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**Stomatal Conductance Depends on Abscisic Acid Production in Both Guard Cells and in  
Phloem Companion Cells**

Bachelor's Thesis

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## **ABSTRACT**

### **Stomatal Conductance Depends on Abscisic Acid Production in Both Guard Cells and in Phloem Companion Cells**

The question how plants use water is important as water availability is often a limiting factor for plant growth. Stomata are mainly responsible for water loss from plant tissues and, therefore, regulatory mechanisms controlling stomatal apertures are in the research focus of plant water management. Abscisic acid (ABA) has an important role in reducing stomatal conductance, however, importance of ABA biosynthesis in guard cells and in phloem is not fully studied. To address this question, we generated transgenic plants with restored ABA biosynthesis either in guard cells or in phloem by using tissue-specific promoters. Our study shows that both guard cells and phloem companion cells can produce ABA in quantities which are enough to regulate plant development and stomatal conductance on the level of wildtype plants.

Keywords: ABA, ABA biosynthesis, guard cells, phloem companion cells, stomatal conductance

CERCS: B310 Physiology of vascular plants

### **Õhulõhede juhtivus sõltub nii sulgrakkudes kui ka floeemi kaasrakkudes toodetud abtsiishappest.**

Taimede veekasutus on väga tähtis, sest tihti osutub see nende kasvu pärssivaks teguriks. Õhulõhed on põhilised taimekoe veekaotuse eest vastutajad, mistõttu on nad veekasutuse reguleerimise mehhanismide uurimise keskmes. Abtsiishappel (ABA) on tähtis roll õhulõhede juhtivuse vähendamisel, aga selle sünteesimist sulgrakkudes ja floeemi kaasrakkudes pole veel täielikult uuritud. Selle uurimiseks lõime koespetsiifilisi promootereid kasutades transgeensed taimed millel oli ABA biosüntees taastatud kas sulgrakkudes või floeemi kaasrakkudes. Saadud tulemused näitavad, et sulgrakud või floeemi kaasrakud on võimelised tootma piisavalt ABA-t, et taastada metsiktüüpi taimede areng ja õhulõhede juhtivus.

Märksõnad: ABA, ABA biosüntees, sulgrakud, floeemi kaasrakud, õhulõhede juhtivus

CERCS: B310 Soontaimede füsioloogia

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## **ABBREVIATIONS**

AAO3 - ABSCISIC ALDEHYDE OXIDASE 3

ABA - abscisic acid

ABA2 - short-chain alcohol dehydrogenase

ABA-GE - abscisic acid glucose ester

DTX - MATE efflux family protein

GORK - GATED OUTWARDLY-RECTIFYING K<sup>+</sup> CHANNEL

H<sup>+</sup>-ATPase - ATP dependent H<sup>+</sup>-channel

K<sup>+</sup><sub>in</sub> - K<sup>+</sup> inward channel

K<sup>+</sup><sub>out</sub> - K<sup>+</sup> outward channel

KAT1 - POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1

KAT2 - POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2

NCED - NINE-CIS-EPOXYCAROTENOID DIOXYGENASE

OST1 - OPEN STOMATA 1

PCR - polymerase chain reaction

PP2C - protein phosphatase belonging to class 2C

QUAC1 - QUICK-ACTIVATING ANION CHANNEL 1

sGFP - synthetic green fluorescent protein

SLAC1 - SLOW ANION CHANNEL-ASSOCIATED 1

SUC2 - ARABIDOPSIS THALIANA SUCROSE-PROTON SYMPORTER 2

## INTRODUCTION

Plants use solar energy to assimilate CO<sub>2</sub> from the air as organic matter and provide food and energy for life on Earth. Stomata are small pores on plant surfaces which regulate gas exchange between the plant and the environment. These pores are surrounded by two guard cells which control stomatal apertures by shrinking and swelling. Plants are sessile organisms and they are not able to move away from unfavorable conditions. Thus, fast regulation of stomatal apertures provides a tool for plants to cope with stress conditions. For example, by closing stomata, plants can reduce water evaporation rates, prevent pathogen invasion or restrict toxicant uptake from the air.

Stomatal behavior is defined by many factors, including concentration of phytohormones. One of the most important phytohormones for stomatal regulation is abscisic acid (ABA) that induces stomatal closure as well as regulates plant development. For a long time, it was believed that ABA is synthesized only in the vascular system of plants and transported to other plant organs by specialized transporters and xylem sap. However, it was recently shown that guard cells themselves have the biosynthesis pathway needed for ABA synthesis (Bauer et al., 2013), although this was argued by other researchers (McAdam and Brodribb, 2015). In this study, we aimed to estimate relative importance of ABA synthesis sites in plants, by generating transgenic plants with ABA biosynthesis specifically in guard cells or phloem companion cells.

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

# 1.LITERATURE OVERVIEW

## 1.1.Importance of stomata for plant functioning

Evolutionary adaptation of plants to conditions on drylands includes development of gas-impermeable cuticle to prevent water loss from plant tissues. Plants assimilate carbon from gaseous CO<sub>2</sub> and, therefore, need a flux of CO<sub>2</sub> to mesophyll where it is assimilated in photosynthesis. Stomata are small openings on the surface of plants that control uptake of CO<sub>2</sub> for photosynthesis and evaporation of water. Stomata consist of two highly-specialized guard cells which regulate stomatal apertures and, therefore, gas exchange between plant and the atmosphere. Thus, regulation of opening and closing of stomatal pores is crucial for plant growth and development as stomata regulate the optimal trade-off between CO<sub>2</sub> uptake and water loss. Plants employ two main mechanisms of stomatal adaptation to changing environment: changes of stomatal density on young leaves (Chater et al. 2014) and fast adjustments of stomatal apertures. Stomatal conductance is defined as the rate of CO<sub>2</sub> uptake or water evaporation through stomata per leaf area and time.

Guard cells integrate information about inner and outer stimuli and react by changing their turgor that defines stomatal apertures. Stomatal closure is triggered in response to some environmental factors, for example, darkness period, low air humidity, high soil salinity as well as pathogen attack. High atmospheric CO<sub>2</sub> also induces stomatal closure to prevent unwanted water loss when substrates for photosynthetic carbon fixation are sufficient. Fast reduction of stomatal apertures is also induced in response to accumulation of abscisic acid (ABA), a phytohormone that regulates plant development and is produced in response to stress conditions, for instance, drought. Stomatal closure is associated with water and solute outflow through specialized channels in guard cell membrane, resulting in cell shrinking and reduced stomatal aperture. Stomatal opening is often caused by the opposite factors as those which trigger stomatal closure, including high air humidity, low atmospheric CO<sub>2</sub> content, and illumination. During stomatal opening, guard cells swell as the result of water and solute uptake.

Limited water availability for agriculture is a problem that attracts significant attention as world population is steadily growing and is expected to reach between 9.1 and 10.8 billion by 2050 (United Nations, 2007). Since plants mainly lose water through stomata, studies of stomatal regulation can contribute to engineering plants with better water management.

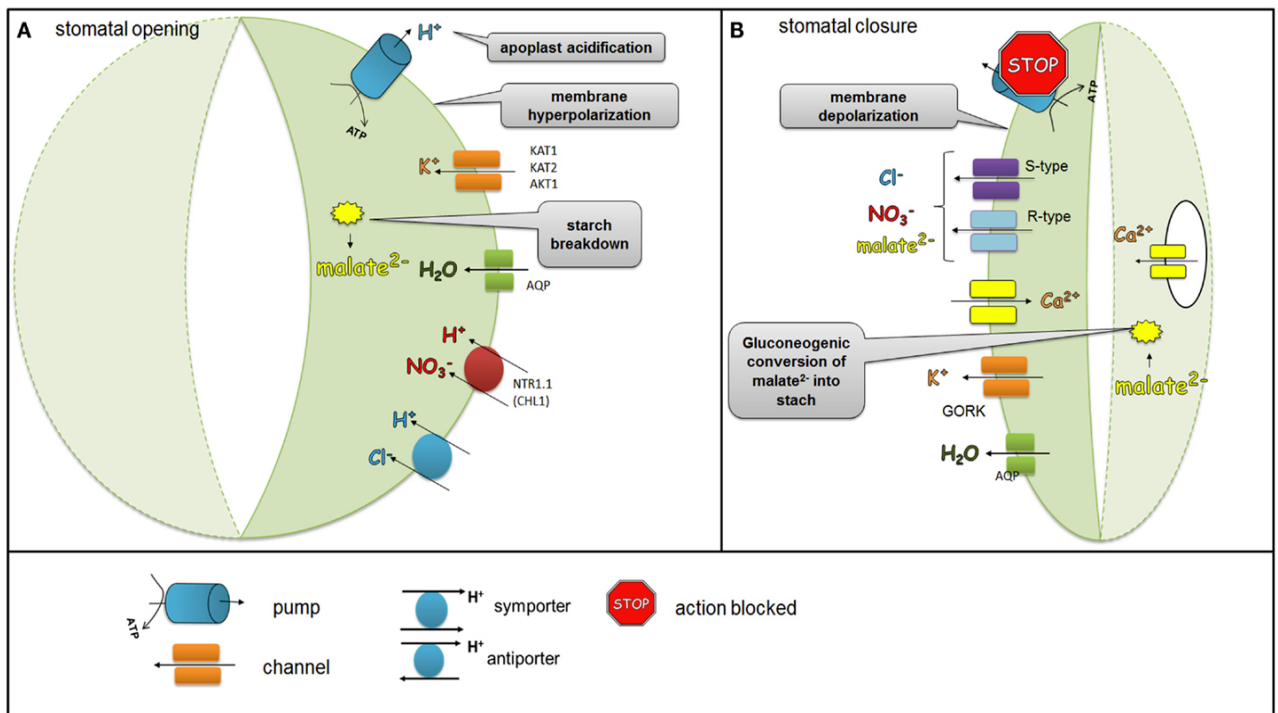
Water use efficiency, the ratio between assimilated carbon and evaporated water, is the parameter which should be optimized in crops for better yield and drought resistance. Stomatal apertures also restrict uptake of some air pollutants, such as ozone (Brosché et al., 2010), so breeding of crops resistant to air pollutions also should include optimization of stomatal functioning.

## 1.2. Stomatal opening

Stomata open to ensure optimal flow of CO<sub>2</sub> to mesophyll, where it is assimilated in photosynthesis. Illumination reduces intercellular CO<sub>2</sub> concentration that can be considered as one of the major regulators of stomatal movements. Stomatal opening is triggered by red and blue light through different mechanisms. The main photoreceptors for blue light in guard cells are phototropins PHOT1 (PHOTOTROPIN 1) and PHOT2 (PHOTOTROPIN 2) that activate plasma membrane H<sup>+</sup>-ATPase (Takemiya et al., 2013). The phototropins PHOT1 and PHOT2 phosphorylate BLUS1 (BLUE LIGHT SIGNALING 1), a serine/threonine protein kinase, which plays a role in stomatal control of photosynthetic CO<sub>2</sub> assimilation under natural light conditions (Takemiya and Shimazaki, 2016). Red light-induced stomatal opening is associated with photosynthesis in mesophyll cells and a reduction of intercellular CO<sub>2</sub> concentration (Roelfsema et al., 2002). In guard cells, phototropins control BAM1 (B-AMYLASE 1) and AMY3 (ALPHA-AMYLASE-LIKE 3) activity, which are related to starch degradation. Starch that is present in guard cells is degraded by the end of the night period to provide energy for fast stomatal opening within the first 30 minutes of day period (Horrer et al., 2016). Thus, stomatal opening consumes energy which is transformed into turgor production.

During stomatal opening, protons are pumped out of the guard cells and are replaced by potassium ions via voltage-gated channels. The activation of guard cell proton pumps, mainly AHA1 (PLASMA MEMBRANE PROTON ATPASE) (Harper et al., 1989; Yamauchi et al., 2016), is the central event in stomatal opening and causes hyperpolarization of the guard cell membrane as well as apoplast acidification. This, in turn, fuels K<sup>+</sup> uptake through K<sup>+</sup><sub>in</sub> channels (K<sup>+</sup> inward channel), including KAT1 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1), KAT2 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2), AKT1 (POTASSIUM TRANSPORTER 1), AKT2/3 (POTASSIUM TRANSPORT 2/3) and AtKC1 (ARABIDOPSIS THALIANA K<sup>+</sup> RECTIFYING CHANNEL 1) (Schachtman et al., 1992; Pilot et al., 2001). It was also

shown that H<sup>+</sup>-ATPase activity positively correlates with the degradation of starch (Horrer, Flüttsch, Pazmino et al., 2016). Potassium influx is balanced by some negatively charged ions, including Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, and malate. Thus, the rise of K<sup>+</sup> leads to uptake of Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, and other osmotically active solutes in the vacuole, generating a water inflow and making the guard cells swell up. Uptake of osmotica is mediated by a number of transporters such as nitrate transporter AtNRT1.1 (ARABIDOPSIS



**Figure 1. Stomatal opening and closure.** Plasma membrane ion channels, pumps and transporters that regulate stomatal opening and closure. During stomatal opening (A) the plasma membrane gets hyperpolarized and K<sup>+</sup><sub>in</sub> channels are activated. The potassium influx is balanced with anions. The ions and water build up in the guard cell and generate turgor, which opens the pore. During stomatal closure (B) the membrane is depolarized, H<sup>+</sup>-ATPase is inhibited and S-type and R-type anion channels activated. GORK transports the potassium ions out of the guard cell. Malate is converted into starch and Ca<sup>2+</sup> concentration rises (Daszkowska-Golec and Szarejko, 2013).

THALIANA NITRATE TRANSPORTER 1), which participates in light-induced opening (Guo et al., 2003), and malate transporter AtABCB14 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE B14) that modulates stomatal response to CO<sub>2</sub> (Lee et al., 2008). The lowered water potential results in water diffusion into guard cells via osmosis. These processes result in guard cell swelling and stomatal opening (Fig. 1A).

### 1.3. Stomatal closure

Stomatal closure is associated with depolarization of guard cell membrane and with activation of ion channels. Membrane depolarization is connected to H<sup>+</sup>-ATPase inhibition to prevent vain energy consumption. Stomatal closure involves anion channels of two types in the plasma membrane of guard cells: R-type (rapid channels) and S-type (slow channels), which differ by time of activation (Roelfsema and Hedrich, 2005). While S-type anion channels show very slow voltage-dependent activation and deactivation, R-type anion channels are activated rapidly by depolarization, deactivated by hyperpolarization, and inactivated during prolonged stimulation (Schroeder and Keller, 1992). The plasma membrane-located SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) protein was recently identified as an S-type anion channel in guard cells. It was shown that it plays the central role in stomatal closure as *slac1* mutants demonstrate dramatically impaired stomatal closure in response to many environmental stimuli (Vahisalu et al., 2008; Negi et al., 2008). It was demonstrated that expression of SLAH3 (SLAC1 HOMOLOGUE 3), another S-type anion channel, can complement SLAC1 functions in the *slac1* mutants. QUAC1 (QUICK-ACTIVATING ANION CHANNEL 1) is an R-type anion channel that is located in membranes of guard cells and activated by malate. Plants which guard cells lack QUAC1 demonstrate partially impaired stomatal response to high CO<sub>2</sub> concentration (Kline et al., 2010; Meyer et al., 2010; Imes et al., 2013). SLAC1 and QUAC1 are activated by protein kinase OST1 (OPEN STOMATA 1) (Meyer et al., 2010; Acharya et al., 2013). It was shown additionally that SLAC1 activity depends on plasma-membrane leucine rich receptor-like protein kinase GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1) (Hua et al., 2012). GORK (GATED OUTWARDLY-RECTIFYING K<sup>+</sup> CHANNEL) is the guard cell outward potassium channel that is activated during stomatal closure (Ache *et al.*, 2000) (Fig. 1B).

ABA is the key regulator of stomatal apertures, whose accumulation is associated with stomatal closure. ABA signaling in guard cells is accompanied by changes in calcium concentration, activation and deactivation of protein kinases and protein phosphatases. When ABA is not present in guard cells, type 2C protein phosphatases (PP2Cs) inhibit OST1 and GHR1, preventing activation of anion channels. ABA binds its receptors PYRABACTIN RESISTANCE1 (PYR1)/PYR1-like (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) which attach and inhibit PP2Cs, resulting in activation of OST1 and GHR1. The family

of PYR/RCARs consists of 14 members whose gradual inactivation results in the dramatic increase of stomatal conductance (Merilo et al., 2013). The active OST1 and GHR1 protein kinases phosphorylate and activate anion channels in the plasma membrane. ABA signaling also involves phosphorylation and activation of transcription factors regulating ABA-responsive genes (Kline et al., 2010; Park et al., 2009; Ma et al., 2009). Anion channels are also activated by  $\text{Ca}^{2+}$  dependent protein kinases, which are activated by elevated calcium concentration in guard cells

$\text{K}^+$ <sub>in</sub> channels (KAT1 and KAT2) are downregulated being phosphorylated by OST1.

Membrane depolarization induces activation of  $\text{K}^+$ <sub>out</sub> ( $\text{K}^+$  outward channel) channel GORK that is voltage-dependent and releases  $\text{K}^+$  from guard cells (Ache et al., 2000).

## 1.4. Roles for ABA in plants and its biosynthesis, storage, and catabolism

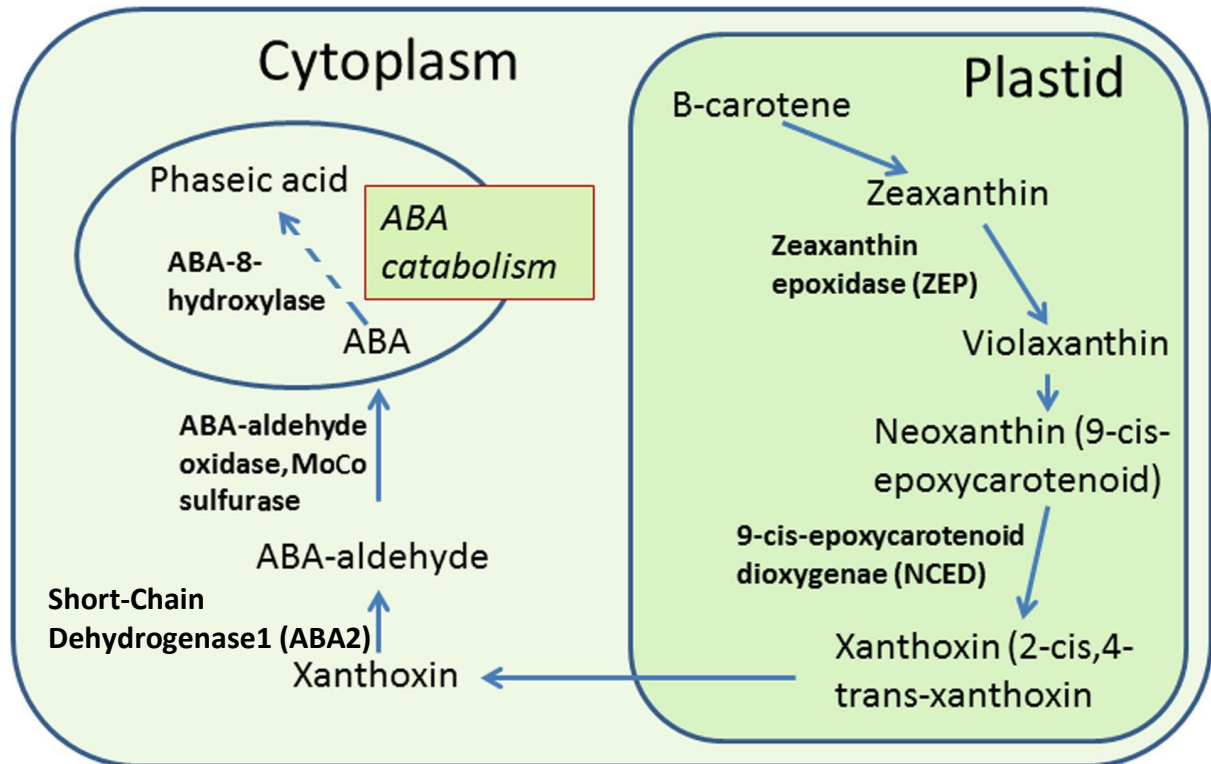
ABA is a phytohormone that plays multiple roles in regulation of plant development, stomatal apertures, and plant adaptation to environmental stresses. ABA acts as a positive and negative regulator of transcription factors, affects RNA processing, phosphorylation and metabolism of second messengers (Finkelstein et al., 2002). Stress tolerance against osmotic stress, drought, high salinity, and low temperature are mediated by ABA accumulation (Ingram and Bartels, 1996; Xiong et al., 2002). ABA is important for induction and maintenance of seed dormancy and it inhibits the transition from embryonic to germination growth (Rodríguez-Gacio et al., 2009). It was suggested that ABA is involved in resistance of plants towards pathogens (Mauch-Mani and Mauch, 2005; Seo and Park, 2010). ABA causes stomatal closure (Mittelheuser and Van Steveninck, 1969) mainly by altering ion fluxes in guard cells. Biological response to ABA depends on its concentration and recipient tissues sensitivity.

ABA is a weak  $\text{C}_{15}$  acid which is formed from  $\beta$ -carotene synthesized from  $\text{C}_5$  precursor isopentenyl diphosphate (Hirai et al., 2000). ABA biosynthesis is started in chloroplasts from  $\beta$ -carotene cleavage by B-CAROTENE HYDROXYLASE 2 with formation of zeaxanthin that is converted to antheraxanthin and further to violaxanthin by ZEP (ZEAXANTHIN EPOXIDASE). Violaxanthin is then converted to neoxanthin followed by its cleavage by NCED (NINE-CIS-

EPOXYCAROTENOID DIOXYGENASE) enzymes resulting in formation of C<sub>15</sub> xanthoxin and C<sub>25</sub> metabolite. The ABA biosynthesis is preceded in cytoplasm where xanthoxin is converted to abscisic aldehyde by ABA2 (SHORT-CHAIN DEHYDROGENASE/ REDUCTASE 1). Finally, abscisic aldehyde is oxidized by AAO3 (ABSCISIC ALDEHYDE OXIDASE 3) resulting in ABA formation (Schwartz et al., 2003) (Fig. 2).

ABA is synthesized *de novo* from carotenoids and by hydrolyzing inactive conjugates. ABA glucose ester is the most common ABA conjugate that has no biological activity. It accumulates in vacuoles and apoplastic space and can be relocated to endoplasmic reticulum during dehydration. It is believed that ABA glucose ester is the form of ABA for storage or transportation (Finkelstein, 2013). ABA recycling from inactive conjugates requires  $\beta$ -d-glucosidases BG1(BETA-1,3-GLUCANASE 1) and BG2(BETA-1,3-GLUCANASE 2) which activities are enhanced by salinity (Lee et al., 2006; Xu et al.,2012). It is believed that ABA conjugate can be transported, although their transporters have been identified yet (Boursiac et al., 2013).

ABA catabolism mainly occurs via by 8',7' and 9' methyl group hydroxylation, employing P450 monooxygenases (Krochko et al., 1998). As products of ABA catabolism, phaseic and dihydrophaseic acids are formed (Fig. 2).



**Figure 2. ABA biosynthesis in plant cells and its catabolism to phaseic acid.** ABA biosynthesis starts from carotenoids cleavage to zeaxanthin and zeaxanthin conversion to violaxanthin by zeaxanthin epoxidase (ZEP). Then, neoxanthin is produced from violaxanthin. The former is converted to xanthoxin by the oxidative cleavage by 9-cis epoxycarotenoid dioxygenase (NCED). Xanthoxin is transported to the cytoplasm and converted to abscisic aldehyde by short-chain dehydrogenase 1 (ABA2). Abscisic aldehyde is finally oxidized to ABA. As products of ABA catabolism, phaseic and dihydrophaseic acids are formed. Enzyme names are shown in bold. Dotted lines indicate more than one reaction (Sharma and Nayyar, 2016 with modifications).

## 1.5.ABA transportation

Although ABA is synthesized in vascular tissue and in guard cells, this phytohormone is omnipresent in vascular plants. ABA exists as an anionic ( $ABA^-$ ) form and a protonated ( $ABA-H$ ) form that does not have a charge and is easily diffusible through biological membranes. These two forms reach equilibrium at pH 4.7 (Boursiac et al., 2013). Some environmental stresses can lead to an increase of pH value in xylem sap, resulting in a reduction of freely diffusible  $ABA-H$  and affecting its redistribution to leaf tissues (Wilkinson and Davies, 1997).

ABA can be transported in both directions between roots and shoots. Plasma membrane-localized transporters for ABA have been identified, including AtABCG40 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G40) that imports ABA into guard cells and AtABCG25 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G25) that functions as ABA exporter. It is also known that members of the NRT1/PTR (NITRATE TRANSPORTER 1) nitrate transporter family (AIT1, ABA-IMPORTING TRANSPORTER 1) and DETOXIFICATION EFFLUX CARRIER 50 (DTX 50) act as ABA transporters. ABCG25 and DTX50, both exporters, are mainly expressed in vascular tissues, although the latter was also found in guard cells (Zhang et al., 2014).

## 1.6.ABA synthesis in guard cells

It has been known that ABA is produced in vasculature of plants, specifically in phloem companion cells (Kuromori et al., 2014). Recently, it was also demonstrated that guard cells are able to synthesize ABA autonomously. Thus, analysis of guard cell transcriptomics showed that this type of plant cells expresses the transcripts of all enzymes belonging to the ABA biosynthesis pathway (Bauer et al., 2013). For example, the authors found that guard cells express ABA2, NCED3 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3) and AAO3 which play important roles in ABA biosynthesis. Transgenic plants with guard cell-specific expression of ABA3 (ALTERED CHLOROPLAST IMPORT 2) in the *aba3-1* genetic background were generated. It was showed that these plants had enhanced resistance to dry air comparing with the parental *aba3-1* mutant, indicating recovered ABA biosynthesis in guard cells (Bauer et al., 2013).

## **2.EXPERIMENTAL PART**

### **2.1. Aims of the study**

As it was shown that guard cells are able to synthesize ABA (Bauer et al., 2013), the relative importance of guard cells and phloem as sites of ABA biosynthesis is of interest. To address this question, we aimed to generate plants with specific production of ABA only in guard cells or only in phloem using an ABA deficient mutant as genetic background in order to estimate ABA production in these plant cells.

Specific aims included:

1. Generation of binary constructs for plant transformation;
2. Plant transformation with these constructs and selection of transgenic plants;
3. Phenotyping of transgenic plants.

## 2.2. Materials and Methods

### 2.2.1 Bacterial strains and plant lines

*Agrobacterium tumefaciens* strain GV3101 from laboratory stocks was used for plant transformation. Genetic manipulations were performed in *Escherichia coli* DH5 *alpha* strain. *Arabidopsis thaliana* *aba2-11* line in the Col-0 genetic background lacking xanthoxin to abscisic aldehyde conversion was used (González-Guzmán, 2002).

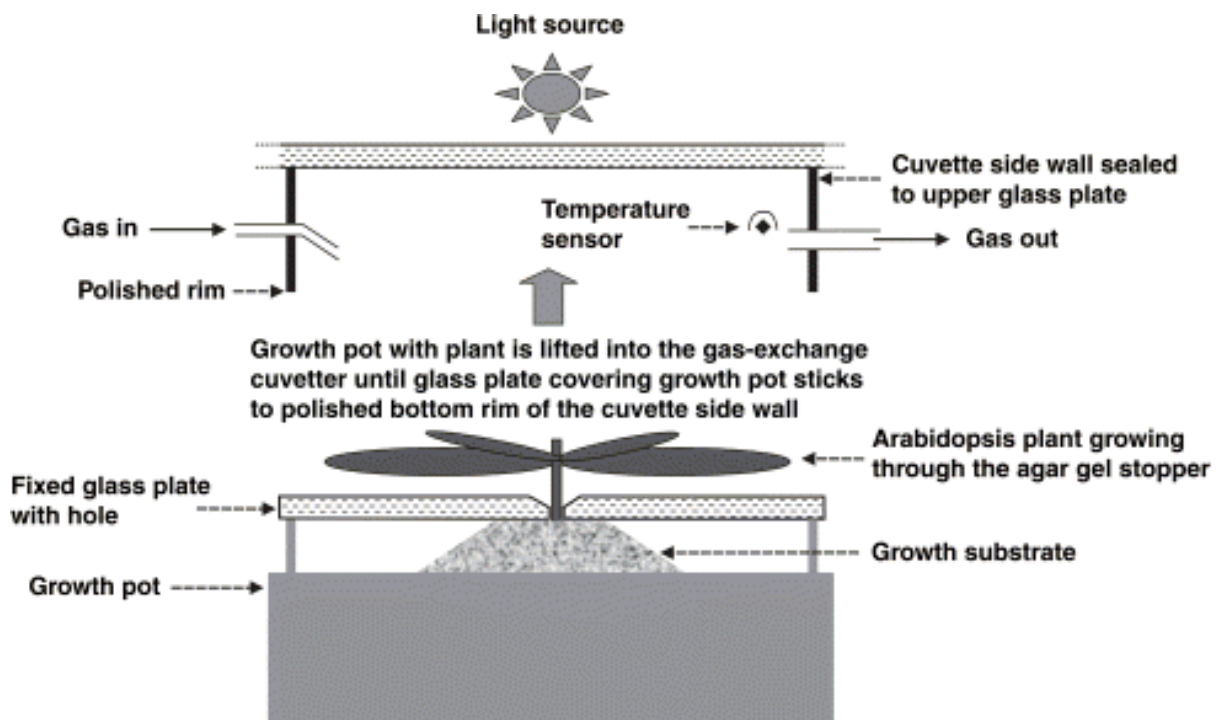
### 2.2.2. Growth conditions and gas exchange experiments

For vernalization, the *A. thaliana* seeds were kept in water and stored at 4°C in darkness for 2-3 days. Then, the seeds were sown in soil containing peat:vermiculite:water (4:3:3, respectively). Pots for gas-exchange experiments contained 250 g of soil and were covered with a glass plate that had a hole in the middle (Kollist et al., 2007). The seeds were sown into the hole on top of the substrate using a pipette. The hole was then covered with a Petri dish to guarantee high air humidity. A week after sprouting, the Petri dish was removed and only one plant was left to grow in each pot. The pots were watered by bottom-watering at least once per week. In 4 days prior experiments, the hole in the glass was closed by grafting wax. This was done to prevent moisture from the soil to interfere assays of stomatal conductance.

Plants were grown in growth chambers at 23°C in the day and 18°C during the night with 70% relative air humidity. The day-night cycle was 12-h light/12-h dark. Plants were illuminated with photosynthetic photon flux density at 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . ABA content measurements were done on plants that grew in growth chambers for 28 days.

A custom made 8-chamber device that enables monitoring of whole-plant rapid-response gas exchange was used to measure stomatal conductance (Kollist et al., 2007) (Fig. 3). The cylindrical chambers are made of stainless steel, have a diameter of 7.8 cm and are 3.5 cm high. The top is covered with a glass plate that is tightly sealed to the stainless steel walls. A pot with a plant was put underneath it and pressed against the walls from underneath by a spring forming a sealed off cuvette. Data about the air difference entering and exiting the chambers were collected every 2 minutes. Standard conditions were ambient CO<sub>2</sub> (~400 ppm), light 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and relative air humidity of ~70%. A gas analyzer (LI-Cor) was

used to collect data about the gas content in the chamber. Stomatal conductance was calculated using a custom program with data gathered by the chamber and projective leaf area (Kollist et al., 2007). The leaf area was determined using ImageJ to analyze the area of the rosette. For scaling, a measured piece of paper was used.



**Figure 3. Cuvette design for gas exchange experiments.** *Arabidopsis thaliana* plants were grown through a hole in a glass plate to isolate the rosette for gas-exchange measurements in the cuvette. The diagram shows the gas-exchange device described in Kollist et al. (2007).

### 2.2.3. Water loss measurements

Water loss was measured from detached leaves. The cut leaves were weighed and left at room temperature, abaxial side up to sit for 2 h and were then weighed again. Then the water loss was calculated as the percentage of the mass difference compared to the starting mass of the leaves.

#### 2.2.4. Generation and verification of transgenic plants

*SUC2* (ARABIDOPSIS THALIANA SUCROSE-PROTON SYMPORTER 2) (Truernit and Sauer, 1995) and *GC1* promoters (Yang et al, 2008) that are specific to phloem companion cells and guard cells, respectively, were amplified using primers shown in Table 1. The synthetic green fluorescent protein (sGFP) coding region (Chiu et al., 1996) was also amplified and cloned downstream of the promoter regions of the *SUC2* and *GC1* genes in BamHI and XbaI restriction sites in the pART7 plasmid. The amplified ABA2 coding region was fused with sGFP using XhoI and BamHI restriction sites. The cloned sequences were sequenced to exclude unwanted mutations. NotI restriction sites were used to cut out the pSUC2::ABA2-sGFP, pSUC2::sGFP, pGC1::ABA2-sGFP, and pGC1::sGFP constructs and cloned them into the pMLBart plasmid.

*A. tumefaciens* GV3101 cells were grown on Luria-Bertani (LB; #L3022, Sigma) medium plates containing gentamycin (15 µg/ mL) at 28°C. Single colonies were grown in 2 mL Yeast Extract Peptone (YEP; 10 g yeast extract, 10 g Bacto peptone, 5 g NaCl, pH 7.0 per 1L) medium with gentamycin (15 µg/ mL) overnight at 28°C on a shaker at 200 rpm. 50 mL YEP medium was inoculated with 2 mL of the overnight culture and incubated at 28°C until OD<sub>600</sub> reached ~0.5. The culture was chilled on ice for 10 minutes and then centrifuged for 10 min at 4000 rpm at 4°C. The pellet was resuspended in 10 mL of ice-cold 0.15 M NaCl and pelleted by centrifugation again for 10 minutes at 4000 rpm at 4°C. The pellet was resuspended in 1 mL ice-cold 20 mM CaCl<sub>2</sub>. The competent cells were put into 1.5-mL tubes and frozen at -80°C.

The plasmids were transformed to *A. tumefaciens* strain GV3101. The transformation was done by heat shock method. Competent *A. tumefaciens* cells (100 µl) were gently mixed with a plasmid and then incubated on ice for 20-30 minutes. Afterwards, the mixture was incubated at 37°C for 3 minutes for the heat shock. 1 mL of LB liquid medium was added and the mixture was incubated at 28°C in a shaker at 200-250 rpm for 1-2 hours. Most of the medium was removed. The cells were spread on LB plates with 25 µg/ml gentamicin and 50 µg/ml spectinomycin as selective markers for the pMLBart plasmid. The plates were incubated at 28°C for 2-3 days.

Agrotransformation of the ABA-deficient *aba2-11* mutant was done using the floral dip method (Bent, 2006). The plants stems were cut back once, after which it took about 10 days for them to start flowering again. Any siliques were removed before the dipping. *Agrobacterium* strains were grown in 5 mL of LB medium containing 50 µmg/ mL spectinomycin and 15 µmg/ mL gentamycin for 48 hours at 28°C on a shaker. 2 mL of the culture were transferred into 200 mL LB medium with the same antibiotics and grown overnight at 28°C on a shaker. Then, the cells were spun down in 50 mL tubes for 10 minutes at 4000 rpm. The supernatant was discarded and the pellet resuspended in 250 mL 5% sucrose solution with 0.05% Silwet L-77. Then, the *aba2-11* plants were dipped into the solution so every flower got wet. The dipped plants were placed on their sides into trays and covered with another tray to keep them in darkness. Wet paper towels were added to maintain humidity. After 24 hours the cover was removed, the plants were put into vertical position and were grown until seeds were mature.

Seeds collected from dipped plants were germinated in soil. The transgenic plants were selected by spraying seedlings with Basta (glufosinate ammonium) herbicide. Plants that survived after Basta spays were studied for sGFP fluorescence. The fluorescence in the vascular system of the mutants with pSUC2::ABA2-sGFP and pSUC2::sGFP was detected using SteREO Discovery.V20 stereo microscope. The 470/40 emission filter was used to excite and the 525/50 emission filter to detect the sGFP. The green fluorescence in guard cells of the pGC1::ABA2-sGFP and pGC1::sGFP transgenic plants were demonstrated under LSM 710 META Laser Scanning Microscope (Carl Zeiss) using a 458-nm beam for sGFP excitation and a 490-570 nm channel for emission of light. We selected 3 lines for each type of transgenic line, which carry only one insertion of T-DNA into plant genome, according to plant segregation after Basta treatments (3 Basta-resistant to 1 Basta-sensitive). These lines were brought to homozygosity.

The presence of the insertions and the genetic background of the transgenic lines were confirmed using PCR with primers shown in Table 1. Genomic DNA was extracted using modified hexadecyltrimethylammonium bromide based method (Healey et al., 2014).

## 2.2.5. Statistical analysis

Statistical analyses were performed with Statistica, version 7.0 (StatSoft Inc., Tulsa, OK, USA) and were considered significant at  $P < 0.05$ . Tukey's HSD test was used to compare individual means.

**Table 1. Primers used in this study**

Target	Sequences	Cloning sites
SUC2 promoter	Forward: AAGAGCTCGCGCCGCTAAAATCTGGTTTCATATTA Reverse: AAAACTCGAGATTTGACAAACCAAGAAAGT	SacI, NotI XhoI
GC1 promoter	Forward: AAAGAGCTCGCGCCGCATGGTTGCAACAGAGAGGATGA Reverse: AAAACTCGAGatttcttgagtagtgatttgaag	SacI, NotI XhoI
ABA2 coding region	Forward: AAAACTCGAGATGTCAACGAACACTGAATC Reverse: AAAGGATCCTCTGAAGACTTTAAAGGAGT	XhoI BamHI
sGFP coding region	Forward: AAGGATCCATGGTGAGCAAGGGCGAGGA Reverse: AAATCTAGATTACTTGTACAGCTCGTCCA	BamHI XbaI
Deletion in the aba2-11 mutant	Forward: GGAGACTATGGTGCGATTGG Reverse: CCTCACCTCCGAGATCATCT	
sGFP in transgenic plants	Forward: AAGGATCCATGGTGAGCAAGGGCGAGGA Reverse: AAATCTAGATTACTTGTACAGCTCGTCCA	
pSUC2::ABA2 region	Forward: ATGCAAAATAGCACACCATT Reverse: AAAGGATCCTCTGAAGACTTTAAAGGAGT	
pGC1::ABA2 region	Forward: GGCTCCAGCGATGAAAGTC Reverse: AAAGGATCCTCTGAAGACTTTAAAGGAGT	

## 2.3. Results and discussion

### 2.3.1. Generation and verification of plants with tissue-specific ABA biosynthesis

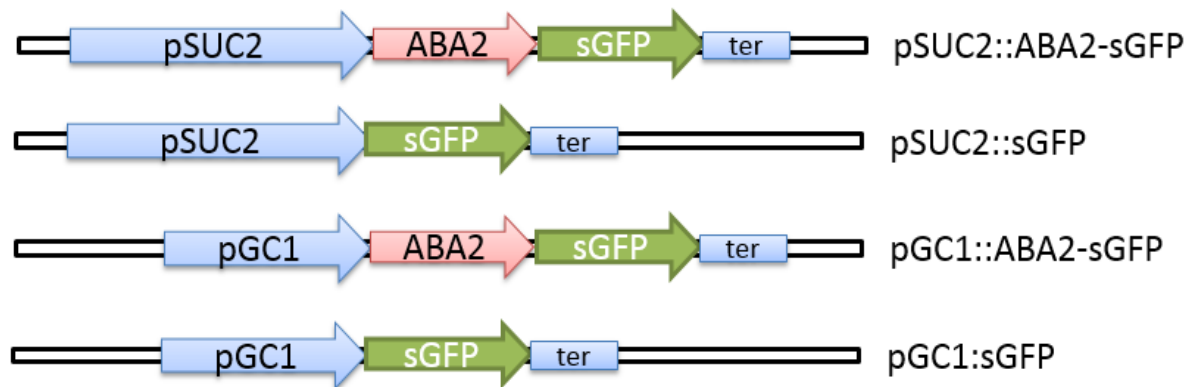
Although foliar ABA is well-known to be synthesized in phloem companion cells, guard cells also were recently shown to be able to produce ABA for stomatal closure (Kuromori et al., 2014; Bauer et al., 2013). In order to generate plants with ABA production in these two sites, we employed the ABA-deficient *aba2-11* mutant as the genetic background for ABA2 expression in a tissue specific manner. The *aba2-11* mutant was chosen due to the high impairment of the ABA biosynthesis pathway, resulting in the higher stomatal conductance and the lower ABA concentration than in other studied ABA-deficient mutants, including the *aba3-1* mutant that was used by Bauer et al. (2013) (the data is not shown). The *aba2-11* mutant does not demonstrate the conversion of xanthoxin to abscisic aldehyde and has a 53-bp deletion in *ABA2* (*At1G52340*). The inactivation of *ABA2* in the *aba2-11* mutant reduced the foliar ABA content to 24% of the wild type level, while the water-stressed *aba2-11* plants demonstrated only 3% of ABA level in the wild type plants (González-Guzmán et al., 2002). Using promoters which are specifically active either in guard cells or phloem companion cells, we aimed to restore ABA biosynthesis only in stomata or in vascular tissue, respectively.

The plasmids for plant transformation were constructed on the basis of pMLBart/pART7 plasmids (Gleave A. 1992) which were effectively used for Arabidopsis transformation before (Yarmolinsky et al, 2013). The cauliflower mosaic virus 35S promoter in the pART7 plasmid was replaced by tissue specific promoters GC1 and SUC2, which are active in guard cells and phloem companion cells, respectively. These promoters were selected based on literature analysis. Truernit and Sauer (1995) showed that the *SUC2* promoter targets expression of the reporter *GUS* gene to phloem of transgenic Arabidopsis plants. Thus, histochemical examination of the pSUC2::GUS plants demonstrated specific staining of phloem tissue in the vasculature of the transgenic plants. The *SUC2* promoter activity was the strongest in the phloem of leaves and was equal in all leaf veins. The *GUS* expression driven by the *SUC2* promoter was also observed in all parts of the developed root system (Truernit and Sauer,

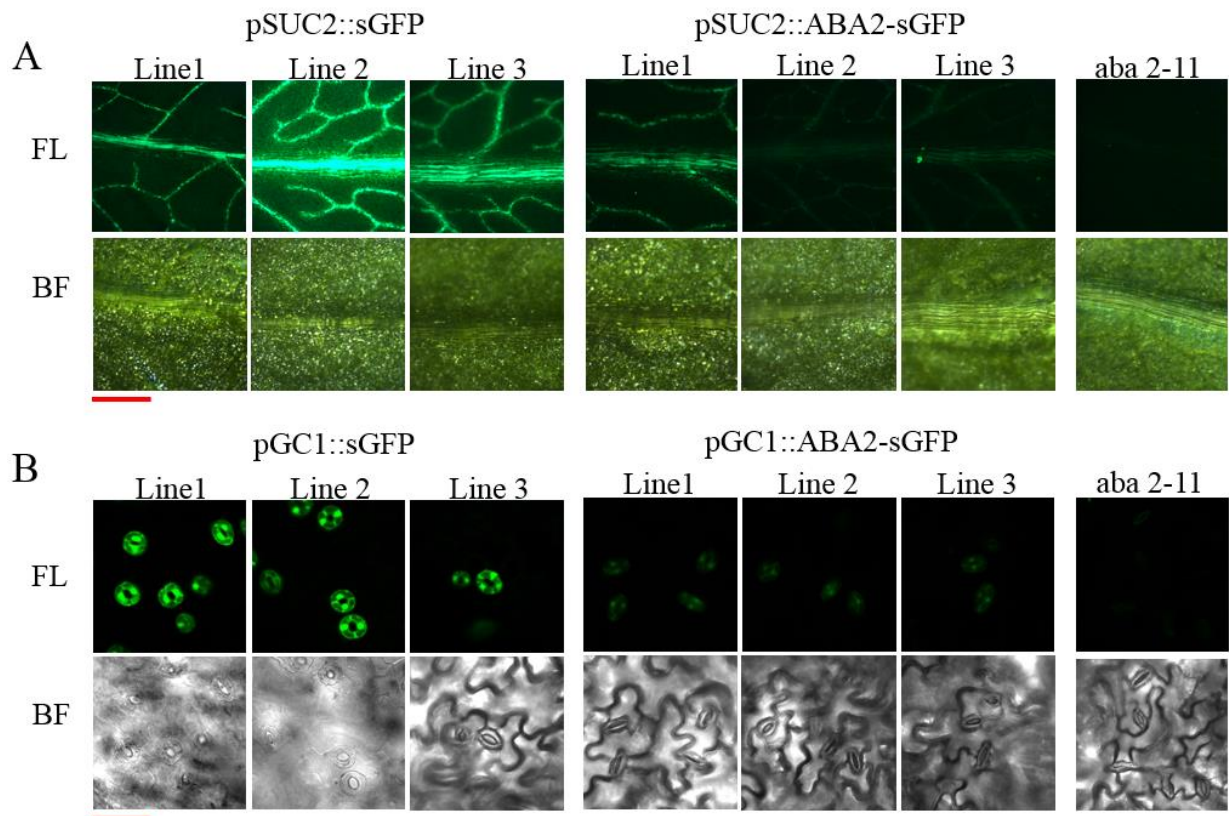
1995). Similarly, Yang et al (2008) reported about isolation of a strong guard cell-specific promoter in *Arabidopsis*. The authors studied available information about transcript expression in guard cells and selected several promoters as potentially guard cell-specific. The further examination showed that the *GC1* promoter had strong and specific activity in guard cells. The T1 pGC1::GUS transgenic plants demonstrated high GUS activity in guard cells while mesophyll cells did not demonstrate significant GUS expression. The *GC1* promoter activity was also efficient for guard cell-specific expression of the YELLOW CHAMELEON 3.60 calcium reporter. No fluorescence was detected in immature guard cells (Yang et al., 2008). These results indicate that the *SUC2* and *GC1* promoters can be used to specifically restore ABA biosynthesis in phloem and guard cells, respectively, in transgenic plants.

The full-length opening reading frame of *ABA2* was cloned and fused with *sGFP* to facilitate selection of transgenic plants. This approach also allowed us to estimate expression of *ABA2* in the transgenic plants. Additionally, we used genetic constructs, which carried only *sGFP* without *ABA2*, to generate control plants. The generated plasmids (Fig. 4) were introduced into *A. tumefaciens* strains which were used for genetic transformation of the *aba2-11* plants employing the floral dip method (Bent, 2006).

Seeds from the dipped plants were collected and germinated. Selection of transgenic plants was done by spraying seedlings with Basta (phosphinotricin). We selected 10-14 transgenic Basta-resistant lines for each construct. Plants, which survived after treatments with Basta, were examined using fluorescent and confocal microscopy. Transgenic lines expressing *ABA2-sGFP* or *sGFP* under the control of the *GC1* promoter demonstrated targeted expression of these proteins in guard cells, while transformation with the pSUC2::*ABA2-sGFP* and pSUC2::*sGFP* constructs resulted in the detectable *sGFP* signal in the vasculature of the transgenic plants (Fig. 5). According to *sGFP* detection, *ABA2-sGFP* expression was much weaker than expression of *sGFP*, pointing at the possibility of post-translational regulation mechanism for *ABA2* protein.

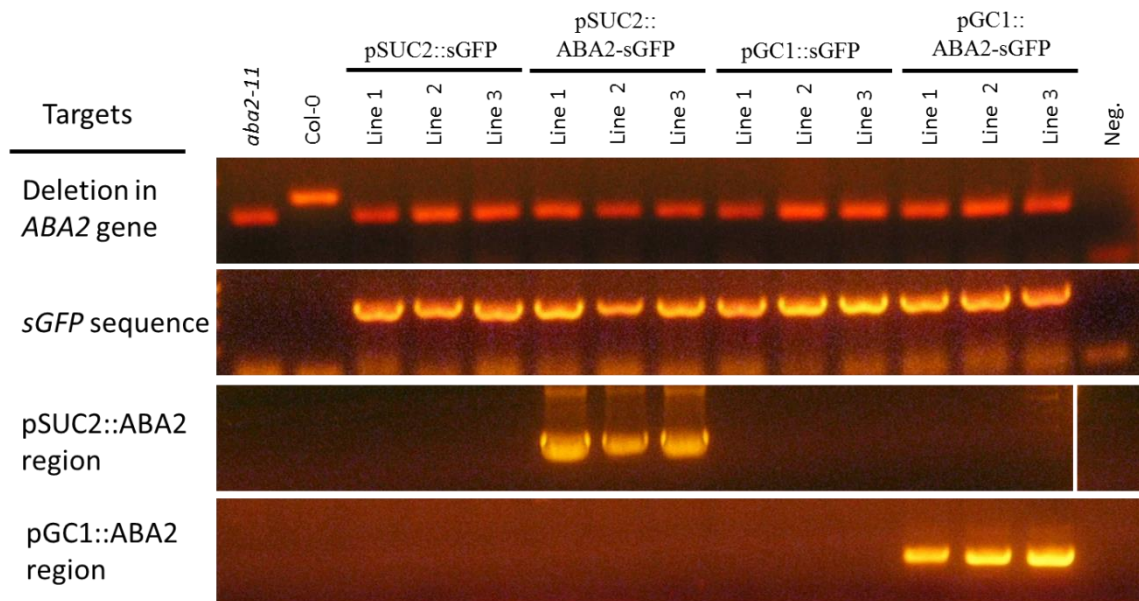


**Figure 4. Schematic diagram showing the structures of the plasmids for generation of transgenic plants used in this work.** Promoter regions of SUCROSE-PROTON SYMPORTER 2 (Truernit and Sauer, 1995) and *GC1* (At1g22690, Yang et al, 2008) are denoted as pSUC2 and pGC1, respectively. The coding region of *ABA2* (AT1G52340) was fused with the coding region of *sGFP* and cloned downstream of the promoters in the pSUC2::ABA2-sGFP and pGC1::ABA2-sGFP plasmids. The pSUC2::sGFP and pGC1::sGFP express only sGFP. Terminator (ter) of octopine synthase was used for transcription termination. The expression cassettes were cloned in pMLBart gene carrying the Basta resistance gene under control of the nos promoter. Element sizes on the diagram do not correspond to the relative sizes of the DNA fragments.



**Figure 5. Selection of transgenic lines used in this study.** A - Fluorescence of ABA2-sGFP and sGFP in the vascular system of plants (pSUC2::ABA2-sGFP and pSUC2::sGFP, respectively) was detected using SteREO Discovery.V20 stereo microscope. A 15-s exposure was used to collect the fluorescent light. The scale bars correspond to 1000  $\mu\text{m}$ . B – The green fluorescence in guard cells of the lines carrying the insertions of pGC1::ABA2-sGFP and pGC1::sGFP was demonstrated by using LSM 710 META Laser Scanning Microscope (Carl Zeiss) as described in the Material and Methods section. The scale bar corresponds to 50  $\mu\text{m}$ . The corresponding brightfield pictures (denoted as “BF”) are presented for every picture showing fluorescence (denoted as “FL”).

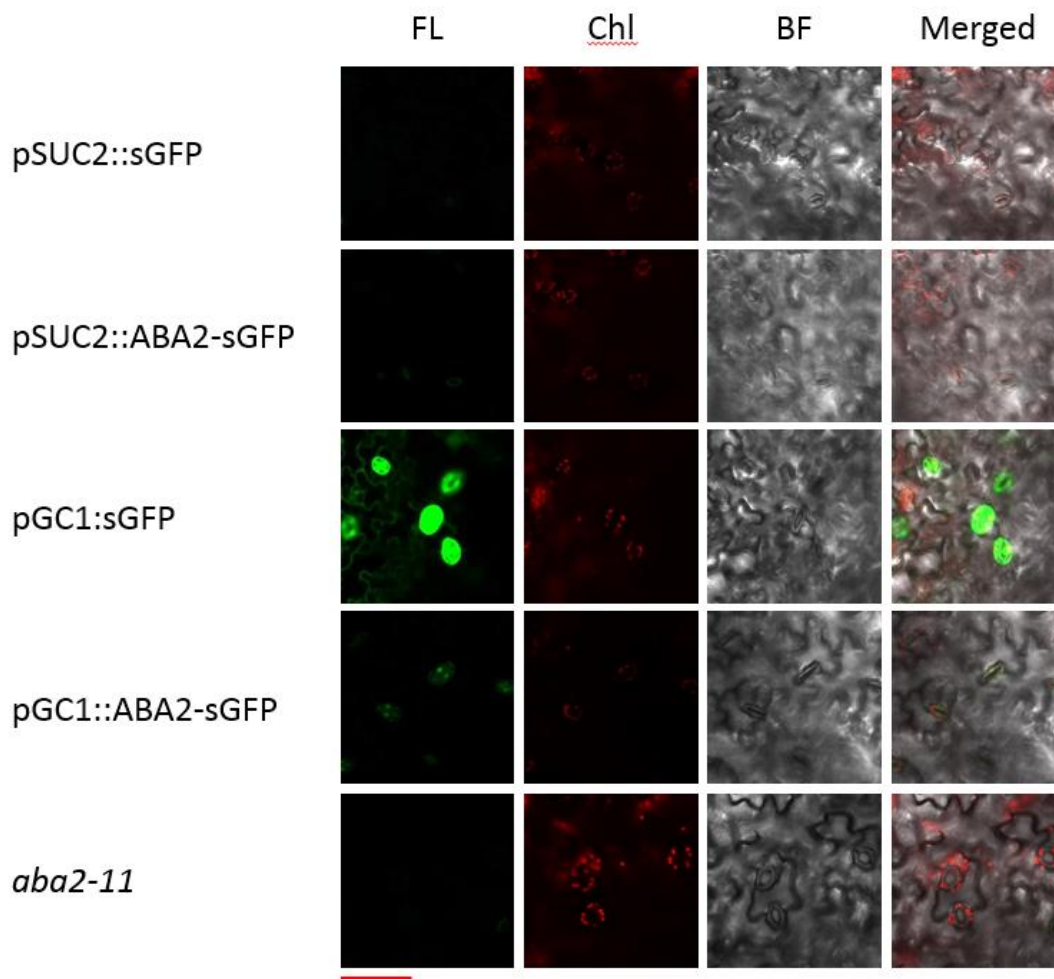
Using Basta treatments, we selected 3 lines for each type of transgenic lines, which carry only one insertion of T-DNA into plant genome. We also confirmed the presence of insertions and *aba2-11* genetic background of the transgenic lines by using PCR (Fig. 6). It was verified that the *ABA2* gene deletion was present in all transgenic lines as well as in the *aba2-11* mutant, but not in Col-0 plants. The presence of the *sGFP* gene was also confirmed by PCR in all generated transgenic lines. PCR with primers complementary to the used promoters and to the *ABA2* gene sequence demonstrated the presence of the insertions with the *ABA2* gene in the genomes of the selected transgenic lines.



**Figure 6. Verification of genotypes of the transgenic lines used in this work.** The deletion in *ABA2* gene was detected in all transgenic lines and in *aba2-11*, excluding the Col-0 plants. The *sGFP* sequence was presented in all transgenic lines. Amplification of *ABA2* gene under the *SUC2* and *GC1* promoters was also successful. Neg. – negative control amplified without genomic DNA. All amplified PCR fragments corresponded to the expected sized according to DNA ladders (not shown). The primer pairs used for PCR are shown in Table 1.

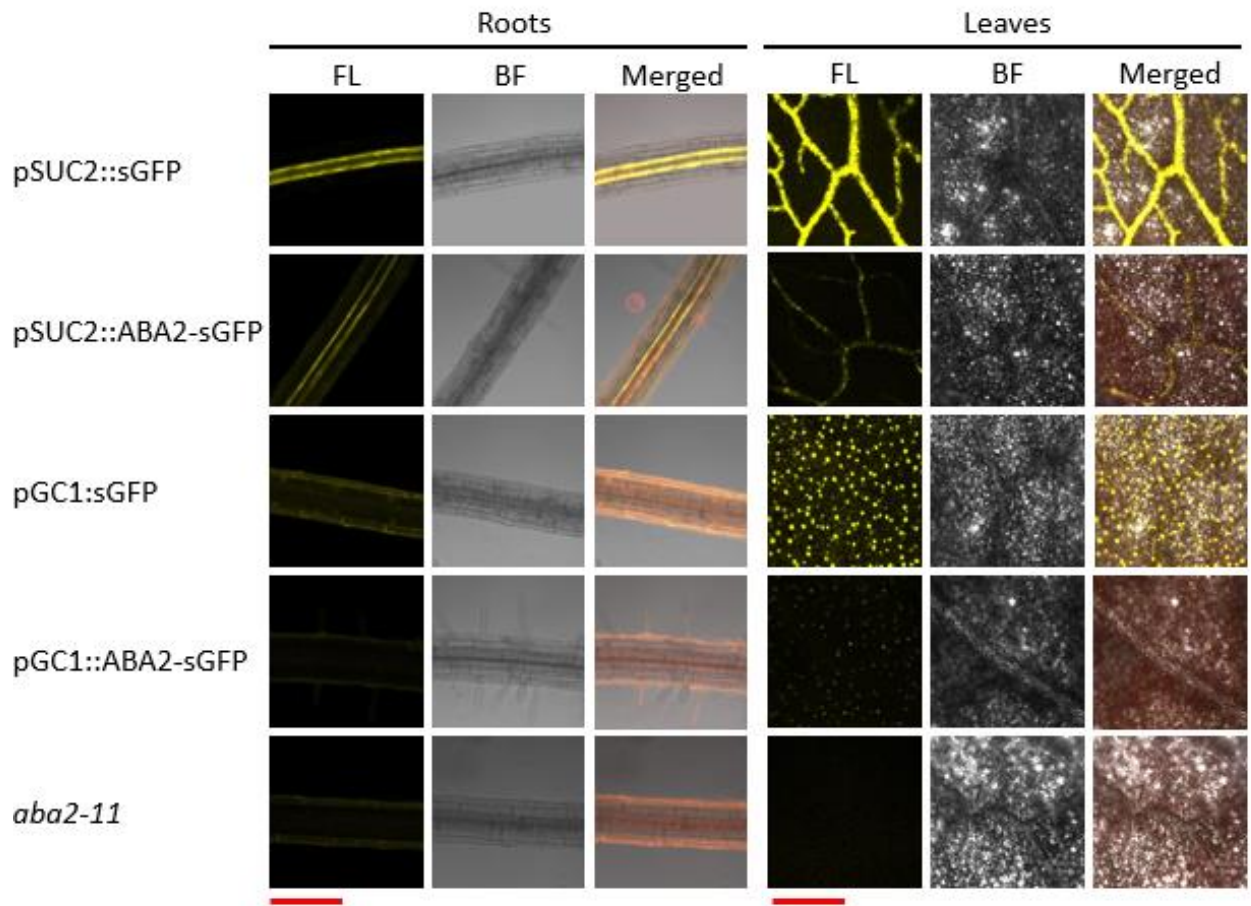
### 2.3.2. Tissue-specificity of *GC1* and *SUC2* promoters

In our study, the tissue-specific *GC1* and *SUC2* promoters were used to find out the relative contribution of guard cells and phloem companion cells into foliar ABA pool. Thus, it was important for us to study the tissue-specific activities of the employed promoters in order to exclude unwanted, “leaking” expression of *ABA2* in other tissues. As our transgenic lines express fluorescent proteins, we used confocal microscopy to study expression patterns of *sGFP* and *ABA2-sGFP* driven by the *GC1* and *SUC2* promoters. In accordance with Yang *et al* (2008), we found that the *GC1* promoter was clearly active in guard cells of the transgenic pGC1::sGFP and pGC1::ABA2-sGFP lines (Fig. 7, 8).



**Figure 7.** The *SUC2* promoter is not active in guard cells. Leaves of transgenic 4-weeks-old plants were analyzed using LSM 710 META Laser Scanning Microscope (Carl Zeiss) as described in the Material and Methods section. FL – fluorescent light, Chl – chlorophyll, BF – bright field, “Merged” was combined from FL, Chl, and BF images. The scale bar corresponds to 50  $\mu$ m.

The vasculature in leaves did not demonstrate any activity of the *GC1* promoter (Fig. 8). However, we found a weak activity of the *GC1* promoter in epithelial cells of roots in the transgenic pGC1::sGFP plants. The *SUC2* promoter was active in vessels of leaves and in roots as described by Truernit and Sauer (1995). As shown in Figure 8, the expression of sGFP under the control of the *SUC2* and *GC1* promoters demonstrate different patterns in roots. Thus, the *GC1* promoter, which seems to be faintly active in epithelial root cells, did not direct expression of sGFP in phloem companion cells where the *SUC2* promoter was active. sGFP signal in roots of pGC1::ABA2-sGFP was detected on the level of autofluorescence in the roots of the *aba2-11* mutant. Thus, fluorescent imaging excludes ABA production in roots of the pGC1::ABA2-sGFP plants.



**Figure 8. The SUC2 and GC1 promoters demonstrate high tissue specificity.** The left insert shows that expression of GFP under the control of the *SUC2* and *GC1* promoters demonstrate different patterns in roots. Plants were grown on plates containing 0.5 MS medium, 1% sucrose, 10 mM MES (pH 5.7), and 0.8% agar and analyzed at the age of 2.5 weeks. Images were taken using LSM 710 META Laser Scanning Microscope (Carl Zeiss). sGFP fluorescence was detected using 20% laser power for all samples excluding pSUC2::sGFP which was photographed using 1% laser power. The right insert shows that activity of *GC1* promoter is not detected in the leaf vessels. Leaves of transgenic 4-weeks-old plants were analyzed using LSM 710 META Laser Scanning Microscope (Carl Zeiss) as described in the Material and Methods section. FL – fluorescent light, Chl – chlorophyll, BF – bright field, “Merged” was merged from FL, Chl, and BF images. The scale bars correspond to 200  $\mu$ m (courtesy of Yarmolinsky).

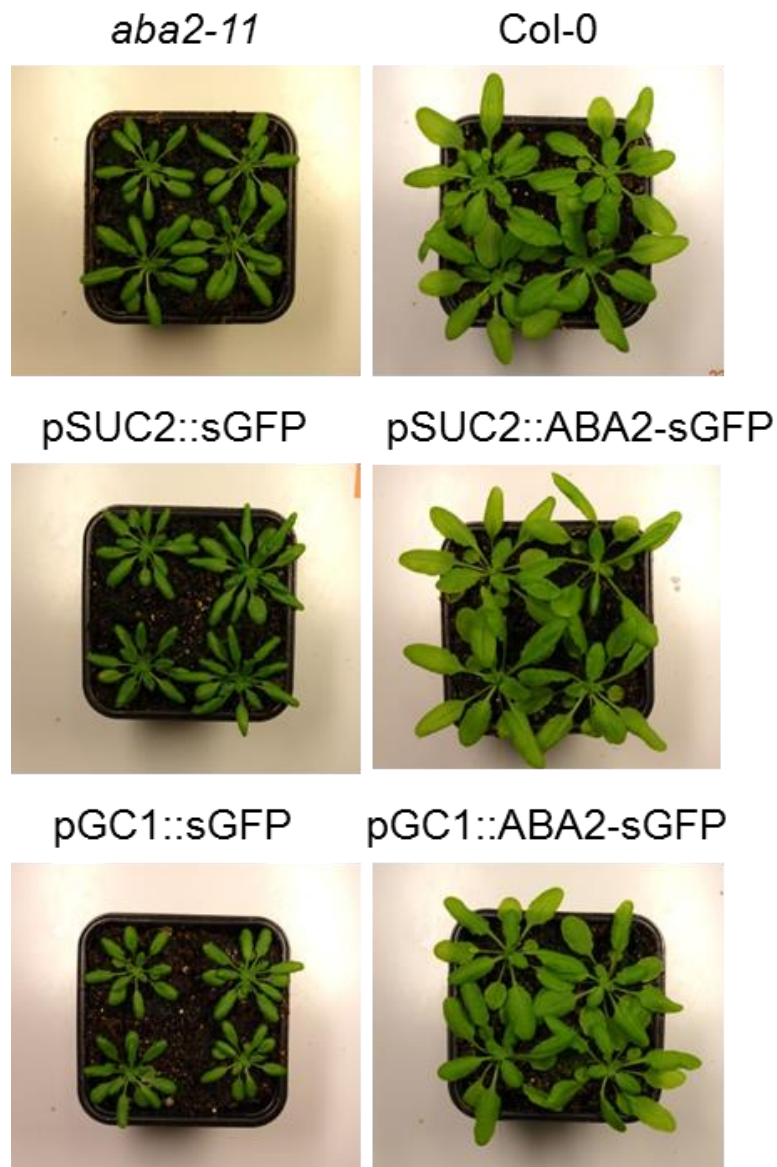
The SUC2 promoter activity was obvious in the vascular tissues of roots and leaves and showed a clearly different pattern from the *GC1* promoter. Thus, the absence of sGFP and ABA2-sGFP expression in guard cells was evident in the pSUC2:: sGFP and pSUC2::ABA2-sGFP lines (Fig. 7).

### 2.3.3. Phenotypes of plants with restored ABA biosynthesis either in guard cells or in phloem

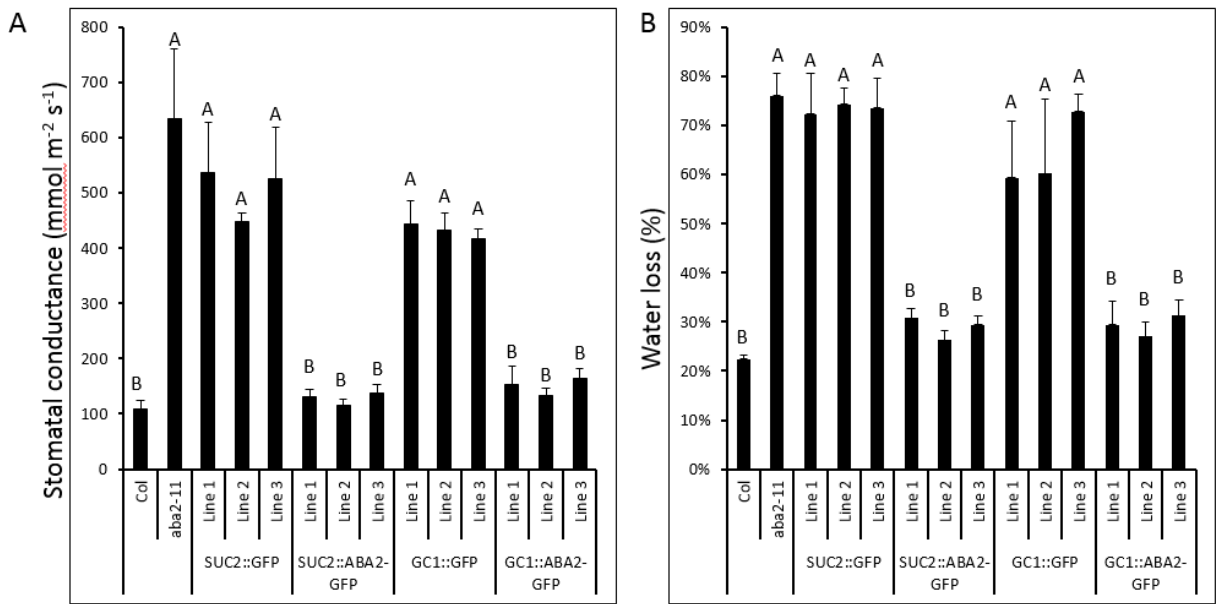
Transgenic lines expressing ABA2-sGFP either in guard cells or in phloem companion cells were compared with the lines carrying *sGFP* under the same promoters as well as with the parental *aba2-11* line and Col-0. Expression of ABA2-sGFP in guard cells or phloem companion cells rescued the visual phenotype of the *aba2-11* mutant. Thus, both pGC1::ABA2-sGFP and pSUC2::ABA2-sGFP had wider leaves and were bigger in size than the *aba2-11* mutant, resembling Col-0 plants. At the same time, the pSUC2::sGFP and pGC1::sGFP lines, lacking the *ABA2* gene, had narrow leaves and reduced plant size, which were characteristic to ABA-deficient mutants. Therefore, ABA production in both guard cells and phloem companion cells of the ABA-deficient mutant rescued plant development in the ABA-deficient *aba2-11* mutant (Fig. 9).

Similarly to the visual phenotypes, stomatal functions in the *ABA2-sGFP* lines were restored. Figure 10 A shows stomatal conductance in the *ABA2-sGFP* lines expressing ABA2-sGFP under the control of the guard cell- and phloem-specific promoters, which was on the same level as in Col-0 plants (about  $100 \text{ mmol m}^{-2} \text{ s}^{-1}$ ). Stomatal conductance in the *aba2-11*, pSUC2::sGFP, and pGC1::GFP lines and sGFP only lines was about 4-6-fold higher than that in the Col-0 and ABA2-sGFP plants.

Water loss from detached leaves is an important characteristic which reflects stomatal functioning and is frequently enhanced in stomatal mutants defective in stomatal closure, for example in the *slac1* mutants (Vahisalu et al, 2008). The pSUC2::ABA2-sGFP and pGC1::ABA2-sGFP lines demonstrated 20-30% of fresh weight loss from detached leaves after 2 h of drying, similarly to the Col-0 plants. The *aba2-11* plants showed dramatically higher water evaporation from detached leaves, which resulted in approx. 80% of fresh weight loss. Expression of pSUC2::sGFP and pGC1::sGFP in the *aba2-11* mutant did not influence water loss which was around 70-60% of fresh weight (Fig. 10 B).

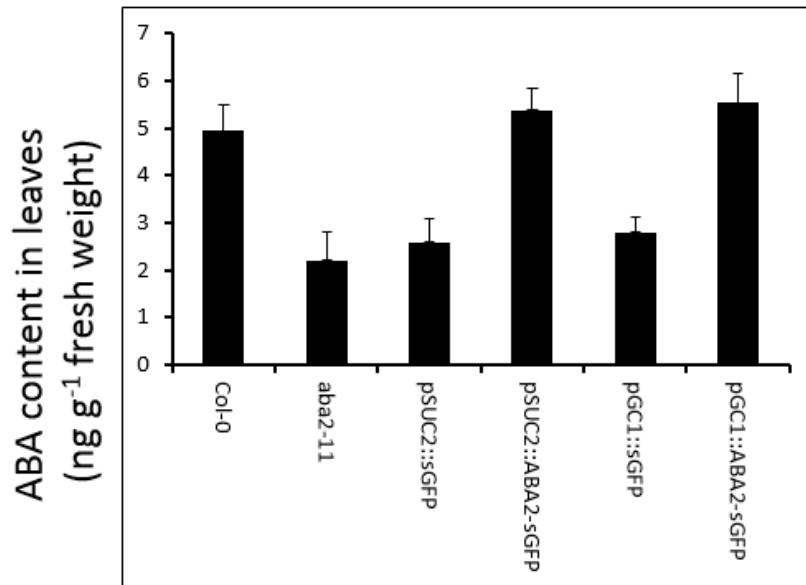


**Figure 9.** Expression of ABA2-sGFP in both phloem companion cells (pSUC2::ABA2-sGFP) and in guard cells (pGC1::ABA2-sGFP) in *aba2-11* recovers the wild type plant appearance. The plants of the transgenic pSUC2::sGFP and pGC1::sGFP lines demonstrated the ABA-deficient *aba2-11* mutant phenotype. Plants were grown in the close vicinity to each other and were photographed at the age of 4 weeks. Representative pictures for each genotype are shown.



**Figure 10.** The pSUC2::ABA2-sGFP and pGC1::ABA2-sGFP lines demonstrate recovered stomatal conductance (A) and water loss from detached leaves (B), while expression of pSUC2::sGFP and pGC1::sGFP did not affect the same parameters. The data on stomatal conductance (A) and water loss (B) are presented as average $\pm$ SE (n=3-4). The values not connected by the same letters are statistically different according to Tukey-Kramer HSD (p<0.05).

Based on the visual phenotypes and stomatal functioning, one can expect that expression of pSUC2::ABA2-sGFP and pGC1::ABA2-sGFP in the *aba2-11* mutant enhanced ABA content to the wild type level. According to our ABA assays (Fig. 11), ABA concentration in the *aba2-11* leaves was reduced to 44% of that in Col-0 plants. The plants expressing only sGFP showed approximately the same level of ABA concentration as the *aba2-11* mutant. Accordingly, the plants with ABA2-sGFP expressed in *aba2-11* under the control of the two different promoters demonstrated restored ABA content in leaves. It is possible to conclude that the rescued visual phenotypes and stomatal functioning in the pGC1::ABA2-sGFP and pSUC2::ABA2-sGFP lines can be explained by the recovered ABA levels in those plants. These results demonstrate that both guard cells and phloem companion cells can be significant sources of ABA in plants.



**Figure 11.** Expression of pSUC2::ABA2-sGFP and pGC1::ABA2-sGFP results in recovered ABA content leaves of the transgenic plants. ABA concentrations were measured in leaves of 4-weeks-old plants. The results were grouped for each genotype; the presented values are average $\pm$ SE (n=12 for pSUC2::sGFP and pSUC2::ABA2-sGFP, n=11 for pGC1::ABA2-sGFP, n=9 for pGC1::sGFP, n=7 for Col-0 and *aba2-11*). The values not connected by the same letters are statistically different according to Tukey-Kramer HSD ( $p < 0.05$ ). The picture is of courtesy of Yarmolinsky.

It was shown by Bauer et al. (2013) for the first time that guard cells can autonomously synthesize ABA (Bauer et al., 2013). However, interpretation of these results is complicated by the facts that the authors used the relatively “weak” *aba3-1* mutant and the *MYC60* promoter that is down-regulated by ABA (Rusconi et al., 2013). Moreover, the authors studied resistance of leaves to low air humidity, which indicates ABA production only indirectly. Bauer et al. (2013) showed that plants with ABA synthesized only in guard cells did not wilt in dry air like the *aba3-1* mutants did, but this does not guarantee that the stomatal closure was ABA dependent. McAdam and Brodribb (2015) disputed the importance attributed to ABA biosynthesis in guard cells, suggesting that ABA could be quickly transported from leaf vasculature to the guard cells (McAdam and Brodribb, 2015). Our results clearly demonstrate that guard cells are able to efficiently produce ABA. The impact of ABA biosynthesis by guard cells is comparable with that in phloem companion cells. It follows from similar phenotypes of the *aba2-11* mutants expressing ABA2 in guard cells or in phloem (Fig. 9, 10).

Kuromori et al., (2014) enhanced ABA production in phloem companion cell of Arabidopsis plants and showed that ABA can be efficiently transported to guard cells, according to higher leaf temperature and reduced water loss from detached leaves. These results point at more closed stomata and indicate ABA transportation to guard cells, however, stomatal conductance was not directly measured by the authors. Our results also indicate the importance of ABA transport and transporters within plants. We showed that guard cells can be the source of ABA that is transported to all plant tissues as shown by recovered visual phenotype in the pGC1::ABA2-sGFP plants in the *aba2-11* genetic background (Fig. 9). Thus, ABA can be transported from vasculature to guard cells and from guard cells to other plant organs, indicating that ABA transportation is well covered in leaves. It remains to be elucidated whether there are other places for ABA synthesis in shoots and why redundant ABA biosynthetic locations exist.

## SUMMARY

Even though it had been previously shown that guard cells are capable to produce abscisic acid, the importance of guard cell-originated ABA is not known. It was believed that ABA is mainly transported to guard cells from the vascular system and that guard cells autonomous ABA biosynthesis could play only a minor role. Finding out the relevance of the two ABA synthesis sites, guard cells and phloem companion cells, is the main goal of this study. We employed generation of transgenic plants in the ABA-deficient background with restored ABA production only in guard cells or phloem companion cells using tissue specific promoters. We used the ABA deficient *aba2-11* mutant with low ABA content and transformed it with constructs expressing fluorescently tagged ABA2 under control of the *GC1* or *SUC2* promoters, which are active in guard cells or phloem companion cells, respectively. Using confocal microscopy, we confirmed tissue specificity of the employed promoters. Plant phenotypes demonstrate that restored ABA biosynthesis in both guard cells and phloem companion cells completely rescues plant phenotypes, including plant appearance, stomatal conductance, and water loss from detached leaves as well as ABA content. Our results show that guard cells and phloem companion cells are significant sources of ABA in plants, although it remains to be elucidated why redundant ABA biosynthetic locations exist. Deeper understanding of ABA biosynthesis can contribute to breeding crops with improved drought resistance.

# KOKKUVÕTE

Õhulõhede juhtivus sõltub nii sulgrakkudes kui ka floemi kaasrakkudes toodetud abtsiishappest

Helen Parik

Resümee

Millist rolli mängib ABA biosüntees taime erinevates kudedes pole veel täpselt teada. Varasemalt on küll näidatud, et sulgrakud on iseseisvalt võimelised ABA-t tootma, kuid teiste kudede rolli ei ole piisavalt uuritud. Käesolevas töös selgitati taime õhulõhede juhtivuse sõltuvust abtsiishappe biosünteesist sulgrakkudes ja floemi kaasrakkudes.

Selleks kasutati vigase abtsiishappe sünteesiga *aba2-11* mutanti. Neil taastati ABA süntees kas sulgrakkudes või floemi kaasrakkudes, kasutades vastavalt kas pGC1 või pSUC2 promooterit. Transgeensete taimede selektsiooniks kasutati resistentsust Bastale ja sGFP-d. Valiku läbinud taimedega tehti gaasivahetuskatseid kasutades eritellimusel valmistatud aparatuuri. Mõõdeti õhulõhede juhtivust ja vee aurustumist. Tulemusi võrreldi Col-0 metsiktüüpi taimedega, millel on normaalne ABA biosüntees. Samuti võrreldi omavahel taimede kasvufenotüüpe.

Töö käigus selgus, et taastatud ABA-sünteesiga *aba2-11* mutantidel oli Col-0-iga õhulõhede juhtivus samal tasemel. Kusjuures sama tulemus saadi nii taimeliinides, mis ekspresseerisid ABA2 sulgrakkude spetsiifilise *GC1* promooteriga kui ka taimeliinides, mis ekspresseerisid ABA2 valku floemispetsiifilise *SUC2* promooteriga. Tavalisel *aba2-11* oli aga kuni 6 korda kõrgem juhtivus. Samuti oli suur vahe ka vee aurustumisel, defektse ABA-sünteesiga taimedel aurustus 4 korda rohkem vett. Vaadeldes taimede välimust oli selgelt näha, et ABA-t ainult sulgrakkudes või floemis sünteesivatel taimedel oli metsiktüübiga sarnane välimus, teistel oli rosett märgatavalt väiksem.

Nende tulemuste põhjal võib väita, et sulgrakkude ja floemi kaasrakkude toodetud abtsiishape on sama tähtsusega õhulõhede juhtivuse muutmisel. Sulgrakud suudavad iseseisvalt toota piisaval hulgal abtsiishapet ja taimedel on hea ABA transpordisüsteem. Tulevikus tuleks uurida miks on taimedel välja kujunenud mitu ABA biosünteesi asukohta.

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