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Roles of the ABCG21 and ABCG22 Transporters in Stomatal Functioning

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Roles of the ABCG21 and ABCG22 Transporters in Stomatal Functioning

Abstract:

Stomata are specialised pores that mediate gas exchange and water loss in plants by changing their aperture. A number of ATP-binding cassette transporters are known to be involved in regulation of stomatal movements. Thus, ABCG22 has been shown to mediate stomatal closure in low air humidity conditions, but roles for ABCG22 and for another functionally related ABC transporter, ABCG21, in stomatal regulation has not been fully clarified. In this study, we demonstrate that ABCG22 is involved in stomatal movements induced by several environmental factors, such as low air humidity, ozone pulse, and CO2, while the *abcg21* mutant showed wildtype stomatal responses to these stimuli. In addition, generation of transgenic plants expressing modified *ABCG22* has been initiated.

Keywords:

Õhulõhe, sulgrakud, õhulõhede juhtivus, ABC-transporterid, ABCG22, ABCG21

CERCS: B310 Physiology of vascular plants

ABCG21 ja ABCG22 transporterite roll õhulõhede funktsioneerimises

Lühikokkuvõte:

Õhulõhed on spetsiaalsed poorid, mis oma avatust muutes vahendavad taimede gaasivahetust ja vee aurustumist. On teada, et õhulõhede regulatsioonis mängivad rolli ABCG-transporterid, näiteks ABCG22 vahendab õhulõhede sulgumist madala õhuniiskuse mõjul. ABCG22 ja sellega funktsionaalselt suguluses oleva teise ABCG-transporteri, ABCG21, roll õhulõhede sulgumisel on hetkel ebaselge. Antud töös näitame, et ABCG22 osaleb õhulõhede sulgumises mitmete keskkonnategurite (madal õhuniiskus, osoon ja CO_2) mõjul. Samas *abcg21* mutantide õhulõhed reageerisid samadele stiimulitele sarnaselt metsiktüübiga. Lisaks alustasime transgeensete taimede, mis ekspresseerivad modifitseeritud *ABCG22*, loomist.

Võtmesõnad:

Õhulõhe, sulgrakud, õhulõhede juhtivus, ABC-transporterid, ABCG22, ABCG21

CERCS: B310 Soontaimede füsioloogia

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ABBREVIATIONS

ABA – abscisic acid

ABC – ATP-binding cassette

AtABCG21 – ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G21

AtABCG22 – ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G22

CAM – crassulacean acid metabolism

HT1 – HIGH LEAF TEMPERATURE 1

MAMP – microbe-associated molecular pattern

NBD – nucleotide-binding domain

OST1 – OPEN STOMATA 1

PM – plasma membrane

RTD – rapid transient decrease

SLAC1 – SLOW ANION CHANNEL-ASSOCIATED 1

SLAH3 – SLAC1 HOMOLOGUE 3

T-DNA – transfer-DNA

TMD – transmembrane domain

YFP – yellow fluorescent protein

INTRODUCTION

Plants support life on Earth by producing oxygen and organic matter as a result of photosynthesis. They began conquering the land around 500 million years ago (Morris *et al.*, 2018), transforming it and allowing animals to spread and flourish. Moving onto the land brought a new challenge for plants: the need to preserve water.

Water loss and gas exchange are mediated by stomata – pores on the surface of leaves. Each stoma is surrounded by two guard cells that are able to swell and shrink to open or close the pore. Plants respond to various environmental signals by stomatal movement, adjusting to its surroundings and threats. As an example of defensive mechanisms, stomata can close within minutes of exposure to air pollutants or pathogens.

While the ability of plants to open and close their stomata has been known for decades, many processes involved in stomatal movement are not fully understood yet. The recent advancements in research leads to new discoveries and extend our knowledge about stomatal behaviour. One such research identified ABCG22, an ABC transporter that plays an important role in stomatal closure in dry air (Kuromori *et al.*, 2011). In this study, the ABCG22 and ABCG21 transporters are further studied by generation of transgenic plants and conducting a series of gas exchange experiments with their mutants.

The current study was performed in The Plant Signal Research Group at the Institute of Technology.

1 LITERATURE REVIEW

1.1 Roles of stomata in plant processes

The process of photosynthesis in algae and plants is the major part of the oxygen and carbon cycles and results in production of not only oxygen, but also nearly all organic matter in the Earth's biosphere. (Field *et al.*, 1998) To perform photosynthesis, plants require light, water and carbon dioxide (CO_2). The leaf epidermis is covered by the waxy cuticle, which protects plants from desiccation, but also prevents the CO_2 entrance. The majority of plant gas exchange is performed through stomata (singular "stoma") – specialised pores in the leaf epidermis. The pores allow the uptake of CO_2 and the release of produced oxygen into the atmosphere.

The aperture of a stoma is regulated by two guard cells surrounding the pore. The size and shape of guard cells vary between plant species. However, the main properties and movement mechanics are shared by all stomata: the pore is opened by the reversible swelling and bending of the guard cells. This similarity points to a common evolutionary background of the stomata in plants, although the differences in environment resulted in variety in shape, size and distribution of the pores, as well as regulation mechanisms (Vatén and Bergmann, 2012).

The appropriate mechanisms of stomatal opening and closure in response to environmental signals are required for plant development and vitality. The transpiration stream, while necessary for uptake of nutrients from the soil, has to be tightly regulated to avoid excessive water loss and maintain cellular turgor pressure.

Stomata are vulnerable points of the plant, often acting as entry sites for toxins and pathogens. Ozone, an air pollutant that mostly enters plants through stomata (Kerstiens and Lendzian, 1989), causes 8-14% global yield loss for soybean, 3.9-15% for wheat, and 2.2-5.5% for maize (Avnery *et al.*, 2011). In developing regions with rising ozone levels, yield losses of wheat are predicted to increase up to 53% by 2050 (Tai and Val Martin, 2017). Pathogenic fungi and bacteria that infect plants through stomata can cause up to 80% yield losses (Fisher *et al.*, 2012).

The variation in stomatal density and stomatal movements results in differences how plants adapt to limited water availability. Understanding of mechanisms that define water use efficiency in crops is an important step toward solving the problem of rapidly rising water usage for crop production.

1.2 Triggers and mechanisms of stomata regulation

Stomatal movements are caused by changes of turgor pressure, generated by water moving into and out of the large vacuoles in the guard cells. This type of cells responds to environmental or internal (hormonal) signals. Water potential is adjusted by transporting ions across the vacuolar and cell membrane through ion channels, ion pumps and transporters. (Daszkowska-Golec and Szarejko, 2013)

Water potential in guard cells can be described as the difference between the turgor pressure affecting the cell wall and the osmotic pressure of the cell (H. Kollist *et al.*, 2014). Enrichment of guard cells with ions increases the osmotic pressure, lowering the cell's water potential and initiating the uptake of water into the vacuoles until a steady state is reached.

Signal reaction pathways may differ between plant species. In the following overview, conditions and triggers of stomatal movements will be described based on *Arabidopsis thaliana*, a model organism plant utilising C_3 carbon fixation metabolic pathway.

1.2.1 Stomatal opening

The majority of plants, with CAM and C_4 plants being the exceptions, open their stomata during daytime to provide photosynthesising mesophyll cells with CO_2. The photosynthetic process results in accumulation of transitory starch in guard cells and mesophyll. Transitory starch is used as a carbon deposit when photosynthesis rate is high, and as a carbon source when photosynthesis rate is low or stopped in darkness (Sean *et al.*, 2010). In guard cells, starch is rapidly degraded to osmotically active monosaccharides promoting the quick increase of stomatal aperture during the first 30 minutes of exposure to light. (Horrer *et al.*, 2016)

As the first step in opening a stoma, ATP is used to pump out protons from the cytosol into the apoplast through plasma membrane (PM) H^+-ATPase and from the cytosol into the vacuole through vacuolar H^+-ATPases and pyrophosphatases. The hyperpolarisation of the PM drives the uptake of K^+ through K^+ inward-rectifying channels from the apoplast into the cytosol. The influx of K^+ is counter-balanced by several types of anions. Cl^- and NO^{3-} are taken up from the apoplast, with NO^{3-} being transported by *AtNRT* nitrate transporters (Guo *et al.*, 2003). $Malate^{2-}$ is released during the degradation of starch stored in the cells or is uptaken from the apoplast by the ABCB14 transporter (Lee *et al.*, 2008). The majority of the ions are transported across the tonoplast and stored in the vacuole: K^+ is transferred

through NHX transporters; malate^{2-} and a part of Cl^- ions are transferred through aluminium-activated anion channels; NO^{3-} and Cl^- are transferred through chloride antiporters. The buildup of ions leads to the uptake of water through aquaporins into the vacuole, increasing the cell's turgor pressure and leading to the guard cell swelling and bending, opening the pore (Figure 1A) (Daszkowska-Golec and Szarejko, 2013; Kollist *et al.*, 2014).

The stomatal aperture is increased when the plant is exposed to the air with low levels of CO_2. Guard cells react to intercellular CO_2 concentration – specifically, to its content in the substomatal cavity – rather than to external CO_2 concentration (Ainsworth and Rogers, 2007). Exposure to red and blue light is another strong trigger for stomatal opening. The reaction of stomata to photosynthetically active red light might be indirect and connected with the levels of CO_2 in the apoplast near the guard cells (Roelfsema *et al.*, 2002). When a large area of a leaf is illuminated by red light, CO_2 levels in the substomatal cavity is reduced. This change is detected by the guard cells and triggers the stomatal opening mechanisms (Matrosova *et al.*, 2015). HIGH LEAF TEMPERATURE 1 (HT1) protein kinase is an essential regulator of response to CO_2. The *ht1* mutants exhibit strongly impaired stomatal opening responses to both red light and low CO_2 in *A. thaliana* (Matrosova *et al.*, 2015).

Blue light signalling results in activation of H^+-ATPase in the PM of guard cells. Activated blue light receptors, phototropins *phot1* and *phot2,* bind to serine/threonine-protein kinase BLUE LIGHT SIGNALING1, whose activity is necessary for the activation of H^+-ATPase (Kinoshita *et al.*, 2001). PROTEIN PHOSPHATASE Type 1, consisting of catalytic and regulatory subunits, is a component of blue light signalling, playing a role in signal transduction from phototropins to PM H^+-ATPase. (Aoki *et al.*, 2019) More recent research has proposed a novel protein kinase BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION to be involved in blue light-dependent stomatal opening (Hayashi *et al.*, 2017).

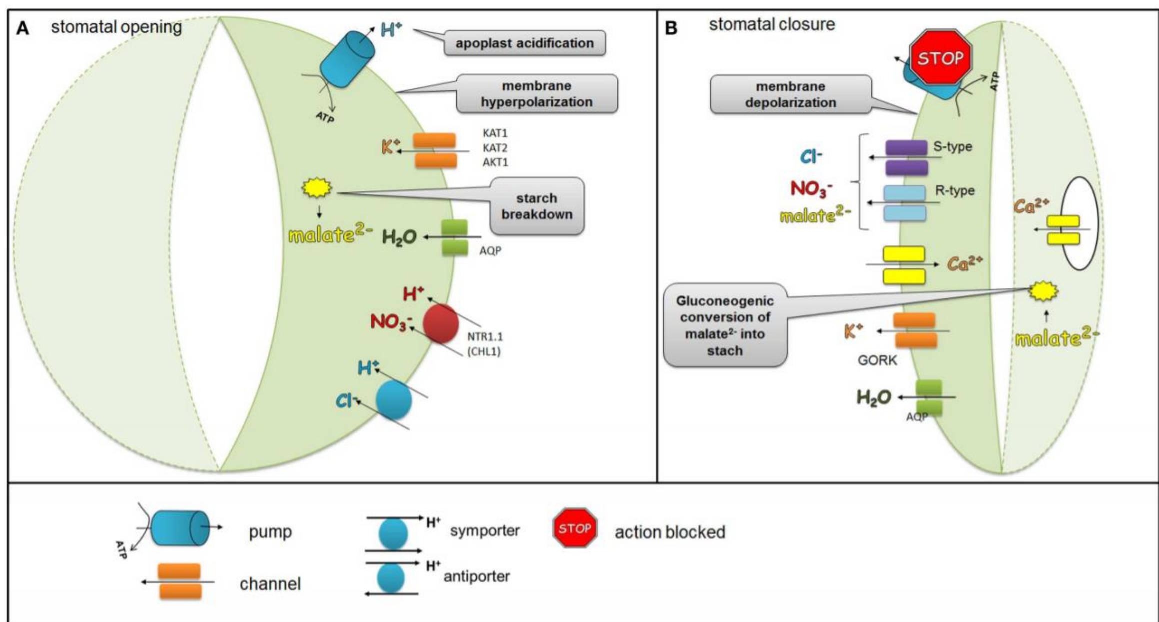

Figure 1. Mechanisms of stomatal opening and closure. **(A)** Protons are pumped out through H^+-ATPase, which leads to hyperpolarization of PM and activation of K^+ inward-rectifying channels. Influx of K^+ is balanced by Cl^-, NO^{3-} and malate^{2-} anions. Buildup of ions leads to water accumulation through aquaporins, generating turgor pressure and opening the pore. **(B)** During stomatal closure, inhibition of H^+-ATPase causes depolarization of PM, which causes activation of K^+ outward rectifying channels. Anions move out of the cell through S-type and R-type channels, malate^{2-} is conversed into starch. Turgor pressure is reduced and the pore closes (Daszkowska-Golec and Szarejko, 2013).

1.2.2 Stomatal closure

Most plants close their stomata in darkness to prevent water loss while photosynthesis is not active, although endogenous biological rhythms may cause some plants to close the stomata in the evening (Webb, 2003). Rapid stomatal closure is an important defence mechanism which can be activated by several pathways. In addition to darkness and high CO_2, stomatal closure is triggered by air pollutants, plant pathogens, unfavourable temperatures, and ultraviolet-B light (Schulze *et al.*, 1973; Tossi *et al.*, 2014; Willmer and Fricker, 1996).

There are two types of anion channels found in plasma membrane of guard cells: slow (S-type) anion channels, which are activated in seconds, and rapid (R-type) anion channels, which respond in milliseconds (Schroeder and Keller, 1992). The anion channels have to be activated by phosphorylation to be functional. The S-type anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) is crucial for stomatal closure in response to high CO_2 concentration, low air humidity, and the air pollutant ozone (O_3) (Vahisalu *et al.*, 2008).

SLAC1 and SLAC1 HOMOLOGUE 3 (SLAH3) expressed in Arabidopsis guard cells are also important for stomatal closure in response to recognition of microbe-associated molecular patterns and abscisic acid (ABA) (Guzel Deger *et al.*, 2015). QUAC1 acts as an R-type anion channel in guard cells (Meyer *et al.*, 2010).

ABA is a phytohormone that regulates multiple processes in plant development and response to environmental changes, including stomatal closure. Also referred as a "drought hormone", ABA acts as an inducer of S-type and R-type anion channels, and as an inhibitor of PM H^+-ATPases in guard cells, initiating stomatal closure (Jezek and Blatt, 2017). The OST1 protein kinase mediates ABA-triggered stomatal closure (Mustilli *et al.*, 2002). During stomatal closure, Cl^- and NO^{3-} are moved to the apoplast through the anion channels; amount of $malate^{2-}$ in the cells is decreased through the R-type channels and by gluconeogenically converting malate into starch (Willmer and Fricker, 1996). The resulting depolarization of the PM drives activation of K^+ outward rectifying channels, such as guard cell outward rectifying K^+ channel. The tonoplast is depolarised through the two-pore K^+ channels, resulting in efflux of ions stored in the vacuole. The Ca^{2+} channels allow the influx of Ca^{2+} from the apoplast and the vacuole into the cytoplasm. These processes result in efflux of water out of the guard cells, reducing the turgor pressure and leading to the pore closure (Figure 1B) (Jezek and Blatt, 2017).

1.3 ABC transporters

ATP-binding cassette (ABC) transporters form a protein superfamily and can be found in cellular and organelle membranes of all living organisms, from prokaryotes to humans (Henikoff *et al.*, 1997). Initially found to be involved in detoxification of various substances (Hyde *et al.*, 1990), ABC transporters are known to be vital for a wide range of processes, from organism development and nutrient transport to responses to environmental signals (Martinoia *et al.*, 2002). Plant genomes encode more ABC transporters than animal genomes. The *A. thaliana* genome encodes 130 ABC transporters that perform import and export of secondary metabolites, hormones, lipids, and metals as well as regulate ion channels and reactions to pathogens (Verrier *et al.*, 2008). However, only a small part of the ABC transporters has been functionally analysed, while many substrates for ABC transporters remain to be elucidated in future research (Kretzschmar *et al.*, 2011). The ABC transporter functions are mostly studied via the reverse genetics approach; however, mutations in ABC transporters may cause changes in downstream metabolic pathways, making conclusions about their substrate more complicated (Lefèvre and Boutry, 2018).

Additionally, many ABC transporters recognise several substrates (Wright *et al.*, 2018) and can be involved in various processes.

1.3.1 Structure

ABC transporters consist of the hydrophobic transmembrane domains (TMDs) and the cytosolic nucleotide-binding domains (NBDs). NBDs contain the conserved sequence motifs involved in ATP binding and hydrolysis. One of these motifs is an amino acid “signature” found only in ABC transporters (Lefèvre and Boutry, 2018). The full-size proteins contain two TMDs and two NBDs encoded by a single gene, while the half-size proteins contain only one TMD and one NBD. Since two NBDs and two TMDs are required to perform transport of a substance, the half-size ABC transporters form homo- or heterodimers to be functional (Verrier *et al.*, 2008). The domain structure, size, and orientation in the membrane allows to divide all ABC transporters into eight subfamilies, seven of which have been found in plants, with the exception of ABCH (Verrier *et al.*, 2008). The general mechanism of ABC transporter functioning is shown in Figure 2. Details of ABC transporter mechanism are yet to be determined, with multiple models being proposed (Kang *et al.*, 2011; Wright *et al.*, 2018).

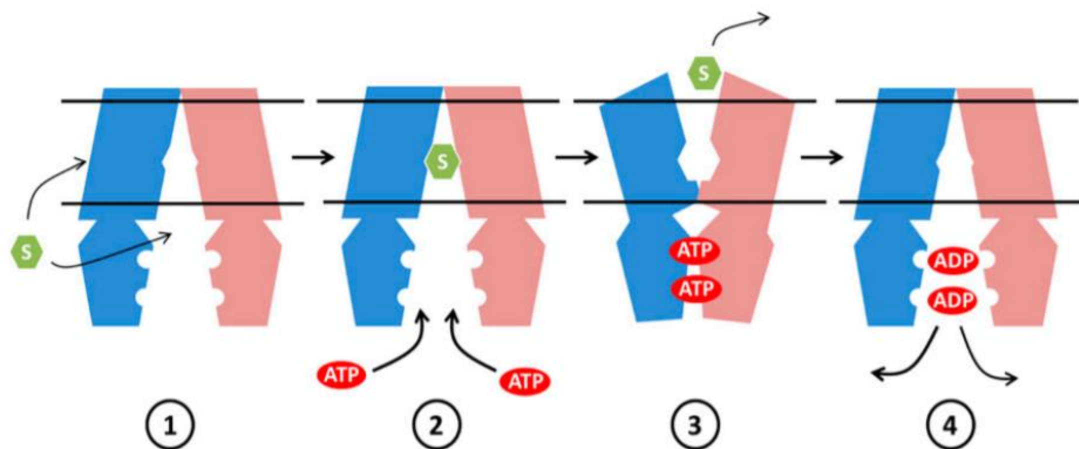

Figure 2. The general scheme of ABC transporter functioning. The substrate pocket formed by TMDs recognises and binds the substrate molecules. The detection and binding of the substrate activates the binding of ATP molecules. ATP binding changes the conformation of the transporter: NBDs dimerise, orienting TMDs to the other side of the membrane, releasing the transporting substrate. ATP hydrolysis releases the NBD dimer, returning the protein to the initial conformation (Lefèvre and Boutry, 2018).

1.3.2 ABCG subfamily

ABCG transporters are characterised by the reverse (NBD-TMD) domain organisation. In *A. thaliana* it is the largest ABC protein subfamily consisting of 15 full-size and 28 half-size transporters, all of which, with the exception of ABCG19 and ABCG28, have been localized in the plasma membrane (Verrier *et al.*, 2008). The ABCG transporters are involved in many various processes important for the plant's development and survival.

Stomatal regulation is known to be influenced by AtABCG25, the ABA exporter (Kuromori *et al.*, 2010, p. 25); AtABCG40, the ABA importer found in guard cells, and possibly the camalexin transporter (Gräfe and Schmitt, 2021), and AtABCG21/AtABCG22 (Kuromori *et al.*, 2017).

1.4 AtABCG22

1.4.1 ABCG22 Characterization

In 2010, Kuromori *et al.* described the AtABCG25 protein which plays a role in ABA transport and signalling (Kuromori *et al.*, 2010). Further research of phylogenetically related ABC transporters led to analysis of a closely related protein whose mutant line had a phenotype suggesting a disruption of ABA pathway (Kuromori *et al.*, 2011). The analysed *At5g06530* gene encodes the AtABCG22 transporter belonging to the same phylogenetic branch as AtABCG25 (Figure 3). The mutant with the transposon insertion in the third exon of *AtABCG22* was designated *abcg22-1* (Kuromori *et al.*, 2004, 2011).

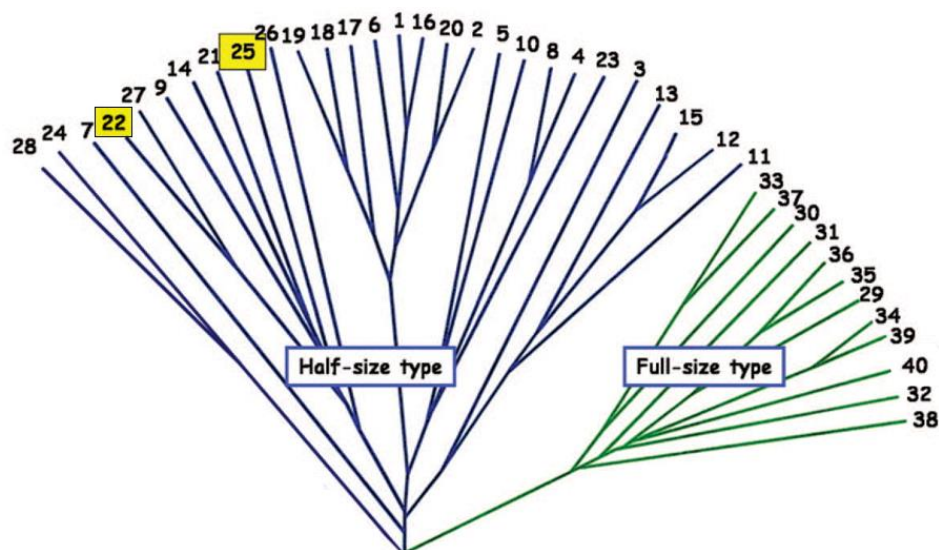

Figure 3. Phylogenetic relationships of AtABCG subfamily members. AtABCG22 and AtABCG25 are indicated with yellow boxes.

The *abcg22-2* mutant is another allele of *AtABCG22.* It was generated by a transfer-DNA (T-DNA) insertion into the first exon of the gene in *A. thaliana* accession Columbia (Col) line.

Kuromori *et al.* (2011) analysed these mutants and showed that under normal growth conditions the *abcg22* knock-out mutants are phenotypically identical to wild type Arabidopsis. Thermographic imaging showed a lower leaf temperature in both *abcg22* mutants, suggesting increased transpiration and more open stomata, what was confirmed with water loss experiments with detached leaves (Figures 4b and c). Measurements of stomatal aperture showed that the stomata of *abcg22* are more open than those in wild type. The *abcg22* plants were more susceptible to drought, wilting faster than wild type plants without water supply. Thus, the conducted experiments indicated that the AtABCG22 transporter plays a role in stomatal regulation.

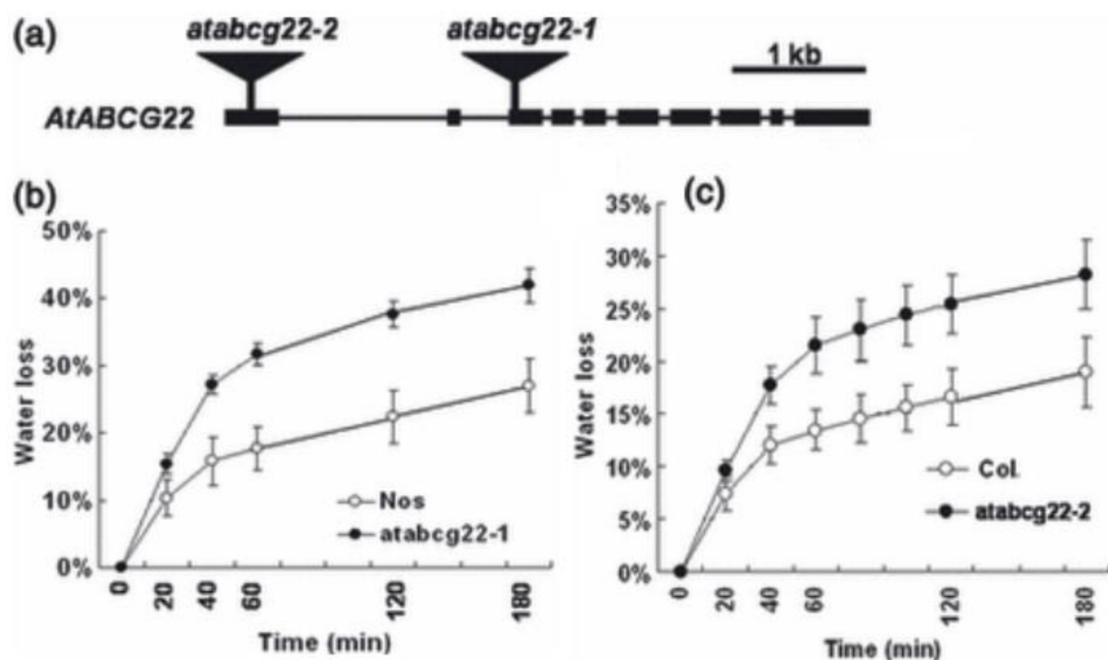

Figure 4. Characterization of the *abcg22* mutants. (a) The structure of the AtABCG22 gene and the positions of the insertions in the *abcg22* mutants. Black boxes represent exons, lines represent introns. The *atabcg22-1* transposon insertion is located in the third exon of the gene, and *atabcg22-2* T-DNA insertion is located in the first exon. (b, c) Comparison of the water loss from detached leaves in *atabcg22* with wild type Nossen (Nos) and Columbia (Col) plants (Kuromori *et al.*, 2011).

Kuromori *et al.* studied the expression patterns of *AtABCG22* in different plant tissues by using semiquantitative RT-PCR. This gene was expressed in aerial organs, most prominently

in the leaves. Further analysis by using transgenic plants expressing β-glucuronidase under control of the native ABCG22 promoter suggested localisation of ABCG22 in guard cells.

Kuromori *et al.* (2011) studied the subcellular localization of AtABCG22 transporter by constructing a recombinant gene which produces a yellow fluorescent protein (YFP) fused with ABCG22. The protein expression was studied in onion cells and tobacco BY2 culture cells through visualization by confocal imaging. The observed yellow fluorescence has shown that the YFP-ABCG22 recombinant protein was localized in the cell membrane (Figure 5).

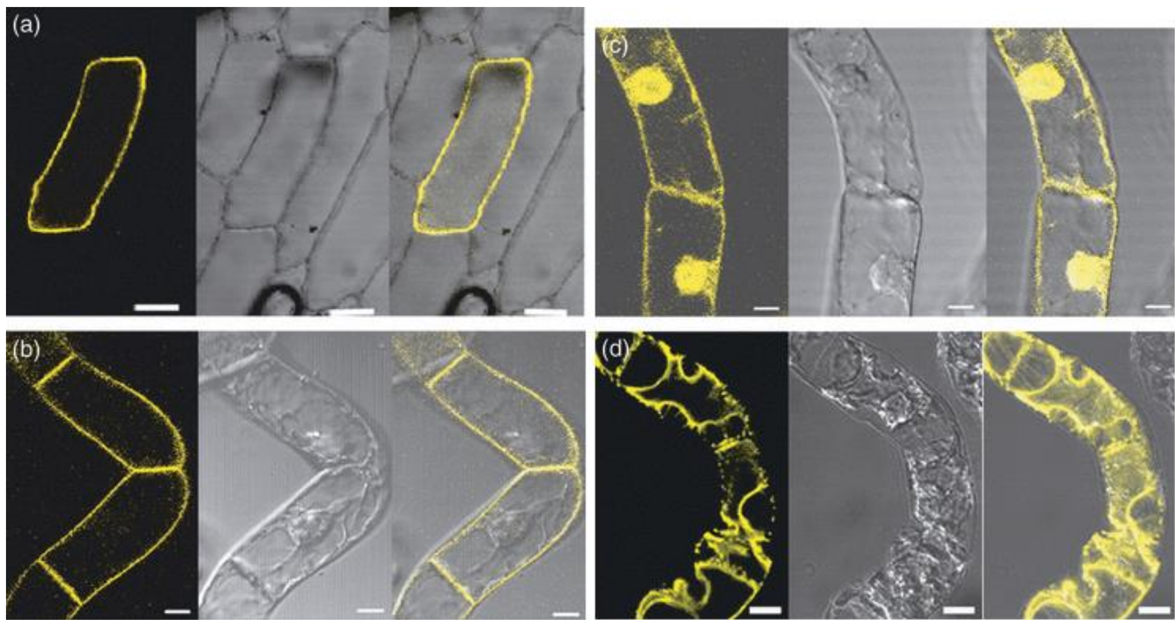

Figure 5. YFP fluorescence in onion (a) and tobacco BY2 culture (b-d) cells. (b) and (c) compare localization of YPF-ABCG22 fused protein with YPF alone, showing that *YPF-ABCG22* is expressed outside of the cell nucleus. Plasmolysis with 8M mannitol (d) separated the recombinant protein from the cell wall. (Kuromori *et al.*, 2011)

In a series of gas exchange experiments done by (Merilo *et al.*, 2015) *abcg22* plants showed significant delay in initiation of fast stomatal response incused by low air humidity. Additionally, stomatal closure in response to exogenous ABA appeared to be intact, indicating that ABA is not the substrate of ABCG22.

Later, a double mutant lacking ABCG22 and another ABCG transporter ABCG21 was generated. In contrast to the *abcg22* mutants, the double *abcg22/abcg21* mutant displayed wild type stomatal phenotypes, including leaf temperature and water loss from detached

leaves. This indicates that ABCG21 might transport the same substrate as ABCG22 but in opposite direction (Kuromori *et al.*, 2017).

1.4.2 ABCG22/OST3 phosphorylation by OST1

The *ost3-1* mutant is an *abcg22* mutant line in the Ler-0 genetic background, which was independently identified in a mutant screen performed in Jeffrey Leung' laboratory. The *ABCG22* gene in this mutant features a premature stop codon, resulting in a truncated ABCG22 protein. Furthermore, Jeffrey Leung with colleagues analysed preferable phosphorylation sites for OST1/SnRK2e protein kinase (Vlad *et al.*, 2009). *In silico*, 3 motives of L_{-5}-x-R_{-3}-x-x-$^{S}/_{T}$ (S or T are the phosphorylatable residues serine or threonine, respectively; X is any amino acid) were identified at the N-end of ABGC22. *In vitro* kinase assays demonstrated that OST1 phosphorylates ABCG22 at S17, S59 and S104 as substitution of these serine residues with alanine residues greatly reduced phosphorylation levels, with the proteins with the triple substitutions having the lowest phosphorylation level. Recently, a collaboration between Jeffrey Leung and our laboratory has been established, in the frame of which this study was performed.

2 THE AIMS OF THE THESIS

The aims of this study are:

- Generation and selection of transgenic plants expressing modified *ABCG22* with blocked or mimicked phosphorylation.
- Gas exchange experiments for determining stomatal phenotypes of *abcg22* and *abcg21* mutants.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Agrobacterium tumefaciens strain GV3101 stored in the Plant Signal Research group collection was used for the generation of transgenic plants expressing ABCG22 in the *abcg22* genetic background.

pMLBart is a plasmid, on a basis of which the vectors for for plant transformation were constructed by Dmitry Yarmolinsky. The resulting plasmids provide spectinomycin resistance to *A. tumefaciens* and BASTA herbicide (glufosinate ammonium) resistance to plants. The used plasmids carry either the guard cell-specific GC1 promoter (Yang *et al.*, 2008) or the ABCG22 native promoter. These promoters drive expression of the mVenus fluorescent protein fused with the ABCG22 coding region. Some of the plasmids were designed to express modified *ABCG22* sequences with substitutions of serine at the positions 17, 59, 104 to alanine or glutamate (Lal *et al.*, 2018) to block or mimic phosphorylation, respectively.

Table 1. Genotypes of the model organism *Arabidopsis thaliana* used in this study.

***A. thaliana* line**	**Genotype**	**Mutation**
Col-0	Wild type	-
abcg22-2	Loss-of-function for ABCG22; Col-0 genetic background	T-DNA mutant with the insertion in the coding region
abcg22-3	Loss-of-function for ABCG22; Col-0 genetic background	T-DNA mutant with the insertion in the coding region
abcg21	Loss-of-function for ABCG21; Col-0 genetic background	T-DNA mutant with the insertion in the coding region
ost1-3	Loss-of-function for OST1; Col-0 genetic background	T-DNA mutant with the insertion in the coding region
Ler-0	Wild type	-
ost3-1 / abcg22_Leu471	Truncated ABCG22 protein; Ler-0 genetic background	a premature stop codon in the coding region

3.1.2 Growth conditions

A. thaliana plants were grown in soil containing 2:1 peat:vermiculite under the following conditions: 70% relative humidity, 10 hour day – 14 hour night cycle, 23°C during the day and 18°C during the night, 250 μmol m^{-2} s^{-1} illumination.

Plants used for the gas exchange experiments were grown in half-filled 10×10×6 cm growth pots covered with 10×10×0.25 cm glass plates with a conical hole in the middle. The holes were 0.3 cm in diameter. The plants were grown through the hole in the glass plate (Kollist *et al.*, 2007). Prior to the gas exchange experiments, the holes were sealed with grafting wax.

3.1.3 Preparation of *Agrobacterium tumefaciens* strains for plant transformation

The *A. tumefaciens* cells from a stock at -80°C were grown on gentamycin-containing (25 μg/ml) LB (Luria-Bertani) medium plates for 2 days at 28°C. Single isolated colonies were used to inoculate 2 ml of yeast extract peptone medium (YEP; 10 g bacto peptone, 5 g NaCl, 10 g yeast extract, no pH adjustments) with 25 μg/ml gentamycin in 15-ml glass tubes. The tubes were incubated overnight on a shaker (200 rpm) at 28°C. The overnight culture was used to inoculate 50 ml of YEP with gentamycin; the inoculated YEP was incubated at 28°C until its optical density at 600 nm reached approximately 0.5. The obtained culture was chilled on ice for 10 minutes, then centrifuged in 50-ml falcon tubes at 4000 rpm for 20 min at 4°C. After discarding the supernatant, the cell pellet was resuspended in 10 ml of ice-cold 0.15M NaCl. The suspension was centrifuged at 4000 rpm for 5 min at 4°C. After discarding the supernatant, the cell pellet was resuspended in 1 ml ice-cold 20 mM $CaCl_2$. The suspension of competent cells was aliquoted into pre-chilled 1.5 ml tubes, frozen in liquid nitrogen, and stored in -80°C freezer.

The plasmid transformation of *A. tumefaciens* cells was done by the heat shock protocol. One μl of a plasmid was added to 100 μl of competent cells which were unfrozen on ice. The cells were incubated on ice for 30 minutes, then at 37°C for 3 minutes, and 2 more minutes on ice. After addition of 1 ml of LB liquid medium, the cells were incubated on a 200 rpm shaker for 2 hours. The cells were then centrifuged at 7000 rpm for 2 minutes to form a cell pellet and to discard the most of the medium. The cells were resuspended in the remaining medium, and the mixture was plated on selection plates: LB plates with 25 μg/ml Gentamicin and 50 μg/ml spectinomycin. The plates were incubated at 28°C for 3 days.

3.1.4 Transgenic plant generation

The transformation of *abcg22-2 A. thaliana* mutants was done by floral dip method. 10 days before the transformation procedure, the stems of the 5-6 weeks-old plants grown in the long day (16 h day/8 h night) were cut to stimulate formation a large number of new stems. Formed siliques were removed before the transformation.

The pre-culture of the transformed agrobacterium cells was grown in 3 ml of liquid LB medium at 28°C on a shaker for 24 hours. 1 ml of the pre-culture was transferred into 50 ml LB medium with 25 µg/ml gentamicin and 50 µg/ml spectinomycin and incubated overnight on a shaker at 28°C. The cells were centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 50 ml of 5% sucrose solution with 125 µl of SILVET L-77 surfactant.

Each stem was dipped into the mixture and gently agitated, so that every flower got in contact with the mixture. The bottom of a tray was lined with wet paper towels. The pots with dipped plants were placed on their sides, with stems facing each other, into the trays, with 6 pots in each tray. The tray with the plants was then covered with another black tray for maintaining humidity. The covered plants were left in a place protected from sunlight for 24 hours. The next day, the plants were uncovered, lifted upright in a new tray, and grown until the seeds matured and were ready for collection.

The collected seeds were sown in 2:1 peat:vermiculite soil. The young plants at the age of 6 days were treated with the commercial BASTA solution diluted 1 to 400. Plants that were resistant to the herbicide (Figure 6) were replanted into individual pots and grown for further analysis.

Figure 6. Selection of transgenic plants by glufosinate ammonium treatment. About 3000 seeds were germinated in soil and the seedlings were repeatedly sprayed with commercial BASTA solution in a day. The picture was taken after the 6th treatment. The green transgenic plants are seen on the background of dying non-transgenic seedlings.

3.1.5 Microscopy analysis

LSM 710 META Laser Scanning Microscope (Carl Zeiss) was used to examine stomata of transgenic plants. 514 nm laser beam was used for excitation of mVenus, and 518-565 nm channel for light emission. Fluorescence in the transgenic plants with GC1 promoter was detected with 10% laser power and 0.99 AU pinhole. Since fluorescence in the plants with the ABCG22 promoter was significantly weaker, 20% laser power and 7.24 AU pinhole were used.

3.1.6 Gas exchange experiments

The gas exchange experiments were made using a custom-made eight-cuvette rapid-response system which allows measurement of stomatal conductance of intact *A. thaliana* rosettes, as described by (Kollist *et al.*, 2007) (Figure 7). Each cuvette is a cylinder with 0.5 cm thick stainless steel walls. The cylinders are 3.5 cm high and have 7.8 cm inner diameter. Upper part of a cuvette is a rectangular glass place sealed to the steel cylinder and fixed to a frame.

The lower part of a cuvette is formed by the glass cover of a growth pot when it is inserted into the system. The pot is pressed to the polished lower rim of the cylinder by a spring underneath. The formed air-tight seal isolates the plant inside of the cuvette.

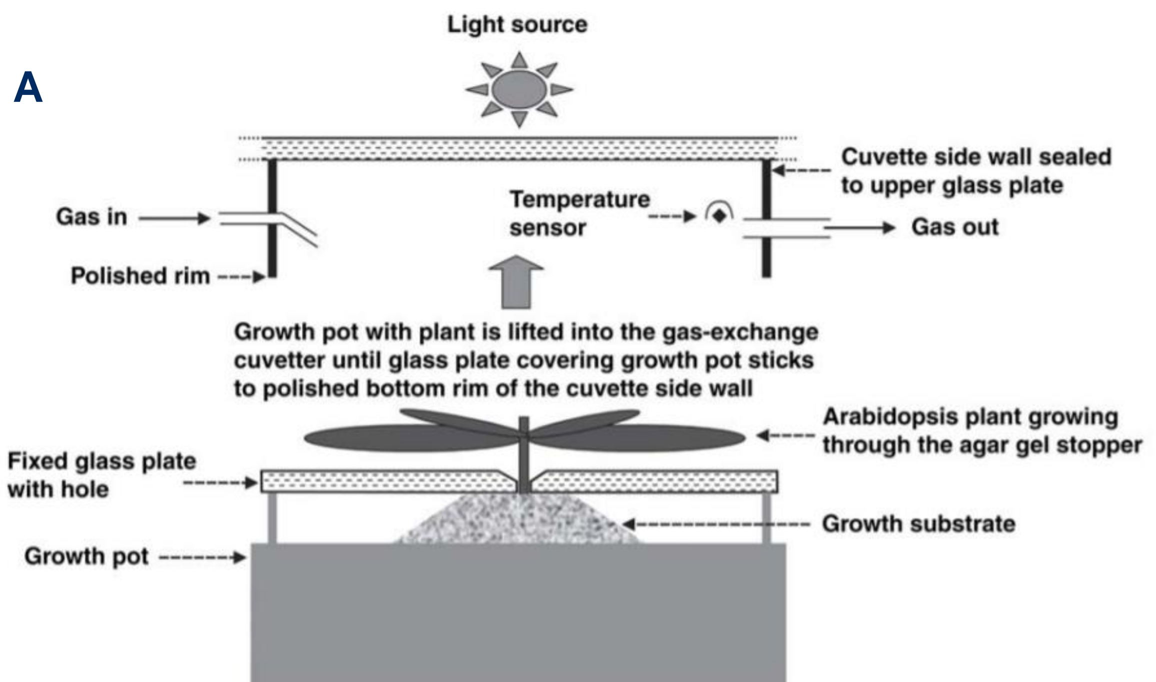

Figure 7. (A) Cuvette for *A. thaliana* gas exchange experiments. (Kollist *et al.*, 2007) **(B)** 8-chamber system used for gas exchange experiments.

Gas enters the cuvette through an input port in the cuvette wall and leaves through an output port, creating an air vortex over the rosette. Water vapour and CO_2 concentration are measured by LiCOR Li-7000 gas analyser with the readings stored on a computer. Stomatal conductance of each plant was measured every 4 minutes.

Readings about the gas entering and leaving the cuvette are processed by a custom application (Kollist *et al.*, 2007) and saved for further analysis.

For each gas exchange experiment done for this stidy, the plants were placed into the system and incubated for 1 hour for stabilization under standard conditions before the studied stimulus was applied. Standard conditions in the cuvettes are: ~400 ppm ambient CO_2, ~70% relative air humidity, 250 µmol m^{-2} s^{-1} light, 24°C.

3.2 RESULTS AND DISCUSSION

3.2.1 Generation and selection of transgenic plants expressing modified forms of ABCG22

The ABCG22 transporter has been established as an important element in rapid stomatal closure in response to drought (Kuromori *et al.*, 2011). Recent studies by Jeffrey Leung (unpublished) suggested that ABCG22 is phosphorylated by OST1 kinase. In order to study the functional interactions between ABCG22 and OST1 in more details, transgenic plants with modified *AtABCG22* were generated.

The plasmids used for transformation of *A. tumefaciens* cells were made by D. Yarmolinsky. The pGC1 (At1g22690) promoter was used in some of the plasmids. It is a strong promoter preferably expressed in guard cells and used for research of gene expression in guard cells of *A. thaliana* (Yang *et al.*, 2008). In order to achieve the wild type pattern of ABCG22 expression in transgenic plants, the native *pABCG22* promoter was also used.

The ABCG22 coding sequence in the plasmids was modified. Serine at the positions of 17, 59 and 104 were substituted to alanine to obtain $ABCG22^{S17A/S59A/S104A}$, which was designated as ABCG22(AAA). $ABCG22^{S17E/S59E/S104E}$ with glutamate substitutions at the same positions were designated as ABCG22(EEE). The substitutions with alanine at the phosphorylation sites block phosphorylation by protein kinases, while glutamate at the same positions is expected to mimic phosphorylation due to its negative charge.

The plasmids were named as follows:

- pGC1::mVenus-ABCG22(WT)
- pGC1::mVenus-ABCG22(AAA)
- pGC1::mVenus-ABCG22(EEE)
- pABCG22::mVenus-ABCG22(WT)
- pABCG22::mVenus-ABCG22(AAA)
- pABCG22::mVenus-ABCG22(EEE)

The plant transformation was done via floral dip method (Bent, 2006) used on flowering *abcg22-2* plants. The collected seeds were germinated. The seedlings were treated with commercial BASTA solution to select plants that successfully underwent transformation and were resistant to the herbicide. The T1 plants were replanted and grown for 4 weeks until they were suitable for examination through microscopy.

3.2.2 Transgenic plant microscopy analysis

To select T1 plants with strong activity of pGC1 and pABCG22 promoters, all plant specimens were examined with confocal microscopy. Many plants of the transgenic pGC1::mVenus-ABCG22 lines showed strong fluorescence in PM of guard cells, in a accordance to (Yang *et al.*, 2008). However, some lines also showed a weaker expression of the transgene in pavement cells (Figure 8A).

Fluorescence in the transgenic pABCG22::mVenus-ABCG22 lines appeared to be weaker than in the lines with the *GC1* promoter and required a higher laser power and a thicker optical slice. Fluorescence in guard cells was observed in multiple plants (Figure 8B), indicating successful transformation.

In continuation of this study, the selected transgenic plants with strong expression of mVenus-ABCG22 will be analysed further to select lines with a single T-DNA insert. Those will be brought to homozygosity. The homozygous T3 plants will be used for gas exchange experiments to examine stomatal phenotypes of plants with modified *AtABCG22* sequence and study the dependence of ABCG22 function on phosphorylation by OST1.

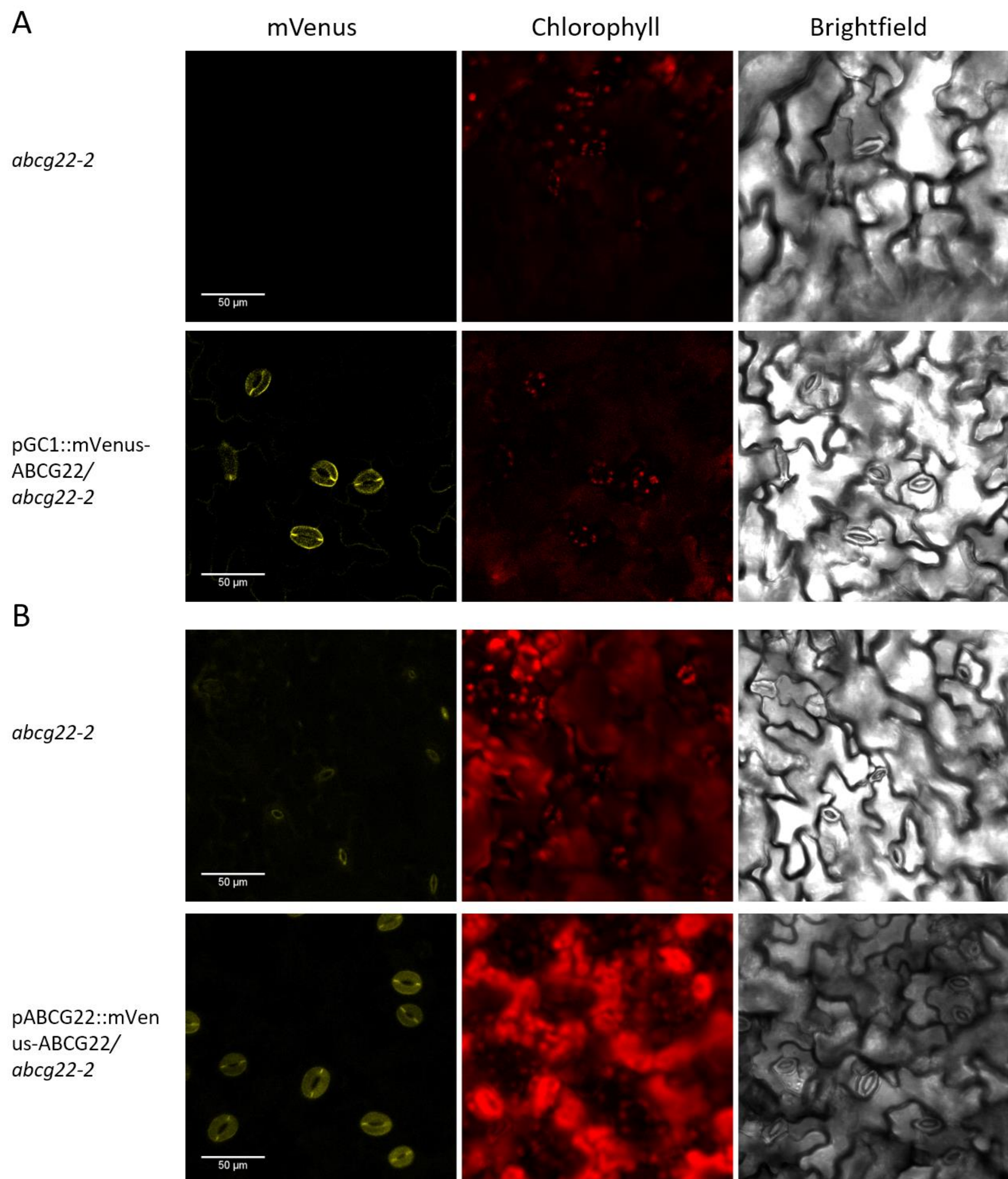

Figure 8. Expression of mVenus-ABCG22 driven by *pGC1* (A) and *pABCG22* (B) promoters in guard cells of transgenic plants. All images were taken with LSM 710 META Laser Scanning Microscope (Carl Zeiss). The *abcg22-2* line was used for control.

3.2.3 ABCG22 is required for rapid stomatal responses to fluctuations in air humidity

Reduced air humidity induces fast stomatal closure to restrict enhanced water evaporation. Although the exact mechanism of this type of stomatal closure is still unknown, a central role for protein kinase OST1 has been demonstrated (Merilo *et al.*, 2018). Moreover, a role for ABCG22 in initiation of stomatal closure in dry air was shown recently (Merilo *et al.*,

2015). It was decided to study whether the *abcg21* mutant, which might transport the same substance as ABCG22 in opposite direction (Kuromori *et al.*, 2017), would have an affected stomatal reaction to low air humidity. Stomatal reactions in the *ost3-1* mutant with truncated ABCG22 to low air humidity were not studied previously.

Air humidity in the gas exchange system with the plants was rapidly reduced from 70% to 20-30%. Stomatal conductance of each plant was then measured for 60 minutes (Figure 9).

The wild type plants demonstrated rapid stomatal closure as a drought adaptation mechanism, reaching the lowest values 15 minutes after the humidity reduction. The *abcg21* mutants' reaction was identical to the wild type. The *ost1-3* mutants showed only minor decrease in stomatal conductance as expected (Jalakas *et al.*, 2018).

The stomatal closure of *abcg22-2* and *abcg22-3* in response to low air humidity was delayed, however, stomatal conductance in these mutants reached the wild type values 30-45 minutes after the air humidity was reduced. These observations replicates the results obtained in the similar experiments previously conducted by Merilo *et al.* (Merilo *et al.*, 2015). Similar behaviour of stomata in *ost3-1* further confirms that ABCG22 plays a role in rapid stomatal response to reduced air humidity.

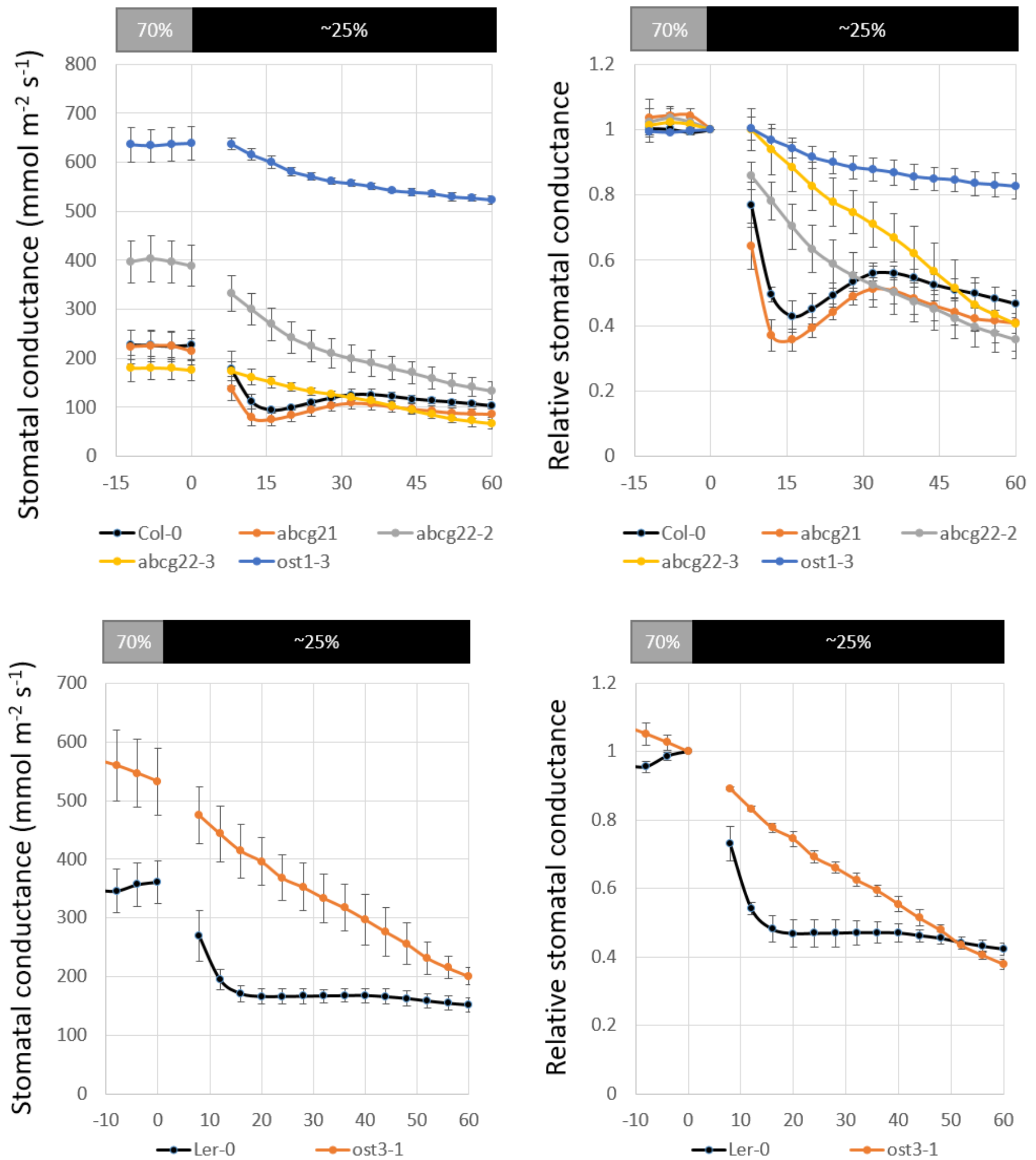

Figure 9. Stomatal responses to the reduction of air humidity in the studied plant lines. The *abcg22* mutants (*abcg22-2, abcg22-3, ost3-1*) demonstrate slower stomatal closure in dry air, compared to the wild type plants (Col-0, Ler-0). In 30-45 min after the air humidity reduction, the stomatal conductance of *abcg22* is similar to that of wild type. The stomatal response to low air humidity in *abcg21* is identical to than in wild type. The air humidity level was reduced at time 0.

To further investigate the role of ABCG22 in stomatal responses to air humidity levels, the experiments were prolonged to record the stomatal opening during the recovery of the plants from dry air. The air humidity in the cuvettes was returned from low (20-30%) to normal

level (70%) and the stomatal conductance of the plants was recorded over the course of an hour (Figure 10).

Stomata of the wild type and *abcg21* plants started to open immediately after the increase in air humidity. The stomatal conductance increased at high rate over 15-20 minutes, after which it slowed down but continued to rise after one hour. The *ost1-3* mutant demonstrated no significant reaction to the stimuli.

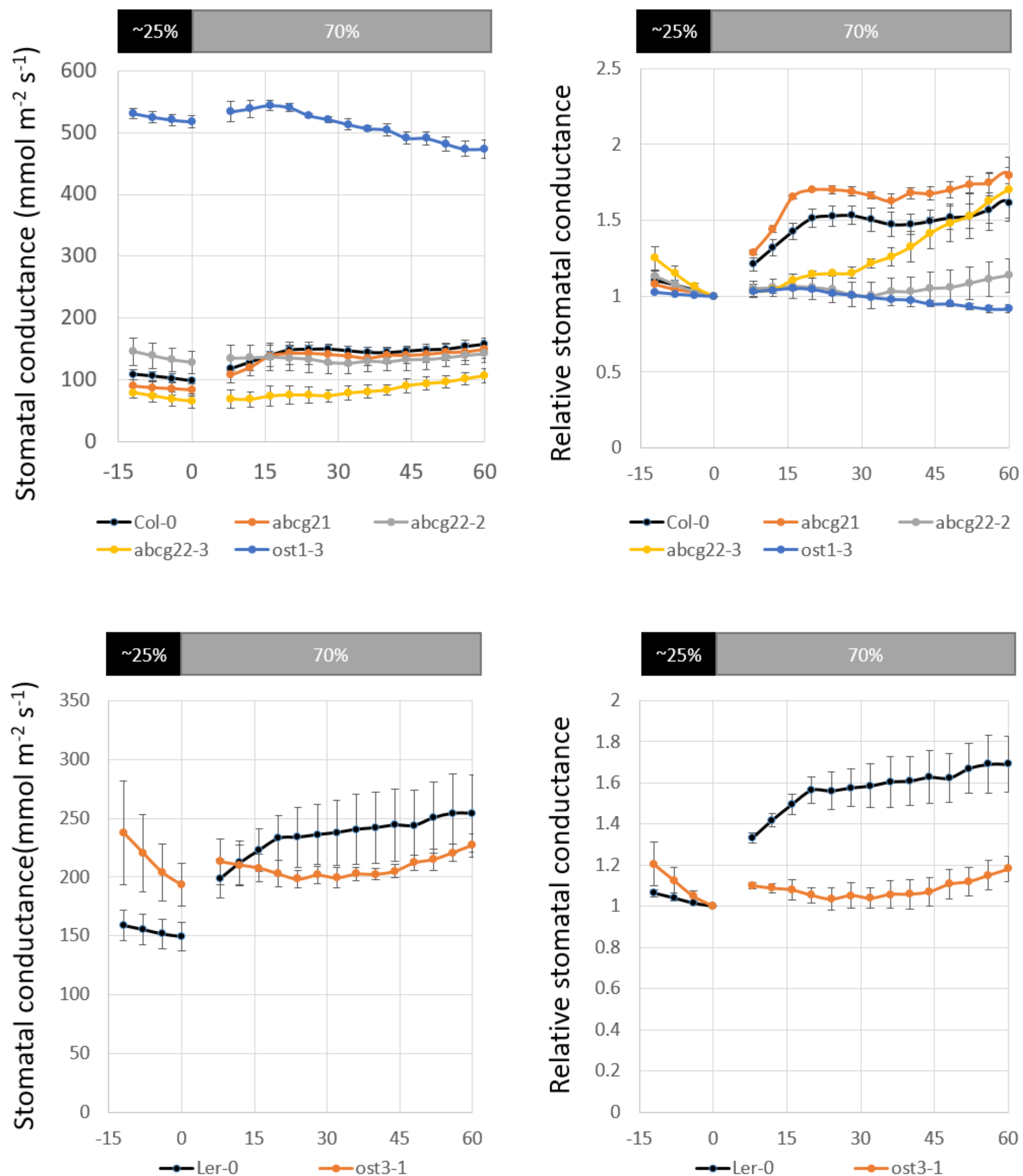

Figure 10. Stomatal response to increase of air humidity from low to normal. Responding opening of stomata in *abcg22* is delayed. According to the relative values, response of *abcg22-2* and *ost3-1* is also partially impaired.

All three *abcg22* mutants displayed delayed opening of the stomata in humid air. Thus, our results indicate that ABCG22 could be involved in both stomatal closure and opening in response to changing air humidity levels.

3.2.4 Ozone-induced stomatal closure is affected by ABCG22

Exposure to atmospheric ozone leads to production of reactive oxygen species in plants' apoplast (Kangasjärvi *et al.*, 2005). The plants react to ozone exposure with rapid transient decrease (RTD) in stomatal conductance (Kollist *et al.*, 2007). The process is regulated by SLAC1 and OST1 and requires ABA signalling pathway to be intact (Vahisalu *et al.*, 2010). Mutant plants with inactive OST1 do not show changes in stomatal conductance in response to short periods of ozone exposure (Merilo *et al.*, 2013). Since ABCG22 might be functionally related to OST1, experiments were conducted to explore the possible role of ABCG22 in RTD.

Stabilized plants were subjected to an ozone pulse: exposure to 450 ppb ozone for 3 minutes, after which the conditions were returned to initial (Figure 11). The wild type and *abcg21* plants demonstrated RTD within 10 minutes after the application of ozone, after which the stomata were slowly opening. The *ost1-3* mutants did not respond to the stimulus. The *abcg22-2* and *abcg22-3* plants showed partially impaired stomatal closure with the reduced magnitude. Additionally, stomatal aperture of *abcg22-2* and *abcg22-3* began increasing only 20-25 minutes after the ozone pulse.

The *ost3-1* line demonstrated a different reaction to ozone exposure. Similarly to the *abcg22*-2 and *abcg22-3* mutants, stomatal conductance in *ost3-1* did not decrease rapidly. However, the closure continued for 45 minutes after the application of ozone, with the resulting relative stomatal conductance decrease being stronger than in Ler-0.

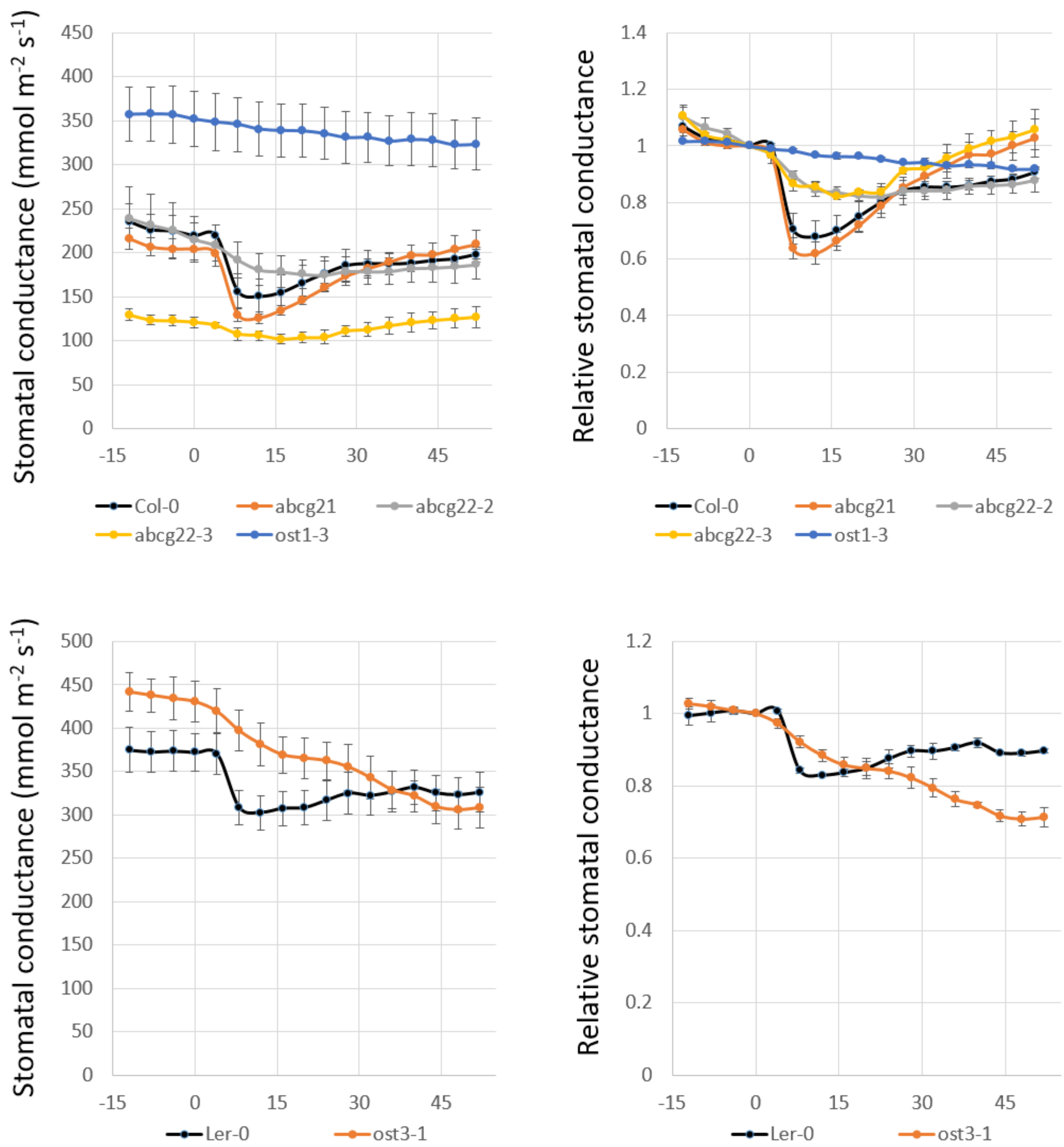

Figure 11. Stomatal response to an ozone pulse in the studied plant lines. The *abcg22* mutants in the Col-0 genetic background show impaired response to ozone exposure. Stomatal response in the *ost3-1* mutants is delayed, but relatively stronger than in Ler-0.

The behaviour of the ABCG22 loss-of-function mutants indicates that ABCG22 transports a substance which affects in stomatal response to atmospheric ozone.

3.2.5 Mutants for ABCG22 have partially impaired reactions to CO_2 fluctuations

To optimize the balance between water loss and CO_2 intake, plants regulate stomatal conductance in response to changes in CO_2 level. High CO_2 concentration in substomatal cavities triggers rapid stomatal closure, while its low concentration triggers stomatal opening (Engineer *et al.*, 2016). The OST1 activity enhances the ABA-independent pathway in CO_2-induced stomatal closure (Hsu *et al.*, 2018), even though high CO_2 level does not trigger increase in OST1 activity (Zhang *et al.*, 2020).

In an experiment conducted by Merilo *et al.*, 2015 a partially impaired response in *abcg22-3* was recorded. In this study, two types of experiments were conducted to research the potential role of ABCG22 in CO_2-induced stomatal movements.

First, the CO_2 concentration in cuvettes was increased from the ambient level (about 400 ppm) to 800 ppm for one hour (Figure 12). The wild type plants reacted immediately by closing their stomata over the next 10-15 minutes, after which the stomatal conductance remained at the same level for the rest of the experiment. The response of *abcg21* mutants was similar to the wild type, possibly with a slightly enhanced magnitude of stomatal closure. The *ost1-3* demonstrated very slow gradual stomatal closure, matching the data from the experiments conducted by Hsu *et al.*, 2018. Thus, the *abcg22* mutants exhibited near-wild type reaction, with only minor impairment in the rate of stomatal conductance reduction in these experiments.

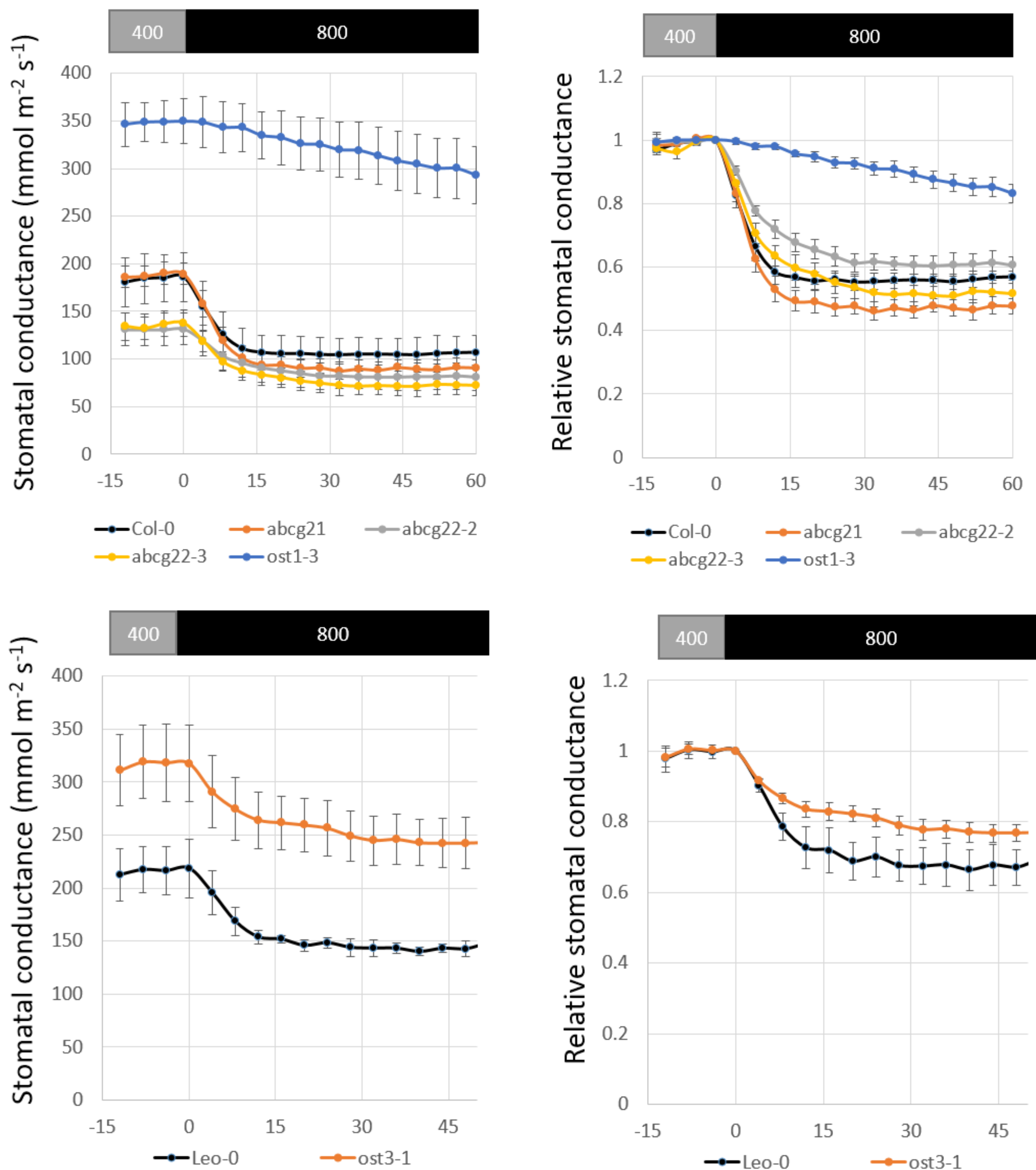

Figure 12. Stomatal response to elevated CO_2 concentration. The *abcg22* plants do not have a delay in stomatal response, and have a weak impairment in closure.

To further research the response of *abcg22* to CO_2 levels, another type of experiments was conducted. In contrast to the previous experiments, the plants were subjected to low CO_2 (90 min) with the consecutive increase in CO_2 concentration (400 – 100 – 800 ppm). The stomatal opening in response to low CO_2 level was similar in wild type, *abcg22* and *abcg21* plants. When the CO_2 was increased from 100 to 800 ppm, the reaction of all *abcg22* mutants

was initially identical to the wild type and *abcg21*, but the rate of stomatal closure dropped 12-15 minutes after application of high CO_2 (Figure 13).

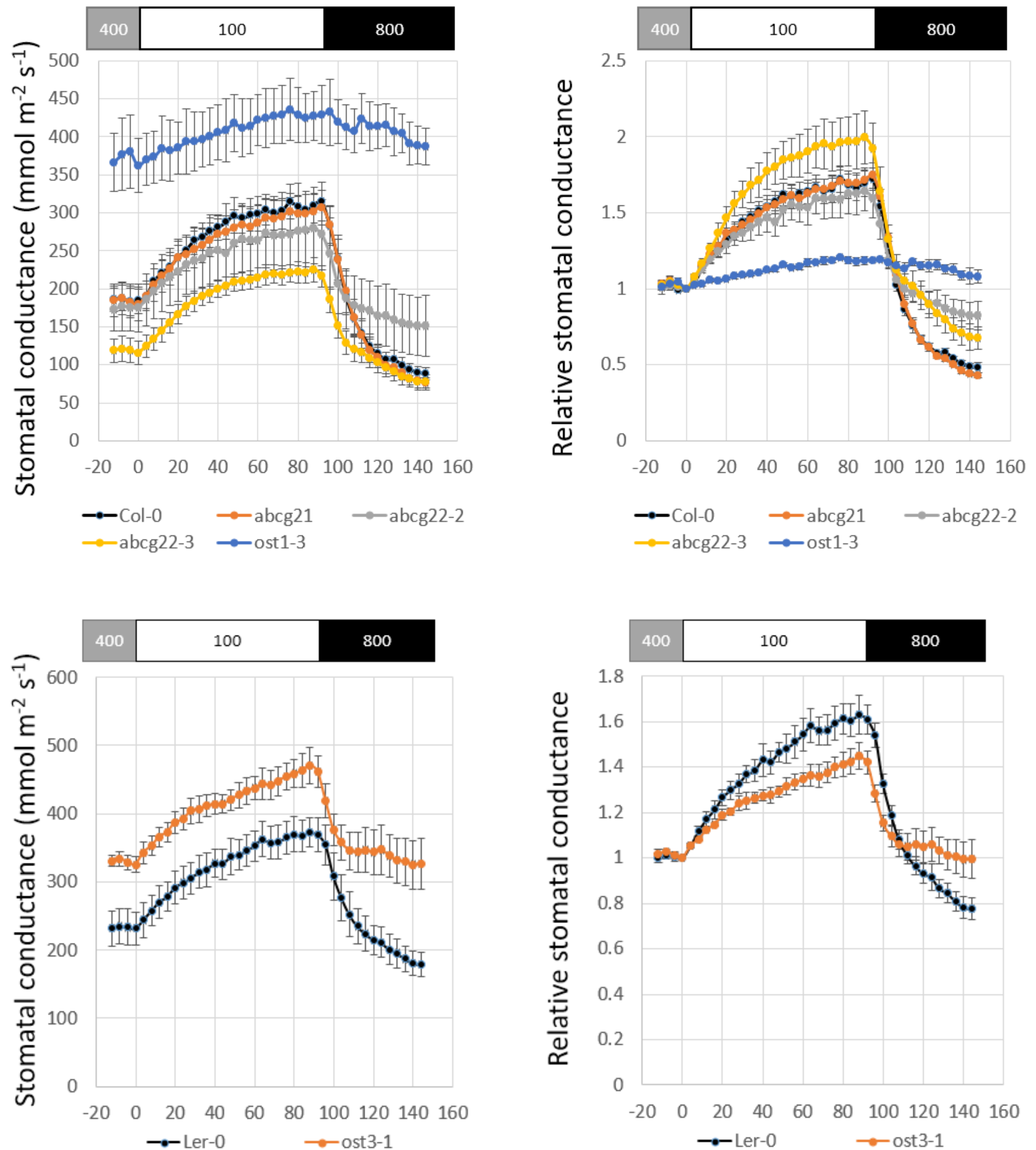

Figure 13. Stomatal response to changing CO_2 concentration levels. Both *abcg22* and *abcg21* showed wild type phenotype when subjected to 100 ppm CO_2. The response to CO_2 increase from 100 ppm to 800 ppm was immediate in *abcg22* and *abcg21* as well as in wild type plants. 15 minutes after the CO_2 increase, stomatal closure in *abcg22* plants rapidly slowed down.

In order to compare 2 types of the experiments (with and without low CO_2 treatments), changes in stomatal conductance were calculated (the difference in stomatal conductance at

the end of the experiments and at the beginning) (Figure 14). It seems that the *abcg22* mutants demonstrated a reduced magnitude of high CO_2-induced stomatal closure after the pre-treatment with low CO_2. This impaired response in the *abcg22* mutants suggests that ABCG22 is still involved in the reaction mechanisms to rapid CO_2 fluctuations. It is possible that an unknown substrate of ABCG22 might be accumulated when the atmospheric CO_2 is reduced, and released by ABCG22 upon increase in CO_2 concentration. In the *abcg22* mutants, this process is inhibited, leading to the impairment of stomatal closure under high CO_2.

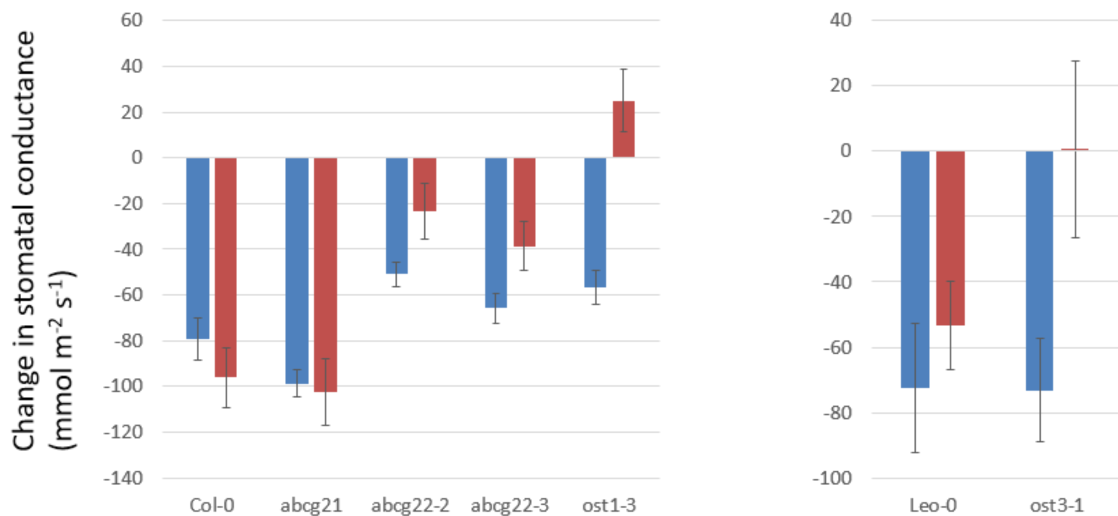

Figure 14. Change of stomatal conductance in 60 minutes after application of high CO_2 concentration. Blue bars depict the stomatal response to increase of CO2 from 400 ppm to 800 ppm, red bars depict the stomatal response to 400 ppm – 100 ppm – 800 ppm CO_2 change.

3.2.6 Stomatal closure in response to darkness might be impaired in mutants for ABCG22

Darkness-induced rapid stomatal closure is mediated through two pathways. The lack of blue light deactivates phototropins and consequently PM H^+-ATPase, stopping the efflux of protons from guard cells (Inoue and Kinoshita, 2017). Simultaneously, the inactive photosynthesis leads to an increased concentration of CO_2 in the substomatal cavity, activating CO_2-induced stomatal closure (Roelfsema *et al.*, 2002). In the *ost1* loss-of-function mutants only the signalling pathway mediated by CO_2 is impaired, while the intact blue light response pathway causes stomatal closure in darkness (Merilo *et al.*, 2013). As the darkness response partially depends on OST1 functioning, the reactions of the *abcg22* loss-of-function mutants to darkness were monitored.

The cuvettes with plants in the gas exchange device were covered for one hour. Immediate rapid stomatal closure was demonstrated by wild type, *abcg21* and *abcg22* plants, with the stomatal conductance being stabilized after 15-20 minutes in Col-0, *abcg22-2,* and *abcg22-3*. The observed rapid response in the *abcg22* mutants corresponds to the results of the experiments previously conducted by (Kuromori *et al.*, 2017). However, darkness-triggered stomatal closure in the *abcg22-2* plants appeared to be impaired (Figure 15). The *ost3-1* mutants' stomatal phenotype was identical to Ler-0.

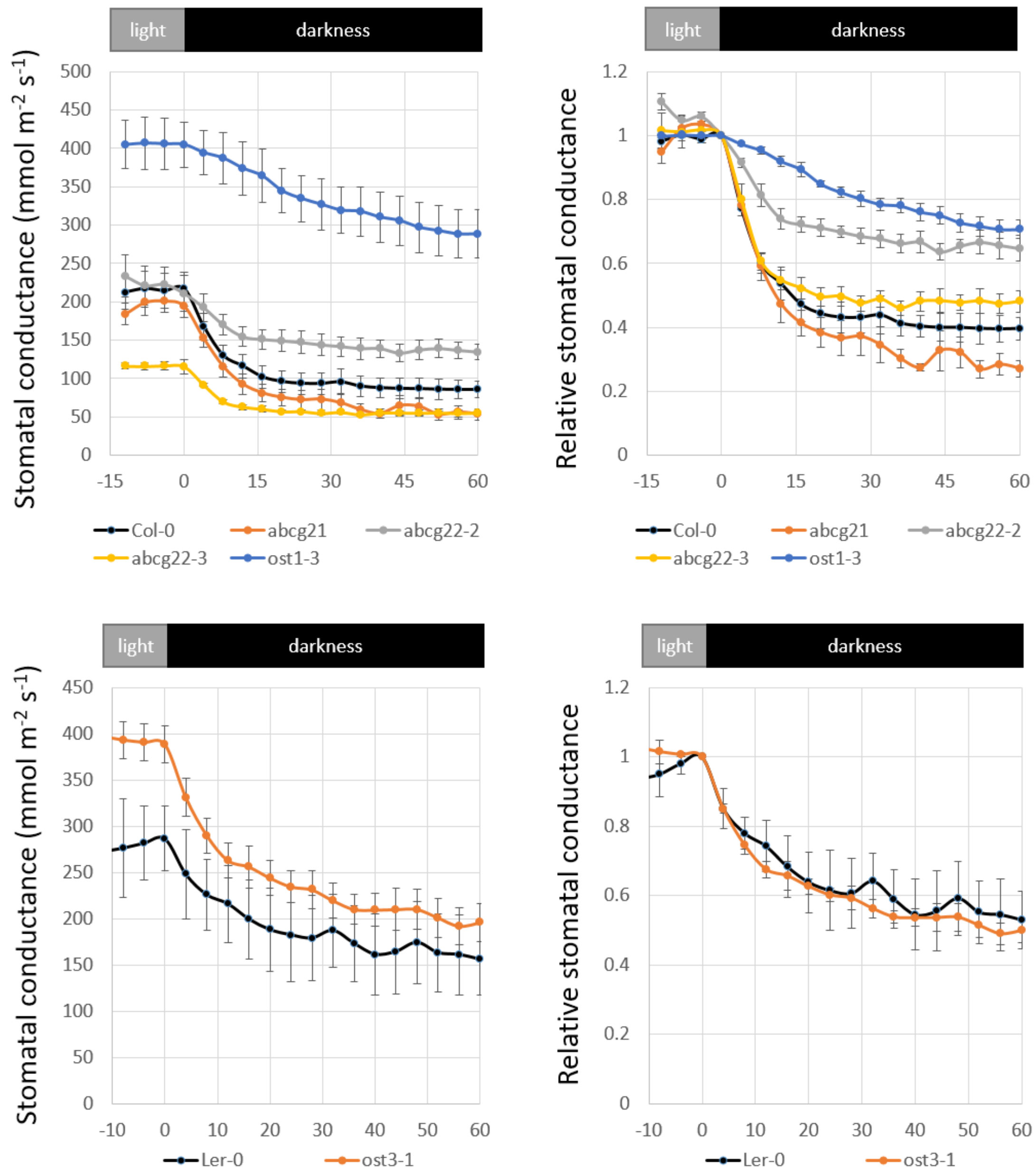

Figure 15. Stomatal response to reduced light intensity. ABCG22-deficient mutants respond to darkness as fast as wild type and *abcg21*. Changes in stomatal conductance of *abcg22-2* and possibly *abcg22-3* are impaired. Changes in relative stomatal conductance of *ost3-1* are identical to the wild type Ler-0.

Reason for darkness-induced closure being impaired only in *abcg22-2* currently remains unknown and requires further research. An involvement of ABCG22 in reaction to darkness is possibly similar to that in CO_2-induced stomatal movements. Further experiments on the *abcg22* mutants might be needed to examine the role of ABCG22 in the process of darkness-induced stomatal closure.

3.3 CONCLUSIONS

During experiments conducted prior to this work, AtABCG22 transporter was found to play a role in rapid stomatal response induced by dry air (Merilo *et al.*, 2015). The gas exchange experiments described here suggest that a substrate transported by ABCG22 affects not only stomatal closure induced by low air humidity but also stomatal opening induced by transition of plants from dry to humid air. Additionally, we showed that ABCG22 is involved in stomatal closure induced by an ozone pulse and changes in CO_2. A possible impairment of stomatal response to darkness in *abcg22* might be connected to a role of ABCG22 in CO_2-induced stomatal closure.

Our gas exchange experiments demonstrated some differences between mutants with T-DNA inserts (*abcg22-2* and *abcg22-3*) and the premature stop codon mutant *ost3-1*. These differences could have been caused by the mutation types (as the ost3-1 mutant still expressed truncated ABCG22) or the genetic backgrounds of the mutants (Col-0 vs. Ler-0).

The *abcg21* mutants had only slightly enhanced responses to the studied stimuli, but mostly it demonstrated near-wild type stomatal phenotypes. Since Kuromori *et al.* (2017) found that the lack of *abcg21* partially rescues leaf temperature and water loss in *abcg22* mutants, it would be of great interest to study gas exchange phenotypes in the double *abcg21 abcg22* mutant.

Identifying the substrate of ABCG22 and its interaction with OST1 would be the main goal of future research. The transgenic plants generated during this work will be brought to homozygosity. These plants will be used in gas exchange experiments to study an effect of blocked or mimicked phosphorylation on stomatal phenotype. The transgenic plants will be also used for generation of double mutants lacking key stomatal regulators, which might provide additional information about the role of ABCG22 in stomatal movements.

SUMMARY

Plants can adapt to changes in the environment by opening or closing their stomata. Regulation of stomatal movements involves a complex network of pathways that allows guard cells to respond to various environmental factors. Many elements of those pathways are being researched in order to improve our understanding of plants. ABCG22, one of many ATP-binding cassette transporters encoded by the plant genome, was analysed and discovered to mediate stomatal closure in response to drought. A closely phylogenetically related ABCG21 transporter was shown to have a functional relationship with ABCG22. However, the exact roles of ABCG21 and ABCG22 in stomatal regulation are still unclear. Recent research has shown that ABCG22 functioning might be strongly dependent on phosphorylation by OST1 protein kinase.

During this study, stomatal behaviour of *abcg22* and *abcg21* mutants was analysed through a series of gas exchange experiments conducted using a custom system built in Tartu University. The experiments showed evidence of ABCG22 affecting stomatal closure in response to low air humidity, an ozone pulse, and low CO_2. Behaviour of abcg21 mutants was similar to that of wild type plants. Identifying the substrate of ABCG21 and ABCG22 is a necessary future step towards understanding the acting mechanisms of these transporters and their functional relationship.

To gain better understanding of ABCG22 functioning pathway, plants expressing ABCG22 with blocked or mimicked phosphorylation sites are required. This project was initiated as a part of this study. Generation of transgenic plants expressing modified *ABCG22* has been successfully performed and selected through confocal microscopy.

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