2* #10-1 and #12-1/WT) were generated for comparison. Self-grafted plants composed of WT scion and WT rootstock (WT/WT) were used again as a control. Following a 2-h incu-

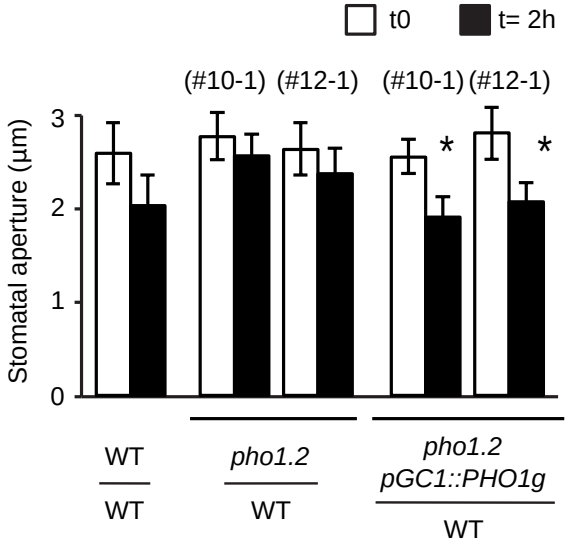


Figure 5.9: **Complete responsiveness of guard cell complemented *pho1.2* is co-dependent on shoot Pi sufficiency during plant development.** Induction of stomatal closure under light following treatment with 10 µM ABA, after 0 and 2 h, on phosphate sufficient micro-grafts composed of either WT, *pho1.2* NTS, or *pho1.2* p*GC1*::*PHO1*g scion micro-grafted onto WT rootstock (WT/WT, *pho1.2*/WT and *pho1.2* p*GC1*::*PHO1*g/WT, respectively). n = 5 independent experiments; average ± SE. Asterisks above the columns indicate stomatal aperture fold changes that are statistically different from the corresponding NTS (*pho1.2*) control values ($P < 0.05$).

bation with 10 µM ABA, stomatal closure was assessed in all micro-grafted plants (figure 5.9). In the *pho1.2* NTS controls, stomata in *pho1.2* NTS/WT micro-grafts failed to close in response to ABA treatment. However, in contrast to what was observed for p*GC1*::*PHO1*g lines, stomata in p*GC1*::*PHO1*g/WT micro-grafts were responsive to ABA treatment and a reduction in stomatal aperture width was seen. This indicates that a restoration of ABA-induced stomatal closure in *pho1* is dependent on both guard cell-specific *PHO1* expression and shoot phosphate sufficiency during plant development.

PHO1 expression in guard cells influences Pi accumulation in guard cells

Induction of *PHO1* in leaf mesophyll protoplasts is known to trigger Pi efflux from cells (Arpat *et al.*, 2012). We were therefore interested in investigating how *PHO1* expression in guard cells can alter their Pi content. Purified guard cells were prepared using epidermis isolated through leaf tissue blending, which was subsequently partially digested using a dialyzed cellulase. We isolated purified guard cell preparations from the guard cell-complemented transgenic lines *pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1, and their corresponding NTS lines, and in parallel from the p*GC1*::*PHO1*RNAi #6-6 and #10-3 transgenic RNAi lines and their corresponding NTS lines. Plants were kept in the dark to keep stomata closed before and during sampling.

Overall, guard cell Pi content was found to be lower in the *pho1.2* lines than in the *Columbia* lines (figure 5.10). Interestingly, one of the guard cell-complemented transgenic line *(pho1.2* p*GC1*::*PHO1*g #10-1) displayed a significant lower Pi content than its NTS counterparts. Conversely, both p*GC1*::*PHO1*RNAi lines presented significant higher guard cell Pi contents than their respective WT NTS. Together, these results show that *PHO1* expression in guard cells has an effect on guard cell Pi homeostasis.

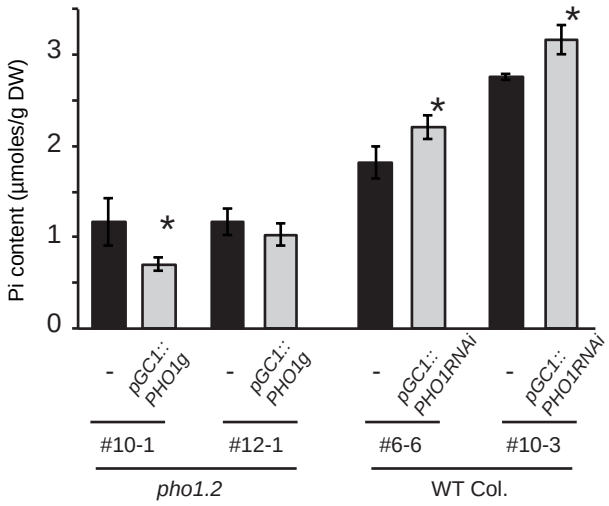


Figure 5.10: ***PHO1* expression influence Pi accumulation in guard cells.** Pi content in µmoles per grams of dry weight (DW) of purified guard cells preparations, in the guard cell-complemented transgenic lines *pho1.2* p*GC1*::*PHO1*g #10-1 and #12-1, and their corresponding NTS (-) #10-1 and #12-1, and in the p*GC1*::*PHO1*RNAi #6-6 and #10-3 transgenic lines and their corresponding NTS (-) #6-6 and #10-3. n=3 biological replicates; average ± SE. Asterisks above the columns indicate a value statistically different from the corresponding NTS control value ($P < 0.10$).

Discussion

In this chapter, we used a guard-cell-targeted gene expression approach to investigate the importance of the specific expression of *PHO1* in guard cells on the mediation of the stomatal response to ABA. We used two complementary approaches: the repression of *PHO1* in guard cells using RNAi, and the targeted expression of *PHO1* in *pho1* guard cells.

The expression of an RNAi construct consisting of inverted repeats of a *PHO1* gene-specific tag under the control of p*GC1* allowed a strong reduction of *PHO1* transcripts levels in *Columbia* guard cells, without affecting the levels of other *PHO1* homologues (figure 5.1 and 5.2). Down-regulation of *PHO1* in stomata did not trigger any morphological or developmental changes, but correlated with a dampening of the stomatal movements response to ABA, during both the induction of stomatal closure and the repression of stomatal opening under light (figure 5.3). This result confirms that the specific expression of *PHO1* in stomata directly plays a role in guard cell ABA signaling.

Part of ABA signaling is modulated by transcriptional and post-transcriptional control. Although it is generally assumed that gene expression modulation is a late event in the signal transduction cascade, it is likely that over the period of plant growth, RNA metabolism and membrane transport regulations become tightly integrated to regulate the guard cell movement response (Sirichandra *et al.*, 2009*a*). Several examples of mutations affecting transcription factors activity or mRNA processing have been shown to directly alter the guard cell response to ABA (Nilson and Assmann, 2007). It was therefore of interest to decipher whether the compromised stomatal response of the guard cells *PHO1*RNAi lines was linked to an alteration of the tran-

scriptional response to ABA. Interestingly, transcript levels measurements of a few ABA-markers showed that expression levels changes induced by ABA were similar between the p*GC1*::*PHO1*RNAi lines and their respective wild type (figure 5.4). This finding suggests that *PHO1* down-regulation in guard cells does not affect the global transcriptional response of ABA-induced and -repressed marker genes and that *PHO1* presumably does not act through the regulation of the ABA-activated transcriptional response. It also further confirms that *PHO1* is not involved in ABA perception and early signaling events, but is involved downstream in the signaling cascade.

We then investigated the effect of targeted *PHO1* expression in the guard cells of the mutant *pho1*, in order to test whether *pho1* stomatal phenotype could be rescued by the sole expression of *PHO1* in guard cells while maintaining a phosphate-deficient background. The p*GC1* promoter was originally isolated upstream of one the most highly expressed genes in guard cells (Yang *et al.*, 2008). As a consequence, driving *PHO1* expression under the control of p*GC1* resulted in a strong guard cell specific over-expression, with *PHO1* transcript levels more than 20 times more abundant than wild type levels (figure 5.6 compared to *PHO1* levels in figure 3.4 on page 44). The guard cell-complemented transgenic lines *pho1.2* p*GC1*::*PHO1*g presented an clear improvement of the ABA response during repression of stomatal opening under light (figure 5.7a). But surprisingly, the induction of stomatal closure by ABA was still inhibited in these lines (figure 5.7b). Incubation of the epidermal peels in phosphate buffer was not sufficient to restore the induction of stomatal closure, suggesting that the inhibition is not simply due to the lack of available phosphate anions (figure 5.8). However, using micro-grafting to restore Pi sufficiency in leaves of these lines enabled the ABA induction of stomatal closure, indicating that the phosphate starvation response inhibited this stomatal response (figure 5.9). Therefore, while ABA inhibition

of stomatal opening is dependent on guard cell *PHO1* expression and independent of the leaf Pi status, ABA induction of stomata closure is dependent on both leaf phosphate sufficiency and guard cell expression of *PHO1*. It is understood that the phosphate starvation response induces profound changes in plants, in particular a rapid modification of shoot carbohydrate metabolism. Low Pi availability triggers restricted carbon assimilation and accumulation of sugars in the leaves; genes involved in starch biosynthesis and degradation as well as in carbohydrate transport, show altered patterns of expression (Hammond and White, 2008). In the *pho1* guard cell complemented lines, it is likely that phosphate deprivation alters the carbohydrate metabolism during light-induced stomatal opening, triggering an accumulation of osmotically active sugars that could potentially counteract stomatal closure following ABA application. In contrast, at the end of the night, closed stomata assumably do not present this accumulation of sugars, a difference that could account for the normal inhibition of stomatal opening by ABA as observed in the *pho1* guard cell complemented lines.

As a note, while expression of p*GC1*::*PHO1*g in *pho1.2* stomata resulted in a large *PHO1* over-expression in guard cells, stomatal responsiveness to ABA was restored but did not be appear to be particularly exacerbated in comparison to the wild-type response (figure 5.7a and 5.9). While we did not measure PHO1 protein levels in the guard cells of *pho1.2* p*GC1*::*PHO1*g lines, recent reports suggest that in some conditions, *PHO1* over-expression can trigger higher PHO1 protein level without an increase in Pi export activity (Stefanovic *et al.*, 2011; Liu *et al.*, 2012). Both these studies reported that *PHO1* over-expression in wild-type roots results in higher level of PHO1, but triggers only a moderate increase of Pi translocation to the shoots, which suggests the presence in roots of posttranslational control of PHO1 activity. Indeed,

Liu *et al.* (2012) found that PHO1 protein levels are regulated at the post-translational level by PHO2, a ubiquitin-conjugating E2 enzyme, and they mentioned the possible presence of additional factors, regulated by PHO2, that could modulate the activity of PHO1. In guard cell, PHO2 appears to be expressed at high levels in guard cells compared to mesophyll cells (267456_at, according to microarrays data from Yang *et al.*, 2008), which suggests that such posttranslational control of PHO1 activity by PHO2 could theoretically take place in stomata.

Stomatal opening and closing are driven by ion fluxes, which are responsible for changes in guard cell turgor and volume. Therefore guard cells accumulate ions, and mutants affected in ion transport can display changes in guard cell ion composition. As examples, it was shown that mutation in *SLAC1* (*SLOW ANION CHANNEL- ASSOCIATED 1*) was accompanied by an over-accumulation of organic/inorganic anions in guard cell protoplasts (Negi *et al.*, 2008), and that *chl1* mutants showed reduced nitrate accumulation in guard cells during stomatal opening (Guo *et al.*, 2003). Recent reports point for a role of PHO1 in Pi efflux from cells (Stefanovic *et al.*, 2011; Arpat *et al.*, 2012). It was therefore of interest to investigate the potential influence of *PHO1* on guard cell inorganic phosphate accumulation. Initial attempts to quantify Pi concentration in guard cell protoplasts were unsuccessful because protoplasting enzyme cocktails provided large amounts of phosphate contamination, and therefore required extensive dialysis that greatly affected their effectiveness. We thus performed epidermis purification following the “blender method”, which yields relatively pure enriched guard cell preparations (Geiger *et al.*, 2011), combined with a partial digestion to remove the remaining epidermal and mesophyll cells, using a dialyzed cellulase. This method allowed isolation of sufficient amounts of enriched guard cell material for phosphate content assays. The *pho1* lines presented overall lower guard cell Pi con-

tent than the WT lines, presumably due to *pho1* intrinsic shoot phosphate deficiency that does not provide sufficient amount of phosphate for proper accumulation in leaf cell vacuoles (Rouached *et al.*, 2011). Interestingly, down-regulation of *PHO1* in the RNAi lines correlated with a slightly higher accumulation of Pi in guard cells, while complementation of *pho1* was associated with a slight reduction in guard cell Pi content (figure 5.10). These results suggest that *PHO1* regulates guard cell homeostasis on some level, and are consistent with a potential role for *PHO1* in mediating Pi efflux in guard cells.

Building on the results of this chapter, we conclude that the specific expression of *PHO1* in guard cells mediates of the stomatal movement response to ABA, in a signaling event that does not affect the transcriptional response to ABA, but likely involves modulation of phosphate homeostasis. Further investigations will be required to understand the mechanisms behind *PHO1* regulation of guard cell phosphate homeostasis, and how *PHO1* and phosphate influence together the guard cell response to ABA. This point will be further discussed in the next chapter (*Chapter 6 - Conclusion and perspectives*).

Experimental procedures

Plant material and growth conditions *Arabidopsis thaliana* wild type and mutants plants were from the Columbia ecotype (wild type Col). The mutants *pho1.2* has been previously been described by Poirier *et al.* (1991), Delhaize and Randall (1995) and Hamburger *et al.* (2002). Seed was sown directly onto potting compost contained in 7cm diameter pots and vernalized for 2 days at 4°C. Plants were grown for 4 to 6 weeks under short-day growth conditions (18 °C, 60 % relative humidity, 10 h light/ 14 h dark, 100µE$m^{-2}s^{-1}$).

Guard-cell-specific *PHO1* RNAi A 203 bp region localized in the 3'UTR of *PHO1* (gene sequence tag GST CATMA3c57344, Sclep *et al.*, 2007) was PCR amplified using primers *PHO1gst-F* and *PHO1gst-R* (5'-agg gac tca gac ggt taa aca aag-3' and 5'-gag cgt tta aca gtt gta gaa tcca-3'). This fragment, flanked by *att*B sites, was inserted between *att*P sites of the entry vector pDONR201 and subsequently cloned by GATEWAY LR recombination into pB7GWIWG2(II) RNAi binary destination vector (Karimi *et al.*, 2002). In parallel, the 1.2 kb guard cell promoter p*GC1* of *At1g22690* (Yang *et al.*, 2008) was PCR amplified using primers *pGC1-F1* and *pGC1-R1* (5'-CTCGAG tag tga ttt tga agt agt gtg-3' and 5'-GAGCTC GGGCCC GCGGCCGC atg gtt gca aca gag agg atg aatt-3', restriction sites in capitals). The CaMV 35S cassette was excised from vector pART7 (Gleave, 1992) with SacI/XhoI double digestion and replaced with p*GC1*. The *PHO1* GST sense/intron/antisense construct in pB7GWIWG2(II) (*PHO1*RNAi) was PCR amplified with flanking XhoI sites and inserted into the XhoI site of pART7, creating pART7-p*GC1*::*PHO1*RNAi. The final p*GC1*::*PHO1*RNAi::ocs3' expression cassette was excised with Bsp120I and inserted into the NotI site of binary vector pGreenII-KAN (kanamycin selective marker in plants, Hellens *et al.*,

2000). This binary vector was used to transform the *Agrobacterium tumefaciens* strain (GV3101) already containing the helper plasmid pSOUP. The resulting *A. tumefaciens* strain was used to transform *A. thaliana* (Columbia) by the floral dip method according to Clough and Bent (1998). Transformants were selected on half-strength MS containing 40 µg/mL kanamycin (Sigma) and 1% w/v sucrose, according to Harrison *et al.* (2006). Independent T1 transformants were verified by PCR using primers specific to pGC1::*PHO1*RNAi::ocs3', and an appropriate 3:1 resistance to sensitivity segregation ratio was confirmed in the T2 generation. A number of homozygous transgenic lines were indentified in the T3 generation and, based on reduced *PHO1* mRNA levels in guard cell protoplasts as detected by real-time quantitative RT-PCR (qRT-PCR), two were selected for future stomatal assays (p*GC1*::*PHO1*RNAi #6-6 and #10-3). Non-transformed segregates (NTS), sharing the same T1 parental plant, were isolated simultaneously for both transgenic lines (WT #6-6 and #10-3).

Guard-cell-specific complementation of *pho1.2* The 1.2 kb guard cell promoter p*GC1* of *At1g22690* (Yang *et al.*, 2008) was PCR amplified using primers *pGC1-F2* and *pGC1-R2* (5'-GGCGCGCC ttc ttga gta gtg att ttg aag tag tgtg-3' and 5'-AAGCTT atg gtt gca aca gag agg atg aatt-3', restriction sites in capitals). The CaMV 35S cassette within vector pMDC32 (Curtis and Grossniklaus, 2003) was excised with HindII/AscI double digestion and replaced with p*GC1*. The full length *PHO1* genomic sequence (*PHO1g*, 5.4 kb) was PCR amplified using primers *PHO1g-F* and *PHO1g-R* (5'-atg gtg aag ttc tcg aag gag ctag-3' and 5'-acc gtc tga gtc cct gtc aag gaac-3'), flanked with *att*B sites, inserted between *att*P sites of the entry vector pDONR201 and cloned by GATEWAY LR recombination into binary vector p*GC1*-pMDC32. As described above, this binary vector was introduced into the *A. tumefaciens* strain GV3101 which was

then used to transform *A. thaliana pho1.2* as per the floral dip method. Transformants were selected on half-strength MS medium containing 60 µg/mL hygromycin (Sigma) and 1% w/v sucrose, according to Harrison *et al.* (2006). Again, independent T1 transformants were verified by PCR and an appropriate 3:1 segregation ratio was confirmed in the T2 generation. Homozygous transgenic lines *pho1.2* p*GC1*::*PHO1g* #10-1 and #12-1 and their NTS counterparts *pho1.2* #10-1 and #12-1 were indentified in the T3 generation. These transgenic lines were selected for future stomatal assays based on *PHO1g* mRNA expression in guard cell protoplasts as verified by qRT-PCR analysis.

Guard cell and mesophyll cell protoplast preparation Guard cell and mesophyll cell protoplasts were isolated according to Pandey *et al.* (2002). For guard cell protoplasts, mature leaves from 12-24 five-week-old plants (main vein discarded) were blended in cold distilled water for 2 min using a small Waring blender on high speed. Processed tissue was passed through a 100 µm nylon mesh to isolate epidermal fragments and rinsed with water until clear. Epidermal fragments were then submerged in a digestion mixture containing 0.7-1.4 % Cellulysin Cellulase *Trichoderma viride* (Calbiochem), 0.1 % PVP40 (Sigma), 0.25 % BSA (Sigma), 13.75 mL basic medium (104 g/L D-sorbitol (Sigma), 0.5 mM ascorbic acid (Sigma), 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM MES, pH 5.5 with KOH) and 11.25 mL distilled water. Digestions were incubated for 3-4 hours at 27 °C incubated in a shaking water bath in the dark. The extent of cellular digestion was monitored microscopically until completion, upon which digests were passed through a 50 µm nylon mesh and rinsed with basic medium. Isolated epidermal cells were transferred to a second digestion mixture containing 1.5 % Cellulase RS onozuka (Yakult pharmaceuticals, Japan), 0.03 % Pectolyase Y23 (Inter-

chim, France), 0.25 % BSA (Sigma) and 25 mL basic medium. These secondary digestions were incubated slowly shaking for 2 hours at 17 °C and the formation of guard cell protoplasts was monitored microscopically until completion. Digests were passed through a 10 µm nylon mesh and isolated protoplasts were collected in Falcon tubes. Triplicate washes were performed where protoplasts were pelleted at 350 g for 15 min in a swing rotor centrifuge and resuspended in basic medium. The purity of guard cell protoplasts in the final pellet was then verified using a hemacytometer. For mesophyll cell protoplasts, mature leaves from five-week-old plants (main vein discarded) were cut into 1mm strips and submerged directly into a digestion mixture containing 1 % Cellulase R-10 (Serva), 0.4 % Macerozyme R-10 (Serva), 5mM MES, 0.2 % BSA, 0.1 % PVP40 and 25 mL medium (118.9 g/L D-sorbitol, 1 mM $CaCl_2$). Digestions were incubated slowly shaking for two hours in the dark at room temperature. Digests were passed through a 25 µm nylon mesh and the resulting medium containing protoplasts were collected in Falcon tubes. Triplicate washes were performed where protoplasts were pelleted at 200 g for 15 min in a swing rotor centrifuge and resuspended in medium. The purity of mesophyl cell protoplasts in the final pellet was then verified using a hemacytometer.

qRT-PCR on protoplasts Protoplasts were collected by duplicate 15 min centrifugations at 350 g in 1.5 ml tubes and all but 100 µL of supernatant was removed from the pellet. Total RNA was extracted using 1 mL TRIZOL reagent (Invitrogen, www.invitrogen.com) following manufacturer's instructions. Glycogen (Roche, www.roche.com) was used as an RNA carrier. Total RNA was treated with DNase (Qiagen, www.qiagen.com), and purified using a RNAeasy Minelute Cleanup Kit (Qiagen). Reverse transcription was performed using M-MLV reverse transcriptase (Promega,

www.promega.com). qRT-PCR analysis was performed using SyberGreen mix and the reference dye ROX (ABgene, www.abgene.com/) in a Stratagene MPx3000 instrument. Default thermal cycle settings with dissociation step were used. All amplification plots were analyzed using MxPro QPCR software (Stratagene, www.genomics.agilent.com/) with an Rn threshold of 0.1 to obtain Ct values. Transcript abundance was estimated as per the standard curve method (Rutledge and Cote, 2003) observing default settings. Transcript abundance of target genes was normalized against expression of the reference gene *AT1G13320* (Czechowski *et al.*, 2005). The mesophyll-cell-specific gene *At4G26530* was chosen according to microarray data described by Yang *et al.* (2008). Primers used for qRT-PCT are the following: *AT1G13320* (5'-taa cgt ggc caa aat gat gc-3' and 5'-gtt ctc cac aac cgc ttg gt-3'); MYB60 (5'-tca ctt gca agc ctt att ggg t-3' and 5'-tct gct cct ctc atc act gtc a-3'); *At4G26530* (5'-agg cct tga acg acc acc atg tc-3' and 5'-acc tgc agg tgg gac tgt gcg-3'); *PHO1;H1* (5'-tga aac gag cca cct agt gaa-3' and 5'-tgt tcc agc ggc taa cat agc-3'); *PHO1;H2* (5'-gcg tgg ttg caa acg ata ctg-3' and 5'-caa gcg ttg tct gct tgt gc-3'); *PHO1;H3* (5'-cag aca agt cct gct tgt tgg-3' and 5'-ggc aag aac aca aag caa tgc-3'); *PHO1;H5* (5'-cct tca tgc aca gac aga cga-3' and 5'-cca cgg cgg ata atc tct aga-3'); *PHO1;10* (5'-tcc tcc agt gca tac gca gat-3' and 5'-aac gca ttg tat ccg tgt acg-3'); to quantify *PHO1* transcript levels in RNAi lines, primers spanning *PHO1* GST were chosen (5'-acc gta ccg tta ccg ttc ctt ga-3' and 5'ctt cgt ttt gca ctt tgg agc gt-3'). Primers designed to produce an amplicon of similar size were used for the reference gene *AT1G13320* (5'-tgc tga aga cag gca ctg gag ag-3' and 5'-tgc tgc att gcc cat tca gga cc-3'); to quantify expression of the *PHO1* transgene in the guard cell complemented *pho1.2* lines, primers were selected that spanned sequence from the second last exon of *PHO1* to the 3'UTR of the pMDC32 vector backbone (*PHO1-3'-pMDC32* 5'-tcg agg cca ctg gaa ctt tt-3' and 5'-cac ttt gta caa gaa agc tgg gt-3').

qRT-PCR on enriched guard cells preparations Six-week-old plants were sprayed one hour after the beginning of the light cycle with +/- 50 μM ABA and incubated under light for 4 h. Enriched guard cell fractions were promptly prepared by the "blender method" as described (Geiger *et al.*, 2011). RNA was extracted using the RNeasy Plant Mini Kit (www.quiagen.com), subjected to DNA digestion with RNase-free DNase (www.fermentas.de); first-strand cDNA was prepared by use of M-MLV-RT (www.promega.com). All procedures were carried out according to the manufacturers' protocols. Quantitative real time PCR (qPCR) was performed in an Eppendorf realplex2 (http://www.eppendorf.de). Transcript numbers were normalized to molecules of actin2/8 as described elsewhere (Ivashikina *et al.*, 2003). Primers used for qRT-PCR are the following: *Actin 2* (*AT3G18780*) *Actin 8* (*AT1G49240*), *AtACT2/8*F 5'-GGT GAT GGT GTG TCT-3' *AtACT2/8*R 5'-ACT GAG CAC AAT GTT AC-3'; *HAI1* (*AT5G59220*), *HAI1F* 5'-GTT GAA TAG TTT TGA CGA-3', *HAI1R* 5'-GCC GTA TTT AGG ATA AGC-3'; *KIN2* (*AT5G15960*), *KIN2R* 5'-TCA GAG ACC AAC AAG AAT-3' *KIN2R* 5'-CGA TAT ACT CTT TCC CGC-3'; *LEA6* (*AT2G40170*), *LEA6F* 5'-CCA AGA CCT AAA TCA AAC-3' *LEA6R* 5'-ACA ACG AGA CAC TTT AC-3'; *LTP4* (*AT5G59310*), *LPT4F* 5'-GTG AAG TGG GGA ATA AC-3' *LPT4R* 5'-GTG ATT AAT AAG GTA CCG-3'; *MYB60* (*AT1G08810*), *MYB60F* 5'-ATG CTG TGA CAA GAT AGG -3' *MYB60R* 5'-AAA GTT TCC ACG TTT AAT-3'; *RD20* (*AT2G33380*), *RD20F* 5'-GTT ACA CTT CCG AGT T-3' *RD20R* 5'-CAC ACA TTC TTA GTC TTG-3'.

Micro-grafting experiments Plant grafting using collars was performed as per the protocol previously described by Turnbull *et al.* (2002). Seedlings were grown for 5 days at 27°C under 8 h light/16 h dark cycles on 1/2 Murashige Skoog medium pH 5.6 with Gamborg vitamins (Duchefa) containing 1% w/v Sucrose (Acros organics)

and 0.8% w/v Agar (Applichem). Root to shoot grafting was performed using sterile silicone tubing 0.3 mm in diameter. Following grafting, plants were incubated at 27°C for a further 4 to 6 days. Grafts unions were examined for the absence of adventitious roots, and successful grafts were transferred to soil for 4-5 weeks under short-day regiment (18°C, 10 h light/ 14 h dark). Prior to phenotype analyses, graft unions were re-examined to ensure that no adventitious roots had grown from the shoot scion.

Stomatal aperture bioassays The youngest fully expanded leaves from 4-6 weeks old plants were excised in darkness one hour before the beginning of the light cycle, and epidermal strips were prepared immediately as follows: the abaxial epidermis of the leaf was fixed to a microscope slide using liquid medical adhesive B (VM 355-1, Ulrich Swiss), and the resulting epidermal peels were floated in petri dishes containing 50 mL of incubation buffer (KCl 30 mM MES-KOH 10 mM pH 6.5) for 30 min in darkness before being subjected to light (100 $\mu Em^{-2}s^{-1}$, 25°C) and ABA treatment. For stomatal closure experiments, the epidermal peels were exposed to light for two hours prior to any further treatments in order to trigger stomatal opening. ABA was then added to the incubation buffer up to the indicated concentrations. Abscisic acid stock was prepared in ethanol. All stomatal apertures were observed under an optical microscope before digital images were taken and subsequently used to measure aperture width in IMAGEJ (National Institutes of Health). Approximately 40 stomatal apertures were measured for each independent experiment, time point, treatment and genotype. The significance of aperture fold change differences (stomatal aperture at t = 2/ stomatal aperture at t = 0) between genotypes was assessed by noncoupled one-tailed Student's t-test analysis. Values of $P<0.05$ were considered statistically significant. For stomatal aperture assays on transgenic lines (WT p*GC1*::*PHO1*RNAi

and *pho1.2* p*GC1*::*PHO1*g), results were compared to replicated experiments where each genotype was given a blind treatment. In an effort to reduce sampling error, the same area of the epidermal peel (approximately 1 mm^2, defined with a marker on the microscope slide) was used for initial (t = 0) and post-treatment (t = 2 h) measurement of stomatal aperture.

Inorganic phosphate measurements on purified guard cells Cellulysin cellulase from *Trichoderma viride* (Calbiochem) was dissolved in a basic medium without sorbitol (0.1 % PVP40, 0.25 % BSA, 0.5 mM ascorbic acid, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM MES, and 25 mL distilled water, pH 5.5 with KOH) then was subsequently desalted using MicroKros® Hollow Filter Modules for dialysis (SpectrumLabs http://eu.spectrumlabs.com/), against the same basic medium in a 4°C cold chamber, until Pi concentration in the enzyme mixture was lowered below 10 µM. Enriched guard cells preparation were then prepared following a "blender method": before the start of the light cycle, mature leaves from 12-24 five-week-old plants (main vein discarded) were blended in cold distilled water for 2 min using a small Waring blender on high speed. Processed tissue was passed through a 100 µm nylon mesh to isolate epidermal fragments and rinsed with water until clear. Epidermal fragments were then submerged in the desalted digestion mixture, with the addition of 50 g/L D-sorbitol (Sigma). Digestions were incubated for 3-4 hours at 27 °C in a shaking water bath in the dark. Cellular digestion was monitored microscopically until completion, upon which digests were passed through a 50 µm nylon mesh. The purified epidermis were freeze-dried and dry weight (DW) was measured. The cellular content of cells was released in nanopure water at 70°C for 30 minutes. Pi concentration was assayed using an enzyme-coupling fluorescence method Vazquez *et al.* 2003.

6 - Conclusion and perspectives

With the present study, we reported that beside its expression in the root vasculature *AtPHO1* is expressed in guard cells and rapidly up-regulated by abscisic acid, suggesting a potential importance for *PHO1* in stomata (figure 3.1, 3.3 and 3.4). Using several different yet complementary approaches such as mutant analysis (figure 4.2 and 4.3), grafting experiments (figure 4.9 and 4.10), guard-cell specific expression of *PHO1* in a mutant background (figure 5.7 and 5.9) and guard cell-specific down-regulation of *PHO1* (figure 5.3), we demonstrated that this guard cell expression directly mediates the stomatal movement response to ABA.

Although our data suggest that *pho1* phosphate deficiency can affect stomatal movements on some level (figure 5.7, and 5.9), experiments using micro-grafts and RNAi lines demonstrated that the impaired response of stomata to ABA in plants with abolished or reduced expression of *PHO1* occurred even in Pi-sufficient leaves, which confirms that *PHO1* influences the stomatal response regardless of the plant Pi status. In addition, we gathered evidence suggesting that *PHO1* is involved in a signaling step that is independent of the transcriptional response to ABA (figure 5.2), and located downstream of ABA perception and ROS production (figure 4.4). The affected mechanism is likely to be a central signaling event common to ABA-, ROS- and CO_2-induced stomatal movements (figure 4.3, 4.4 and 4.7). We showed that *pho1* is not affected in

several other stomatal movements pathways, thus proving that *PHO1* knock-out does not interfere with the guard cell response via an unspecific mechanical failure (figure 4.5 and 4.6). Finally, we observed that modulation of *PHO1* expression in guard cells affects guard cells Pi content, suggesting that *PHO1* might act through the regulation of guard cell phosphate homeostasis (figure 5.10). Together, our data show that *PHO1* is a novel important player in the response of guard cells to ABA. Since *PHO1* is primarily involved in the efflux of phosphate out of cells (Stefanovic *et al.*, 2011; Arpat *et al.*, 2012), this result gives strong indications of a potential role of phosphate in this process. To our knowledge, this is the first report suggesting the involvement of phosphate and of a phosphate transporter in the guard cell response to ABA.

To date the exact mechanisms behind the involvement of *PHO1* and phosphate in guard cell signaling remain to be elucidated. PHO1 is known to be involved in phosphate export to the apoplast (Stefanovic *et al.*, 2011; Arpat *et al.*, 2012), which is consistent with the role of PHO1 in loading phosphate into the xylem. It is thus tempting to speculate that PHO1 might have a similar function in guard cells, i.e. that ABA, ROS and CO_2 signaling could involve phosphate efflux through PHO1. As a matter of fact, the release of anions to the outside of the guard cells is an important signaling event occurring during ABA-, ROS- and elevated CO_2-promotion of stomatal closure. The anion channel activation induces membrane depolarization which in turns stimulates K^+ outward-rectifying channels. The subsequent release of osmotically active anions and K^+ contributes to cell deflation and stomatal closure (Sirichandra *et al.*, 2009*b*; Negi *et al.*, 2008; Kim *et al.*, 2010). Although the presence of anion currents during ABA, elevated CO_2 and calcium transients response has been known for a long time (Schroeder and Keller, 1992), the identity of the guard cell anion channels has only started to be uncovered in the past few years, with SLAC1 (Negi *et al.*, 2008;

Vahisalu *et al.*, 2008) and SLAH3 (Geiger *et al.*, 2011) as the main candidates for S-type anion channels, and AtALMT12 (QUAC1) as a R-type anion channel candidate permeable to malate^{2-} (Meyer *et al.*, 2010).

Malate^{2-} and Cl^- have long been recognized as two important anions mediating stomatal movements, but other anions are also involved: NO_3^- uptake into guard cells via NRT1.1 was shown to influence stomata opening (Guo *et al.*, 2003), and the anion channel SLAC1 and SLAH3 exhibit conductance for nitrate in addition to chloride (Geiger *et al.*, 2011). In an effort to characterize the main anions involved in stomatal closure, an early study by Schmidt and Schroeder (1994) described the relative slow anion channels permeability of several physiologically significant anions, including Cl^-, Br^-, F^-, I^-, NO_3^- and malate^{2-}. The highest permeability over chloride was attributed to NO_3^-, but all tested anions displayed significant permeabilities as well, suggesting that slow anion channels do not discriminate strongly among anions. Unfortunately, this set of experiments did not include measurements of PO_4^{3-} permeability (Schmidt and Schroeder, 1994). As a matter of fact, while it is theoretically possible that phosphate efflux might act, together with Cl^-, NO_3^- and malate^{2-}, as a signal for membrane depolarization, or might participate in the regulation of osmotic pressure during stomatal closing, to our knowledge the potential importance of phosphate as an osmolyte or a signaling molecule during ABA-regulated stomatal movements has not been fully addressed in the literature, and remains to be elucidated.

Moreover, it is intriguing to note that several studies described an accumulation of phosphorus in guard cells and epidermis of different plant species (Conn and Gilliham, 2010). Outlaw *et al.* (1984) presented a qualitative description of phosphate distribution within *Vicia faba* leaves, and reported that phosphate is more abundant in guard cells and epidermal cells than mesophyll cells; Treeby *et al.* (1987) described

similar phosphate accumulation in *Lupinus luteus* epidermis; Heath *et al.* (1997) also observed accumulation of phosphorus in the stomata of *Thlaspi montanum.* Together, these results raised the question of the biological importance of such phosphate accumulation in plant epidermis and/or stomata. In fact, it was early hypothesized that phosphate might be important for guard cell movements (Outlaw *et al.*, 1984; Treeby *et al.*, 1987). With the aim of identifying the subsidiary elements that play a role in stomatal movements beside the main osmolytes K^+ and Cl^-, Garrec *et al.* (1983) followed the variations of K, Cl, Na, P, Ca, Mg and S elements occurring in the stomatal complex of *Vicia faba* and *Commelina communis,* during stomatal opening and closure. However, they reported that phosphorus did not show large repartition changes in closed versus open stomata, which suggested that phosphate is likely to play a different role than being a major osmolyte for stomatal movements (Garrec *et al.*, 1983).

Nevertheless, the paper of Garrec *et al.* (1983) did not exclude the existence of smaller phosphate concentrations variations, nor did it investigate the potential presence of phosphate fluxes between sub-cellular compartments, which could also theoretically influence stomatal movements through a different signaling mechanism than the sole regulation of osmolarity. Phosphate transport could work as a signal regulating the activity of other signaling components, for example by contributing to membrane depolarization and regulation of voltage-dependent channels. Also, Pi and nucleoside phosphates participate in energy transformation and in numerous enzymatic reactions, with phosphorylation playing a particularly important role in the control of signal transduction pathways (Poirier and Bucher, 2002). Phosphate is also a precursor for the synthesis of ABA signaling molecules such as phosphatidic acid and inositol phosphates (Lemtiri-Chlieh *et al.*, 2000, 2003; Mishra *et al.*, 2006; Nagy *et al.*, 2009; Zhang *et al.*, 2009). The maintenance of appropriate phosphate pools in guard cells

and the regulation of phosphate transport through PHO1 could therefore potentially influence many aspects of guard cell signaling.

Moreover, while strong evidence have been gathered for the role of PHO1 in phosphate export, the general mechanisms behind PHO1 mediation of Pi release to the extracellular space, and its definite site of action, are still not fully understood. Based on localization data of PHO1 in roots cells, onion epidermal cells, tobacco and *Arabidopsis* mesophyll cells, Arpat *et al.* (2012) and Liu *et al.* (2012) discussed a mode of action for PHO1 in mediating Pi transport that could be very different from typical PM-localized transporters. Arpat *et al.* (2012) suggested that only a minor fraction of PHO1 is localized at the plasma membrane and is responsible for Pi export, while the major pool of PHO1 is sequestered in the endomembrane network, with the distribution of PHO1 between the secretory system and the plasma membrane being regulated by Pi homeostasis. Another hypothesis suggested by Arpat *et al.* (2012) and Liu *et al.* (2012) is that Pi export could be first mediated by PHO1 loading Pi into secretory vesicles, followed by the release of Pi to the extracellular space via exocytosis and the rapid recycling of PHO1. Similar hypothesis could be drawn for the action mechanism of PHO1 in guard cells: PHO1 could play a role in phosphate transport directly at the plasma membrane, or could be primarily involved in intracellular remobilization of phosphate. Localization of PHO1 in guard cells could provide a better understanding of PHO1 action site in stomata. In addition, further investigation will be required to characterize phosphate fluxes in and out of guard cells, or within subcellular compartments, that could occur during the guard cell ABA response, as well as the possible role of PHO1 in such phosphate fluxes. If technically possible, electrophysiology measurements using phosphate in the buffer could also help gain insight on this matter.

The maintenance of guard cell phosphate homeostasis through *PHO1* might also be of importance for the appropriate functioning of Ca^{2+} signaling. It is indeed known that elevations in phosphate and calcium can trigger rapid precipitation of calcium phosphate (Poirier and Bucher, 2002; Roelfsema and Hedrich, 2010). Alterations in phosphate transport and relocation could therefore theoretically interfere with the high elevations of calcium that are observed during ABA, ROS and CO_2 signaling. In fact, this tendency to precipitate in the presence of high calcium does not favor the hypothesis of phosphate being a major guard cell osmolyte. However, it is consistent with the need for a tight regulation of phosphate homeostasis in guard cells. Among other roles, $[Ca^{2+}]_{cyt}$ oscillations participate in stomatal movements via the regulation of anion channels activity (Mori *et al.*, 2006; Vahisalu *et al.*, 2008; Siegel *et al.*, 2009; Meyer *et al.*, 2010) and down-regulation of H^+ ATPases and K^+_{in} channels (Kinoshita *et al.*, 1995; Siegel *et al.*, 2009). The calcium-dependent pathways is believed to be responsible for a large part of the stomatal response to ABA and CO_2: in the absence of $[Ca^{2+}]_{cyt}$ elevations, ABA-induced stomatal closure shows only approximately 30% of the normal stomatal closure response (Siegel *et al.*, 2009), and CO_2-induced stomatal closure is also strongly inhibited (Hubbard *et al.*, 2012). Therefore if the *pho1* mutation, by modifying guard cell phosphate pools and transport, interferes with the modulation of the repetitive $[Ca^{2+}]_{cyt}$ transient patterns, this could affect a large part of the stomatal movement response to ABA and CO_2. The monitoring of $[Ca^{2+}]_{cyt}$ transient patterns in *pho1* or in the guard-cell RNAi lines (using for example GFP-based calcium reporter), in response to ABA, ROS, CO_2 and extracellular Ca^{2+}, as well as further characterization of the stomatal response to calcium-related stimuli, could help understand the possible effect of *pho1* mutation or *PHO1* knock-down on calcium signaling.

A challenging task ahead will be to address the potential regulation mechanisms of PHO1 activity in guard cells. Evidence have been gathered on the role of a set of CBL/CIPKs that can phosphorylate PHO1 and regulate its Pi-export activity when co-expressed in *Xenopus* oocytes (M. Jabnoune, personal communication). In addition, research is currently being carried out to identify other calcium dependent kinases (CPKs) that could play a role in such regulation. Incidentally, CBL-CIPKs and CPKs have been shown to be important regulators of guard cells ABA and CO_2 signaling (Cheong *et al.*, 2007; Zhu *et al.*, 2007). It is believed that ABA and CO_2 might enhance the calcium sensitivity of the downstream calcium sensors specific to their signaling pathways (Siegel *et al.*, 2009; Kim *et al.*, 2010). In turn, these calcium sensors can directly regulate ion channels activity (Mori *et al.*, 2006; Cheong *et al.*, 2007; Geiger *et al.*, 2010, 2011). A good example of this mechanism was seen with the characterization of SLAH3 function and regulation (Geiger *et al.*, 2011). Co-expression of SLAH3 with CPK21 in *Xenopus* oocytes was found to mediate anion currents via phosphorylation of SLAH3. *In vitro*, ABA stimulated the phosphorylation of SLAH3 by CPK21 in the presence of ABA receptor–phosphatase complex RCAR1-ABI1, by releasing CPK21 from inhibition by ABI1 and allowing its activation by cytosolic Ca^{2+} (Geiger *et al.*, 2011). It would be interesting to investigate whether PHO1 phosphorylation by CBL/CIPKs and whether its phosphate export activity could be regulated by ABA in a similar mechanism.

To conclude, the involvement of *PHO1* in guard cell signaling opens a new chapter in the study of the role of PHO1 in *Arabidopsis*. Further understanding of PHO1 function in roots and guard cells could bring valuable knowledge in both phosphate transport and guard cell signaling research fields. Studying the role of PHO1 in guard cells could provide a better comprehension of its function in roots and phosphate ac-

quisition. In parallel, a deeper understanding of PHO1 function in the roots could help identify new regulatory mechanisms in the control of stomatal movements and resistance to drought. The comprehension of plant phosphate acquisition and guard cell signaling is of great importance for the development of future breeding and GM approaches, that face the challenge of maintaining crop yields while reducing our reliance on non-renewable inorganic phosphate fertilizers (Vance *et al.*, 2003; Hammond *et al.*, 2004; Vuuren *et al.*, 2010), and coping with the pressing issues of fresh water scarcity and continuous atmospheric CO_2 rise (Schroeder *et al.*, 2001).

7 - Appendix

Stomatal movement response of *pho1;h10* to ABA

In *Arabidopsis*, the *PHO1* gene family is composed of 11 members, designated as *PHO1*, and *PHO1;H1* to *PHO1;H10*. In the phylogenetic tree of *Arabidopsis* PHO1 members, PHO1;H10 belongs to a clade that is distinct from the other homologues (Wang *et al.*, 2004). The homologue *PHO1;H10* is expressed in a variety of tissues, including in the root epidermal/ cortical cells, leaf blade and flowers. *PHO1;H10* was found to have a unique expression pattern with strong induction upon numerous biotic and abiotic stresses, such as wounding, dehydration, cold, salt, pathogen attack, phosphate deficiency, and phytohormones treatments including OPDA and ABA (Ribot *et al.*, 2008*a*,*b*). In an effort to characterize a potential phenotype and a role for *PHO1;H10*, the mutant *pho1;h10* (T-DNA knock-out mutant) was extensively studied by Ribot (2006), under various conditions such as germination assays, water loss and ion uptake measurements. However, *pho1;h10* has so far displayed wild type phenotype during all assays.

Since the *PHO1;H10* was strongly induced upon ABA treatment and dehydration, and was found expressed in guard cells (according to microarray data of Yang *et al.*, 2008), we were interested in characterizing the stomatal response to ABA of the mutant *pho1;h10*. In order to assess any potential functional redundancy between *PHO1* and *PHO1;H10*, double mutants lines *pho1,pho1;h10* were also produced and analyzed in parallel. We analyzed the stomatal movement response in WT, *pho1.2*, *pho1;h10* and the double mutant *pho1.2*,*pho1;h10* following treatment with ABA or H_2O_2.

During ABA-induced stomatal closure (figure 7.1a), ABA repression of stomatal opening under light (figure 7.1b) and H_2O_2 induction of stomatal closure (figure 7.1c), the mutant *pho1;h10* presented stomatal aperture similar to WT. In contrast, the double

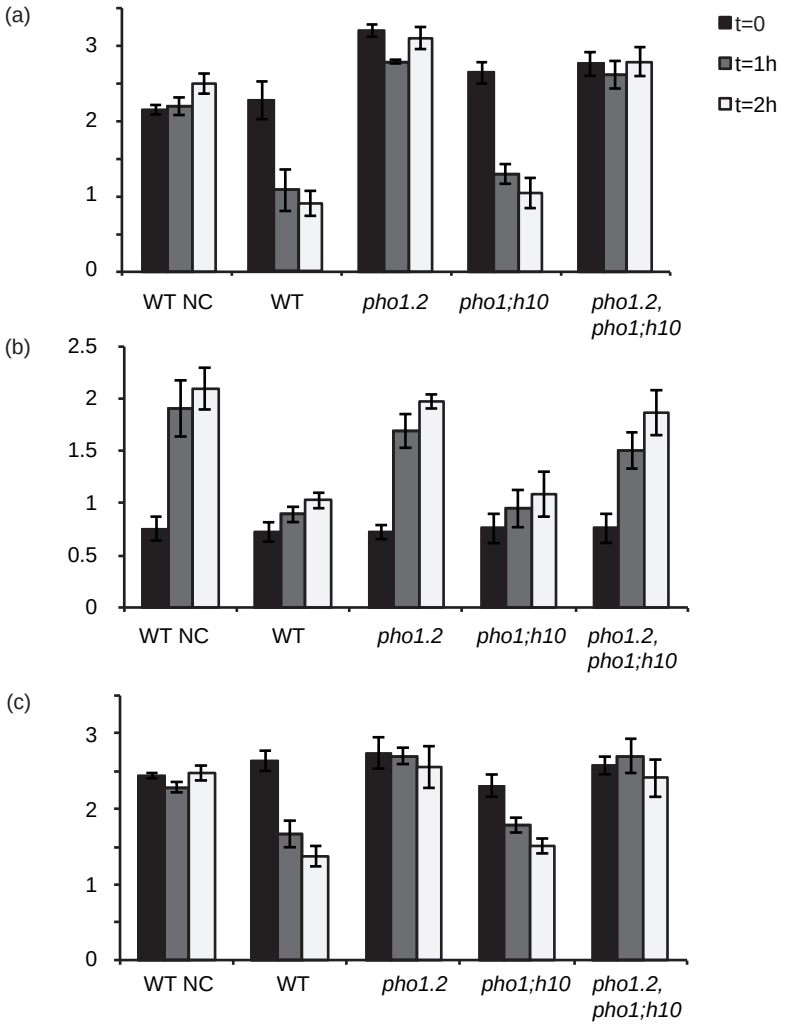


Figure 7.1: **The T-DNA mutant *pho1;h10* responds to ABA in terms of stomatal movements; the double mutant *pho1.2,pho1;h10* recapitulates the phenotypes of *pho1.2*.** (a) Induction of stomatal closure and (b) repression of stomatal opening under light in WT, *pho1.2*, *pho1;h10* and the double mutant *pho1.2,pho1;h10*, in response to 10 μM ABA, after 0, 1, and 2 h. (c) Stomatal closure in WT, *pho1.2*, *pho1;h10* and the double mutant *pho1.2,pho1;h10*, following 0, 1, and 2-h treatments with 100 μM H_2O_2. For the above experiments, n = 3 independent experiments; average ± SE. NC: Negative control (-ABA).

mutant *pho1.2,pho1;h10* presented a strong impairment of the stomatal response to ABA, with aperture similar to *pho1.2* during all treatments and time points, suggesting that the double mutant recapitulates the phenotype of *pho1.2*. These results show that while expressed in guard cells and up-regulated under ABA, *PHO1;H10* does not mediate the stomatal movement response to ABA. To date, the role of *PHO1;H10* remains to be elucidated.

Experimental procedures

Plant material and growth conditions *Arabidopsis thaliana* wild type and mutants plants were from the Columbia ecotype (wild type Col). The mutants *pho1.2* has been previously been described by Poirier *et al.* (1991), Delhaize and Randall (1995) and Hamburger *et al.* (2002). Seed was sown directly onto potting compost contained in 7 cm diameter pots and vernalized for 2 days at 4°C. Plants were grown for 4 to 6 weeks under short-day growth conditions (18 °C, 60 % relative humidity, 10 h light/ 14 h dark, 100 $\mu Em^{-2}s^{-1}$). The homozygous *pho1;h10* mutant line is a T- DNA-insertional knockout line (SALK_034134) isolated by C. Ribot, and was used to generate the double mutants *pho1.2, pho1;h10* lines by cross between *pho1.2* and *pho1;h10*. Double homozygous mutants lines were selected by scoring for *pho1* phenotype and by detection of the *PHO1;H10* insertion by PCR. The *PHO1;H10* complete knock-out was confirmed by Northern hybridization as described in (Ribot *et al.*, 2008*b*).

Stomatal aperture bioassays The youngest fully expanded leaves from 4-6 weeks old plants were excised in darkness one hour before the beginning of the light cycle, and epidermal strips were immediately prepared: briefly, the abaxial epidermis of the leaf was fixed to a microscope slide using liquid medical adhesive B (VM 355-1, Ulrich

swiss), and the resulting epidermal peels were floated in petri dishes containing 50 mL of incubation buffer (KCl 30mM MES-KOH 10mM pH6.5) for 30 min in darkness before being subjected to light (100 $\mu Em^{-2}s^{-1}$, 25°C) and chemical treatments. For stomatal closure experiments, prior to treatments, the epidermal peels were exposed to light for 2 h to trigger stomatal opening. ABA or H_2O_2 were then added to the incubation buffer to indicated concentration. Abscisic acid 10 mM stock was prepared in ethanol. All stomatal apertures were observed under an optical microscope before digital images were taken and subsequently used to measure aperture width in IMAGEJ (National Institutes of Health). Approximately 40 stomatal apertures were measured for each independent experiment, time point, treatment and genotype.

Bibliography

Acharya BR and Assmann SM (2009). Hormone interactions in stomatal function. *Plant Mol Biol*, 69(4):451–462.

Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, and Berkowitz GA (2007). Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. *Plant Cell*, 19(3):1081–1095.

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, Chory J, and Schroeder JI (2000). Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. *Science*, 289(5488):2338–2342.

Arpat B, Magliano P, Wege S, Rouached H, Stefanovic A, and Poirier Y (2012). Functional expression of *PHO1* to the Golgi and *trans*-Golgi network and its role in Pi export. *Plant J*, DOI: 10.1111/j.1365-313X.2012.05004.x.

Assmann SM, Simoncini L, and Schroeder JI (1985). Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. *Nature*, 318(6043):285–287.

Barbier-Brygoo H, De Angeli A, Filleur S, Frachisse JM, Gambale F, Thomine

S, and Wege S (2011). Anion channels/transporters in plants: from molecular bases to regulatory networks. *Annu Rev Plant Biol*, 62:25–51.

Batistic O and Kudla J (2009). Plant calcineurin B-like proteins and their interacting protein kinases. *Biochim Biophys Acta*, 1793(6):985–992.

Bergmann DC and Sack FD (2007). Stomatal development. *Annu Rev Plant Biol*, 58(1):163–181.

Chen YF, Wang Y, and Wu WH (2008). Membrane transporters for nitrogen, phosphate and potassium uptake in plants. *J Integr Plant Biol*, 50(7):835–848.

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim BG, Lee SC, Kudla J, and Luan S (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis*. *Plant J*, 52(2):223–239.

Cho D, Kim SA, Murata Y, Lee S, Jae SK, Nam HG, and Kwak JM (2009). Deregulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term Ca^{2+}-programmed stomatal closure. *Plant J*, 58(3):437–449.

Christmann A, Weiler EW, Steudle E, and Grill E (2007). A hydraulic signal in root-to-shoot signalling of water shortage. *Plant J*, 52(1):167–174.

Ciereszko I, Johansson H, Hurry V, and Kleczkowski LA (2001). Phosphate status affects the gene expression, protein content and enzymatic activity of UDP-glucose pyrophosphorylase in wild-type and *pho* mutants of *Arabidopsis*. *Planta*, 212(4):598–605.

Clough SJ and Bent AF (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*, 16(6):735–743.

Cominelli E, Galbiati M, Albertini A, Fornara F, Conti L, Coupland G, and Tonelli C (2011). DOF-binding sites additively contribute to guard cell-specificity of *AtMYB60* promoter. *BMC Plant Biol*, 11:162.

Conn S and Gilliham M (2010). Comparative physiology of elemental distributions in plants. *Ann Bot*, 105(7):1081–1102.

Curtis MD and Grossniklaus U (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol*, 133(2):462–469.

Cutler SR, Rodriguez PL, Finkelstein RR, and Abrams SR (2010). Abscisic acid: emergence of a core signaling network. *Annu Rev Plant Biol*, 61:651–679.

Czechowski T, Stitt M, Altmann T, Udvardi MK, and Scheible WR (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol*, 139(1):5–17.

De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, and Barbier-Brygoo H (2006). The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. *Nature*, 442(7105):939–942.

Delhaize E and Randall P (1995). Characterization of a phosphate-accumulator mutant of *Arabidopsis thaliana*. *Plant Physiol*, 107(1):207–213.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, and Zhu JK (2009). *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature*, 462(7273):660–664.

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, and Yamaguchi-Shinozaki K (2006). Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci U S A*, 103(6):1988–1993.

Galbiati M, Simoni L, Pavesi G, Cominelli E, Francia P, Vavasseur A, Nelson T, Bevan M, and Tonelli C (2008). Gene trap lines identify *Arabidopsis* genes expressed in stomatal guard cells. *Plant J*, 53(5):750–762.

Garrec JP, Vavasseur A, Michaowicz G, and Laffray D (1983). Stomatal movements and repartition of the elements K, Cl, Na, P, Ca, Mg and S in the stomatal complexes of *Vicia faba* and *Commelina communis*. Electron probe studies. *Zeit Pflanz*, 112:1.

Gaymard F, Pilot G, Lacombe B, Bouchez D, Bruneau D, Boucherez J, Michaux-Ferriere N, Thibaud JB, and Sentenac H (1998). Identification and disruption of a plant shaker-like outward channel involved in K^+ release into the xylem sap. *Cell*, 94(5):647–655.

Geiger D, Maierhofer T, Al-Rasheid KAS, Scherzer S, Mumm P, Liese A, Ache P, Wellmann C, Marten I, Grill E, Romeis T, and Hedrich R (2011). Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. *Sci Signal*, 4:173.

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese A, Wellmann C, Al-Rasheid KAS, Grill E, Romeis T, and Hedrich R (2010). Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities. *Proc Natl Acad Sci U S A*, 107(17):8023–8028.

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KAS, Romeis T, and Hedrich R (2009). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proc Natl Acad Sci U S A*, 106(50):21425–30.

Gleave AP (1992). A versatile binary vector system with a T-DNA organisational

structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol*, 20(6):1203–1207.

Gobert A, Isayenkov S, Voelker C, Czempinski K, and Maathuis FJM (2007). The two-pore channel TPK1 gene encodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis. *Proc Natl Acad Sci U S A*, 104(25):10726–10731.

Gomez-Porras JL, Riano-Pachon DM, Dreyer I, Mayer JE, and Mueller-Roeber B (2007). Genome-wide analysis of ABA-responsive elements ABRE and CE3 reveals divergent patterns in *Arabidopsis* and rice. *BMC Genomics*, 8:260.

Guo B, Jin Y, Wussler C, Blancaflor EB, Motes CM, and Versaw WK (2008). Functional analysis of the *Arabidopsis* PHT4 family of intracellular phosphate transporters. *New Phytol*, 177(4):889–898.

Guo FQ, Young J, and Crawford NM (2003). The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in *Arabidopsis*. *Plant Cell*, 15(1):107–117.

Guo Y, Xiong L, Song CP, Gong D, Halfter U, and Zhu JK (2002). A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in *Arabidopsis*. *Dev Cell*, 3(2):233–244.

Hamburger D, Rezzonico E, MacDonald-Comber Petetot J, Somerville C, and Poirier Y (2002). Identification and characterization of the *Arabidopsis PHO1* gene involved in phosphate loading to the xylem. *Plant Cell*, 14(4):889–902.

Hammond JP, Broadley MR, and White PJ (2004). Genetic responses to phosphorus deficiency. *Ann Bot*, 94(3):323–32.

Hammond JP and White PJ (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *J Exp Bot*, 59(1):93–109.

Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC, and Cottage A (2006). A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods*, 2:19.

Heath SM, Southworth D, and D'Allura JA (1997). Localization of nickel in epidermal subsidiary cells of leaves of *Thlaspi montanum var. Siskiyouense (Brassicaceae)* using energy-dispersive X-Ray microanalysis. *Int J Plant Sci*, 158(2):184–188.

Hellens RP, Edwards EA, Leyland NR, Bean S, and Mullineaux PM (2000). pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol*, 42(6):819–832.

Hetherington AM and Woodward FI (2003). The role of stomata in sensing and driving environmental change. *Nature*, 424(6951):901–908.

Hirsch RE, Lewis BD, Spalding EP, and Sussman MR (1998). A role for the AKT1 potassium channel in plant nutrition. *Science*, 280(5365):918–921.

Hosy E, Vavasseur A, Mouline K, Dreyer I, Gaymard F, Poree F, Boucherez J, Lebaudy A, Bouchez D, Very AA, Simonneau T, Thibaud JB, and Sentenac H (2003). The *Arabidopsis* outward K^+ channel GORK is involved in regulation of stomatal movements and plant transpiration. *Proc Natl Acad Sci U S A*, 100(9):5549–5554.

Hu H, Boisson-Dernier A, Israelsson-Nordstrom M, Bohmer M, Xue S, Ries A, Godoski J, Kuhn JM, and Schroeder JI (2010). Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells. *Nat Cell Biol*, 12(1):87–93.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, and Schroeder JI (2010).

Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes Dev*, 24(16):1695–1708.

Hubbard KE, Siegel RS, Valerio G, Brandt B, and Schroeder JI (2012). Abscisic acid and CO_2 signalling via calcium sensitivity priming in guard cells, new CDPK mutant phenotypes and a method for improved resolution of stomatal stimulus-response analyses. *Ann Bot*, 109(1):5–17.

Ivashikina N, Deeken R, Ache P, Kranz E, Pommerrenig B, Sauer N, and Hedrich R (2003). Isolation of *AtSUC2* promoter-GFP-marked companion cells for patch-clamp studies and expression profiling. *Plant J*, 36(6):931–945.

Joshi-Saha A, Valon C, and Leung J (2011). A brand new START: abscisic acid perception and transduction in the guard cell. *Sci Signal*, 4(201):re4.

Jossier M, Kroniewicz L, Dalmas F, Le Thiec D, Ephritikhine G, Thomine S, Barbier-Brygoo H, Vavasseur A, Filleur S, and Leonhardt N (2010). The *Arabidopsis* vacuolar anion transporter, AtCLCc, is involved in the regulation of stomatal movements and contributes to salt tolerance. *Plant J*, 64(4):563–576.

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, and Lee Y (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proc Natl Acad Sci U S A*, 107(5):2355–2360.

Kang X and Ni M (2006). *Arabidopsis* SHORT HYPOCOTYL UNDER BLUE1 contains SPX and EXS domains and acts in cryptochrome signaling. *Plant Cell*, 18(4):921–934.

Karimi M, Inze D, and Depicker A (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci*, 7(5):193–195.

Kim TH, Bohmer M, Hu H, Nishimura N, and Schroeder JI (2010). Guard cell

signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. *Annu Rev Plant Biol*, 61:561–591.

Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, and Shimazaki K (2001). phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature*, 414(6864):656–660.

Kinoshita T, Nishimura M, and Shimazaki K (1995). Cytosolic concentration of Ca^{2+} regulates the plasma membrane H^+-ATPase in guard cells of *Fava Bean*. *Plant Cell*, 7(8):1333–1342.

Kinoshita T and Shimazaki K (2001). Analysis of the phosphorylation level in guard-cell plasma membrane H^+-ATPase in response to fusicoccin. *Plant Cell Physiol*, 42(4):424–432.

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, Yamamoto A, and Hattori T (2005). Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. *Plant J*, 44(6):939–949.

Kollist H, Jossier M, Laanemets K, and Thomine S (2011). Anion channels in plant cells. *FEBS J*, 278(22):4277–92.

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E, Kamiya A, Moriyama Y, and Shinozaki K (2010). ABC transporter AtABCG25 is involved in abscisic acid transport and responses. *Proc Natl Acad Sci U S A*, 107(5):2361–2366.

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, and Schroeder JI (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J*, 22(11):2623–2633.

Kwak JM, Murata Y, Baizabal-Aguirre VM, Merrill J, Wang M, Kemper A, Hawke SD, Tallman G, and Schroeder JI (2001). Dominant negative guard cell K^+ channel mutants reduce inward-rectifying K^+ currents and light-induced stomatal opening in *Arabidopsis*. *Plant Physiol*, 127(2):473–485.

Lee M, Choi Y, Burla B, Kim YY, Jeon B, Maeshima M, Yoo JY, Martinoia E, and Lee Y (2008). The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO_2. *Nat Cell Biol*, 10(10):1217–1223.

Lee SC, Lan W, Buchanan BB, and Luan S (2009). A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proc Natl Acad Sci U S A*, 106(50):21419–21424.

Lemtiri-Chlieh F, MacRobbie EA, and Brearley CA (2000). Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells. *Proc Natl Acad Sci U S A*, 97(15):8687–8692.

Lemtiri-Chlieh F, MacRobbie EAC, Webb AAR, Manison NF, Brownlee C, Skepper JN, Chen J, Prestwich GD, and Brearley CA (2003). Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proc Natl Acad Sci U S A*, 100(17):10091–10095.

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, and Schroeder JI (2004). Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell*, 16(3):596–615.

Lin WY, Lin SI, and Chiou TJ (2009). Molecular regulators of phosphate homeostasis in plants. *J Exp Bot*, 60(5):1427–1438.

Liu TY, Huang TK, Tseng CY, Lai YS, Lin SI, Lin WY, Chen JW, and Chiou TJ

(2012). PHO2-Dependent Degradation of PHO1 Modulates Phosphate Homeostasis in Arabidopsis. *Plant Cell*. Http://dx.doi.org/10.1105/tpc.112.096636.

Liu X, Yue Y, Li B, Nie Y, Li W, Wu WH, and Ma L (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science*, 315(5819):1712–1716.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, and Grill E (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, 324(5930):1064–1068.

Mao J, Zhang YC, Sang Y, Li QH, and Yang HQ (2005). From the cover: A role for *Arabidopsis* cryptochromes and COP1 in the regulation of stomatal opening. *Proc Natl Acad Sci U S A*, 102(34):12270–12275.

Marten H, Hedrich R, and Roelfsema MRG (2007). Blue light inhibits guard cell plasma membrane anion channels in a phototropin-dependent manner. *Plant J*, 50(1):29–39.

Martinoia E, Meyer S, De Angeli A, and Nagy R (2012). Vacuolar transporters in their physiological context. *Annu Rev Plant Biol*, 63:183–213.

McAinsh M, Clayton H, Mansfield T, and Hetherington A (1996). Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiol*, 111(4):1031–1042.

McCourt P and Creelman R (2008). The ABA receptors – we report you decide. *Curr Opin Plant Biol*, 11(5):474–478.

Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V, Kovach A, Li J, Wang Y, Li J, Peterson FC, Jensen DR, Yong EL, Volkman BF, Cutler SR, Zhu JK, and Xu HE (2009).

A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. *Nature*, 462(7273):602–608.

Melotto M, Underwood W, Koczan J, Nomura K, and He SY (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell*, 126(5):969–980.

Merlot S, Leonhardt N, Fenzi F, Valon C, Costa M, Piette L, Vavasseur A, Genty B, Boivin K, Muller A, Giraudat J, and Leung J (2007). Constitutive activation of a plasma membrane H^+-ATPase prevents abscisic acid-mediated stomatal closure. *EMBO J*, 26(13):3216–3226.

Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, and Giraudat J (2002). Use of infrared thermal imaging to isolate *Arabidopsis* mutants defective in stomatal regulation. *Plant J*, 30(5):601–609.

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD, and Sauer N (2004). Wounding enhances expression of *AtSUC3*, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. *Plant Physiol*, 134(2):684–693.

Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Rasheid KAS, Geiger D, Marten I, Martinoia E, and Hedrich R (2010). AtALMT12 represents an R-type anion channel required for stomatal movement in *Arabidopsis* guard cells. *Plant J*, 63(6):1054–1062.

Meyer S, Scholz-Starke J, De Angeli A, Kovermann P, Burla B, Gambale F, and Martinoia E (2011). Malate transport by the vacuolar AtALMT6 channel in guard cells is subject to multiple regulation. *Plant J*, 67(2):247–257.

Mishra G, Zhang W, Deng F, Zhao J, and Wang X (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. *Science*, 312(5771):264–266.

Miyazono Ki, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira KS, Yamaguchi-Shinozaki K, and Tanokura M (2009). Structural basis of abscisic acid signalling. *Nature*, 462(7273):609–614.

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, Kwak JM, and Schroeder JI (2006). CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. *PLoS Biol*, 4(10):e327.

Mott KA (2009). Opinion: stomatal responses to light and CO(2) depend on the mesophyll. *Plant Cell Environ*, 32(11):1479–1486.

Mudge SR, Rae AL, Diatloff E, and Smith FW (2002). Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in *Arabidopsis*. *Plant J*, 31(3):341–353.

Nagy R, Grob H, Weder B, Green P, Klein M, Frelet-Barrand A, Schjoerring JK, Brearley C, and Martinoia E (2009). The *Arabidopsis* ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. *J Biol Chem*, 284(48):33614–33622.

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, and Iba K (2008). CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature*, 452(7186):483–486.

Nilson SE and Assmann SM (2007). The control of transpiration. Insights from *Arabidopsis*. *Plant Physiol*, 143(1):19–27.

Nishimura N, Hitomi K, Arvai A, Rambo R, Hitomi C, Cutler S, Schroeder J, and Getzoff E (2009*a*). Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science*, 326(5958):1373–1379.

Nishimura N, Sarkeshik A, Nito K, Park S, Wang A, Carvalho P, Lee S, Caddell D, Cutler S, Chory J, Yates J, and Schroeder J (2009*b*). PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in *Arabidopsis*. *Plant J*, 61(2):290–299.

Outlaw W, Tarczynski M, and Miller W (1984). Histological compartmentation of phosphate in *Vicia faba L.* leaflet : possible significance to stomatal functioning. *Plant Physiol*, 74(2):430–433.

Pandey S, Nelson DC, and Assmann SM (2009). Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. *Cell*, 136(1):136–148.

Pandey S, Wang XQ, Coursol SA, and Assmann SM (2002). Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. *New Phytol*, 153(3):517–526.

Pandey S, Zhang W, and Assmann SM (2007). Roles of ion channels and transporters in guard cell signal transduction. *FEBS Lett*, 581(12):2325–2336.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, and Cutler SR (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science*, 324(5930):1068–1071.

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, and

Schroeder JI (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, 406(6797):731–734.

Peiter E, Maathuis FJM, Mills LN, Knight H, Pelloux J, Hetherington AM, and Sanders D (2005). The vacuolar Ca^{2+}-activated channel TPC1 regulates germination and stomatal movement. *Nature*, 434(7031):404–408.

Pilot G, Lacombe B, Gaymard F, Cherel I, Boucherez J, Thibaud JB, and Sentenac H (2001). Guard cell inward K^+ channel activity in *Arabidopsis* involves expression of the twin channel subunits KAT1 and KAT2. *J Biol Chem*, 276(5):3215–3221.

Plaxton WC and Tran HT (2011). Metabolic adaptations of phosphate-starved plants. *Plant Physiol*, 156(3):1006–1015.

Plesch G, Ehrhardt T, and Mueller-Roeber B (2001). Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. *Plant J*, 28(4):455–464.

Plesch G, Kamann E, and Mueller-Roeber B (2000). Cloning of regulatory sequences mediating guard-cell-specific gene expression. *Gene*, 249(1-2):83–89.

Poirier Y and Bucher M (2002). Phosphate transport and homeostasis in *Arabidopsis*. *The Arabidopsis Book*. (Somerville, C.R. and Meyerowitz, E.M. eds). Rockville, MD: American Society of Plant Biologists.

Poirier Y, Thoma S, Somerville C, and Schiefelbein J (1991). A mutant of *Arabidopsis* deficient in xylem loading of phosphate. *Plant Physiol*, 97(3):1087–1093.

Raghavendra AS, Gonugunta VK, Christmann A, and Grill E (2010). ABA perception and signalling. *Trends Plant Sci*, 15(7):395–401.

Razem FA, El-Kereamy A, Abrams SR, and Hill RD (2006). The RNA-binding protein FCA is an abscisic acid receptor. *Nature*, 439(7074):290–294.

Ribot C (2006). Expression analyses of the *AtPHO1* gene family and the involvement of *AtPHO1* in the regulation of stomatal movements. Thèse de doctorat ès sciences de la vie (PhD thesis, Université de Lausanne).

Ribot C, Wang Y, and Poirier Y (2008*a*). Expression analyses of three members of the *AtPHO1* family reveal differential interactions between signaling pathways involved in phosphate deficiency and the responses to auxin, cytokinin, and abscisic acid. *Planta*, 227(5):1025–1036.

Ribot C, Zimmerli C, Farmer EE, Reymond P, and Poirier Y (2008*b*). Induction of the *Arabidopsis PHO1;H10* gene by 12-oxo-phytodienoic acid but not jasmonic acid via a CORONATINE INSENSITIVE1-dependent pathway. *Plant Physiol*, 147(2):696–706.

Roelfsema M, Hedrich R, and Geiger D (2012). Anion channels: master switches of stress responses. *Trends Plant Sci*, 17(4):221–229.

Roelfsema MRG, Hanstein S, Felle HH, and Hedrich R (2002). CO_2 provides an intermediate link in the red light response of guard cells. *Plant J*, 32(1):65–75.

Roelfsema MRG and Hedrich R (2005). In the light of stomatal opening: new insights into 'the Watergate'. *New Phytol*, 167(3):665–691.

Roelfsema MRG and Hedrich R (2010). Making sense out of Ca^{2+} signals: their role in regulating stomatal movements. *Plant Cell Environ*, 33(3):305–321.

Rouached H, Arpat A, and Poirier Y (2010). Regulation of phosphate starvation responses in plants: signaling players and cross-talks. *Mol Plant*, 3(2):288–299.

Rouached H, Stefanovic A, Secco D, Bulak Arpat A, Gout E, Bligny R, and Poirier Y (2011). Uncoupling phosphate deficiency from its major effects on growth and transcriptome via *PHO1* expression in *Arabidopsis*. *Plant J*, 65(4):557–570.

Rubio V, Bustos R, Irigoyen M, Cardona-López X, Rojas-Triana M, and Paz-Ares J (2009). Plant hormones and nutrient signaling. *Plant Mol Biol*, 69(4):361–373.

Rutledge RG and Cote C (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res*, 31(16):e93.

Santiago J, Dupeux F, Round A, Antoni R, Park S, Jamin M, Cutler S, Rodriguez P, and Marquez J (2009). The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature*, 462(7273):665–668.

Sato A, Sato Y, Fukao Y, Fujiwara M, Umezawa T, Shinozaki K, Hibi T, Taniguchi M, Miyake H, Goto DB, and Uozumi N (2009). Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase. *Biochem J*, 424(3):439–448.

Schachtman DP, Schroeder JI, Lucas WJ, Anderson JA, and Gaber RF (1992). Expression of an inward-rectifying potassium channel by the *Arabidopsis KAT1* cDNA. *Science*, 258(5088):1654–1658.

Schauer SE, Schluter PM, Baskar R, Gheyselinck J, Bolanos A, Curtis MD, and Grossniklaus U (2009). Intronic regulatory elements determine the divergent expression patterns of *AGAMOUS-LIKE6* subfamily members in *Arabidopsis*. *Plant J*, 59(6):987–1000.

Schmidt C and Schroeder J (1994). Anion selectivity of slow anion channels in

the plasma membrane of guard cells (large nitrate permeability). *Plant Physiol*, 106(1):383–391.

Schroeder JI and Keller BU (1992). Two types of anion channel currents in guard cells with distinct voltage regulation. *Proc Natl Acad Sci U S A*, 89(11):5025–5029.

Schroeder JI, Kwak JM, and Allen GJ (2001). Guard cell abscisic acid signalling and engineering drought hardiness in plants. *Nature*, 410(6826):327–330.

Schroeder JI, Raschke K, and Neher E (1987). Voltage dependence of K^+ channels in guard-cell protoplasts. *Proc Natl Acad Sci U S A*, 84(12):4108–4112.

Sclep G, Allemeersch J, Liechti R, De Meyer B, Beynon J, Bhalerao R, Moreau Y, Nietfeld W, Renou JP, Reymond P, Kuiper MT, and Hilson P (2007). CATMA, a comprehensive genome-scale resource for silencing and transcript profiling of *Arabidopsis* genes. *BMC Bioinformatics*, 8:400.

Secco D, Wang C, Arpat BA, Wang Z, Poirier Y, Tyerman SD, Wu P, Shou H, and Whelan J (2012). The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. *New Phytol*, 193(4):842–851.

Sheard LB and Zheng N (2009). Plant biology: signal advance for abscisic acid. *Nature*, 462(7273):575–576.

Shen YY, Wang XF, Wu FQ, Du SY, Cao Z, Shang Y, Wang XL, Peng CC, Yu XC, Zhu SY, Fan RC, Xu YH, and Zhang DP (2006). The Mg-chelatase H subunit is an abscisic acid receptor. *Nature*, 443(7113):823–826.

Shimazaki Ki, Doi M, Assmann SM, and Kinoshita T (2007). Light regulation of stomatal movement. *Annu Rev Plant Biol*, 58:219–247.

Shope JC, DeWald DB, and Mott KA (2003). Changes in surface area of in-

tact guard cells are correlated with membrane internalization. *Plant Physiol*, 133(3):1314–1321.

Siegel RS, Xue S, Murata Y, Yang Y, Nishimura N, Wang A, and Schroeder JI (2009). Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K channels in *Arabidopsis* guard cells. *Plant J*, 59(2):207–220.

Sirichandra C, Gu D, Hu HC, Davanture M, Lee S, Djaoui M, Valot B, Zivy M, Leung J, Merlot S, and Kwak JM (2009*a*). Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Lett*, 583(18):2982–2986.

Sirichandra C, Wasilewska A, Vlad F, Valon C, and Leung J (2009*b*). The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. *J Exp Bot*, 60(5):1439–1463.

Stadler R, Buttner M, Ache P, Hedrich R, Ivashikina N, Melzer M, Shearson SM, Smith SM, and Sauer N (2003). Diurnal and light-regulated expression of *AtSTP1* in guard cells of *Arabidopsis*. *Plant Physiol*, 133(2):528–537.

Stefanovic A, Arpat AB, Bligny R, Gout E, Vidoudez C, Bensimon M, and Poirier Y (2011). Over-expression of *PHO1* in *Arabidopsis* leaves reveals its role in mediating phosphate efflux. *Plant J*, 66(4):689–699.

Stefanovic A, Ribot C, Rouached H, Wang Y, Chong J, Belbahri L, Delessert S, and Poirier Y (2007). Members of the *PHO1* gene family show limited functional redundancy in phosphate transfer to the shoot, and are regulated by phosphate deficiency via distinct pathways. *Plant J*, 50(6):982–94.

Szyroki A, Ivashikina N, Dietrich P, Roelfsema MR, Ache P, Reintanz B,

Deeken R, Godde M, Felle H, Steinmeyer R, Palme K, and Hedrich R (2001). KAT1 is not essential for stomatal opening. *Proc Natl Acad Sci U S A*, 98(5):2917–2921.

Torsethaugen G, Pell EJ, and Assmann SM (1999). Ozone inhibits guard cell K^+ channels implicated in stomatal opening. *Proc Natl Acad Sci U S A*, 96(23):13577–13582.

Treeby MT, van Steveninck RF, and de Vries HM (1987). Quantitative estimates of phosphorus concentrations within *Lupinus luteus* leaflets by means of electron probe X-ray microanalysis. *Plant Physiol*, 85(2):331–334.

Trull MC, Guiltinan MJ, Lynch JP, and Deikman J (1997). The responses of wild-type and ABA mutant *Arabidopsis thaliana* plants to phosphorus starvation. *Plant Cell Environ*, 20(1):85–92.

Turnbull CGN, Booker JP, and Leyser HMO (2002). Micrografting techniques for testing long-distance signalling in *Arabidopsis*. *Plant J*, 32(2):255–262.

Ueno K, Kinoshita T, Inoue SI, Emi T, and Shimazaki KI (2005). Biochemical characterization of plasma membrane H^+-ATPase activation in guard cell protoplasts of *Arabidopsis thaliana* in response to blue light. *Plant Cell Physiol*, 46(6):955–963.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, and Yamaguchi-Shinozaki K (2010). Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol*, 51(11):1821–1839.

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, and Shinozaki K (2009). Type 2C

protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc Natl Acad Sci U S A*, 106(41):17588–17593.

Vahisalu T, Kollist H, Wang YF, Nishimura N, Chan WY, Valerio G, Lamminmaki A, Brosche M, Moldau H, Desikan R, Schroeder JI, and Kangasjarvi J (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature*, 452(7186):487–491.

Vahisalu T, Puzorjova I, Brosche M, Valk E, Lepiku M, Moldau H, Pechter P, Wang YS, Lindgren O, Salojarvi J, Loog M, Kangasjarvi J, and Kollist H (2010). Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. *Plant J*, 62(3):442–453.

Vance C, Uhde-Stone C, and Allan D (2003). Phosphorus Acquisition and Use: Critical Adaptations by Plants for Securing a Nonrenewable Resource. *New Phytol*, 157(3):423–447.

Vavasseur A and Raghavendra AS (2005). Guard cell metabolism and CO_2 sensing. *New Phytol*, 165(3):665–682.

Vazquez MJ, Rodriguez B, Zapatero C, and Tew DG (2003). Determination of phosphate in nanomolar range by an enzyme-coupling fluorescent method. *Anal Biochem*, 320(2):292–298.

Versaw W and Harrison M (2002). A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. *Plant Cell*, 14(8):1751–1766.

Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Lauriere C, and Merlot S (2009). Protein phosphatases 2C regulate

the activation of the snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant Cell*, 21(10):3170–3184.

Vuuren DPv, Bouwman AF, and Beusen AHW (2010). Phosphorus demand for the 1970-2100 period: A scenario analysis of resource depletion. *Glo env cha*, 20(3):428–439.

Wang FF, Lian HL, Kang CY, and Yang HQ (2010). Phytochrome B is involved in mediating red light-induced stomatal opening in *Arabidopsis thaliana*. *Mol Plant*, 3(1):246–259.

Wang P and Song CP (2008). Guard-cell signalling for hydrogen peroxide and abscisic acid. *New Phytol*, 178(4):703–718.

Wang RS, Pandey S, Li S, Gookin TE, Zhao Z, Albert R, and Assmann SM (2011). Common and unique elements of the ABA-regulated transcriptome of *Arabidopsis* guard cells. *BMC Genomics*, 12:216–216.

Wang Y, Ribot C, Rezzonico E, and Poirier Y (2004). Structure and expression profile of the *Arabidopsis PHO1* gene family indicates a broad role in inorganic phosphate homeostasis. *Plant Physiol*, 135(1):400–11.

Ward J and Schroeder J (1994). Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell*, 6(5):669–683.

Weiner JJ, Peterson FC, Volkman BF, and Cutler SR (2010). Structural and functional insights into core ABA signaling. *Curr Opin Plant Biol*, 13(5):495–502.

Xue S, Hu H, Ries A, Merilo E, Kollist H, and Schroeder JI (2011). Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. *EMBO J*, 30(8):1645–58.

Yang XJ and Finnegan PM (2010). Regulation of phosphate starvation responses in higher plants. *Ann Bot*, 105(4):513–526.

Yang Y, Costa A, Leonhardt N, Siegel RS, and Schroeder JI (2008). Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. *Plant Methods*, 4:6.

Young JJ, Mehta S, Israelsson M, Godoski J, Grill E, and Schroeder JI (2006). CO_2 signaling in guard cells: calcium sensitivity response modulation, a Ca^{2+}-independent phase, and CO_2 insensitivity of the *gca2* mutant. *Proc Natl Acad Sci U S A*, 103(19):7506–7511.

Yuan H and Liu D (2008). Signaling components involved in plant responses to phosphate starvation. *J Integr Plant Biol*, 50(7):849–859.

Zeng W, Melotto M, and He SY (2010). Plant stomata: a checkpoint of host immunity and pathogen virulence. *Curr Opin Biotechnol*, 21(5):599–603.

Zhang X, Miao YC, An GY, Zhou Y, Shangguan ZP, Gao JF, and Song CP (2001). K^+ channels inhibited by hydrogen peroxide mediate abscisic acid signaling in *Vicia* guard cells. *Cell Res*, 11(3):195–202.

Zhang X, Wang H, Takemiya A, Song Cp, Kinoshita T, and Shimazaki Ki (2004). Inhibition of blue light-dependent H^+ pumping by abscisic acid through hydrogen peroxide-induced dephosphorylation of the plasma membrane H^+-ATPase in guard cell protoplasts. *Plant Physiol*, 136(4):4150–4158.

Zhang Y, Zhu H, Zhang Q, Li M, Yan M, Wang R, Wang L, Welti R, Zhang W, and Wang X (2009). Phospholipase dalpha1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*. *Plant Cell*, 21(8):2357–2377.

Zhou Y and Ni M (2010). SHORT HYPOCOTYL UNDER BLUE1 truncations and mutations alter its association with a signaling protein complex in *Arabidopsis*. *Plant Cell*, 22(3):703–715.

Zhu SY, Yu XC, Wang XJ, Zhao R, Li Y, Fan RC, Shang Y, Du SY, Wang XF, Wu FQ, Xu YH, Zhang XY, and Zhang DP (2007). Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. *Plant Cell*, 19(10):3019–3036.

Acknowledgements

I would like to offer my sincere gratitude to my supervisor Yves Poirier, for giving me the opportunity to carry out my doctoral thesis, be part of his research group, and for creating a perfect working environment. I am particularly grateful for his continuing trust, motivation and guidance throughout this challenging project.

I would like to thank Alain Vavasseur, Nathalie Leonhardt (CEA Cadarache, France) and Enrico Martinoia (University of Zürich, Switzerland) for precious scientific advise and valuable comments, as well as for welcoming me in their laboratories and introducing me to guard cell research. I am also grateful towards Rainer Hedrich and Hubert Bauer (University of Würzburg, Germany) for their help on the guard cell expression profiling, and for constructive comments on the project.

I would like to thank past and present members of the Poirier group for the friendly and supportive environment, in particular Bulak Arpat for his constant help and advice; Pasqualina Magliano and Syndie Delessert for their precious help with the tedious leaf harvesting; and Olivier Emery for his hard work on the high phosphate sensitive mutant screen.

Past and present members of the DBMV: I have truly enjoyed my time here and will miss being part of the department. Thank you all for the great working atmosphere you have provided me over the past five years. A particular warm thank you to my friends Imene Garroum, Guillaume Fabre, Fabian Schweizer, Frederic Mascaux, Kimberley Tilbrook, Julien Alassimone, Lothar Kalmbach, Friederike Brüssow, Jonatan Montpetit, Khan Ghazanfar and Christelle Bonnet, with whom I share the best memories of my time spent in Lausanne. You will all be greatly missed, and I wish you all well in your future science/life projects!

I would finally like to thank the Société Académique Vaudoise, the Bureau de l'Egalité des Chances (UNIL), and the Swiss Plant Science Web (SPSW) project, for their very generous financial support with research and mobility grants.