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Guard cell-specific silencing of Arabidopsis plasma membrane proton pumps

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Tartu 2021

Guard cell-specific silencing of Arabidopsis plasma membrane proton pumps

Abstract:

Stomata are structures in the plant epidermis consisting of two specialized guard cells and a stomatal pore between them. The gas exchange occurs through stomata, making these cells vital for plants. Stomatal aperture is regulated by the activity of plasma membrane proton pumps (AHAs), which is encoded by 11 isoforms in *Arabidopsis.* Previously, mutants with overexpressed or hyperactive single AHAs have been analysed, which have failed to answer the questions about the regulation and functions of proton pumps in guard cells. However, *aha* double mutants demonstrated embryo lethality and the single *aha* loss-of-function mutants have no growth impairments, making the function of AHAs hard to test. To address these problems, transgenic lines where three major AHAs were silenced in the guard cells by a novel amiRNA approach was applied in this thesis.

Keywords: stomata, guard cells, proton pump, amiRNA

CERCS: B310; Physiology of vascular plants

Plasmamembraani prootonpumpade vaigistamine hariliku müürlooga sulgrakkudes

Lühikokkuvõte:

Õhulõhed on struktuurid taime epidermises, mis koosnevad kahest spetsialiseerunud sulgrakust ja nende vahele jäävast õhupilust. Õhulõhede kaudu toimub taimede gaasivahetus, muutes need taimedele eluliselt tähtsaks. Õhulõhede avatust reguleerivad plasmamembraani prootonpumbad, mida on harilikus müüürloogas kokku 11 erinevat isovormi. Varem on analüüsitud taimeliine kus on muudetud üksikute prootonpumpade aktiivsust. Prootonpumpade topeltmutandid on embrüoletaalsed ja prootonpumpade üksikmutantidel puudub spetsiifiline fenotüüp, mistõttu on prootonpumpasid olnud raske uurida. Probleemi lahendamiseks loodi prootonpumpade mutandid, kus sulgrakkudes vaigistati kolm peamist õhulõhedes esinevat prootonpumpa kasutades uudset amiRNA meetodit.

Võtmesõnad: õhulõhed, sulgrakud, prootonpump, amiRNA

CERCS: B310, soontaimede füsioloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ABA – abscisic acid

AHA1-1 – ARABIDOPSIS THALIANA H^+-ATPase 1-11

amiRNA – artificial micro RNA

AtABCB14 – ATP-BINDING CASSETTE B14

NRT1.1 (CHL1) – NITRATE TRANSPORTER 1 (CHLORINA 1)

AKT1 – ARABIDOPSIS THALIANA K^+ TRANSPORTER 1

ALMT – ALUMINUM-ACTIVATED MALATE TRANSPORTERS

DCL1 – DICER-LIKE1

GORK – GATED OUTWARDLY-RECTIFYING K^+ CHANNELS

HT1 – HIGH LEAF TEMPERATURE 1

KAT1; KAT2 – POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1; 2

MPK4; MPK12 – MITOGEN-ACTIVATED PROTEIN KINASES 4; 12

OST1; OST2 – OPEN STOMATA 1; 2

PCR – polymerase chain reaction

PHOT; PHOT2 – PHOTOTROPIN 1; 2

PM – plasma membrane

QUAC1 – QUICK-ACTIVATING ANION CHANNEL 1

ROS – reactive oxygen species

R- type – rapid type

SLAC1 – SLOW ANION CHANNEL ASSOCIATED 1

SLAH3 – SLAC1 HOMOLOGUE 3

S-type – slow type

T-DNA – transfer DNA

INTRODUCTION

Plants remain to be the main sources of food and energy on Earth. First, plants evolved in the oceans, when plants started to colonize land, it was important to adapt and find a way to maintain plants' active metabolism in dry terrestrial conditions. This required evolution of water-impermeable cuticles covering plant leaves and the formation of stomatal pores to maintain an exchange of gases with the surrounding atmosphere. Stomatal pores are formed by two guard cells located on surfaces of plant leaves. Guard cells are adapting to environmental change or other stimuli by opening and closing stomatal pores. Open stomata allow CO_2 uptake for photosynthesis and water evaporation from the plant. Guard cell movement is one of the plant responses to unfavorable conditions. For instance, in response to drought, chemical or physical attack, stomata close their pores.

Nowadays, 80% of Earth biomass consists of plants making it the primary carbonaceous organism. However, over the decades the human population has been increasing in numbers and in its impact on environment which resulted in biomass decrease by half since the start of civilization (Bar-On et al., 2018). At the same time, human water consumption has been increased eight-fold within past hundred years and due to this, it is likely that water will be the most limited natural resource for plant growth.

Stomatal opening is triggered by plasma membrane proton pumps. Therefore, the identification of its function can contribute to crop production, overall plant stress resistance and growth. Current research provides information on important functional characteristics of major proton pumps.

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

1 LITERATURE REVIEW

1.1 Stomata

The gas exchange between the plant and its environment is regulated through special microscopic pores that can be found on the aerial green surfaces of higher plants. Each pore is bounded by a pair of guard cells that originate from epidermal stem cells. The complex is comprised of two guard cells, and the opening between them is distinctively called stoma, in plural stomata. Guard cells can control the aperture size of stomata by changing their shape according to demand. Stomata are separated from each other by at least one neighboring epidermal pavement cell (Figure 1). Stomatal opening and closure are also influenced by adjacent cells since the ions needed for stomatal movements come from the surrounding apoplast (Nadeau and Sack, 2002; Nadeau, 2009; Pillitteri and Torii, 2012).

Most of the available information about stomata is based on research where *Arabidopsis thaliana* is used as a model organism. In *Arabidopsis,* stomata appear kidney-shaped and are more frequent on the abaxial side of the leaves. This pattern helps to avoid their direct exposure to heat and wind. The guard cell wall consists of radially orientated cellulose microfibrils and pectin-rich hemicellulose matrix, which support changes in stomatal size and shape. Cellulose microfibers provide stiffness, while pectin is important for the flexibility of the guard cell wall (Jones et al., 2005; Marom et al., 2017).

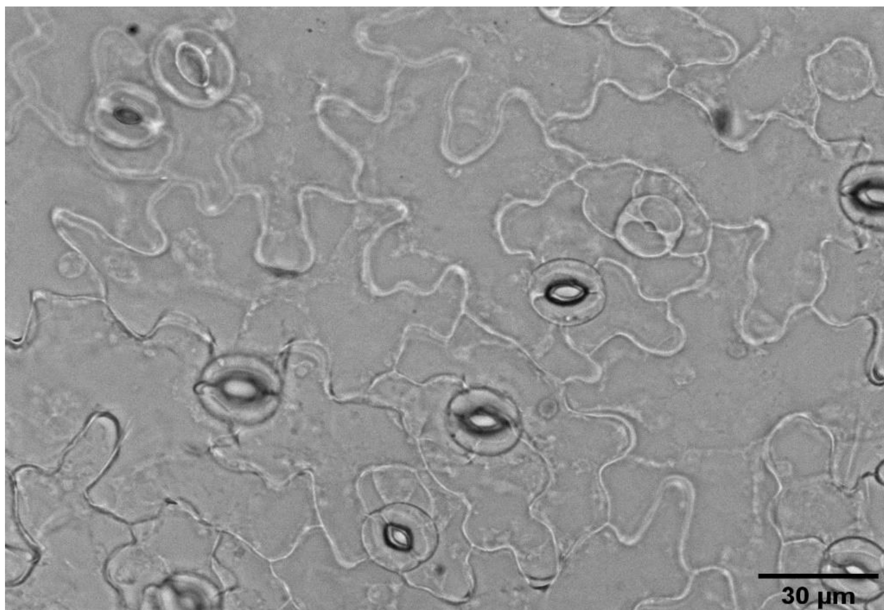

Figure 1. Arabidopsis stomata under the microscope. Stomata of wild-type plant Col-0 have been visualized after incubating epidermal strips under the light in the opening buffer containing KCl.

The evolution of stomatal regulation is part of the adaptation process that allowed plants to move from water to land (Ziegler, 1987). Through stomatal pores, the CO_2 required for photosynthesis is accumulated from the atmosphere. However, CO_2 diffusion into leaves occurs in parallel with water evaporation through stomata as the relative air humidity is usually lower in the surrounding atmosphere than inside the leaves. Consequently, the main function of guard cells is to maintain and fine-tune the balance between CO_2 uptake and transpirational water loss under different environmental conditions. There are many factors that can affect stomatal movements, such as changes in CO_2 concentrations, water deficiency, pathogen attack, ozone exposure, and concentration of different hormones in the plant tissues (Kim et al., 2010; Schroeder et al., 2001b).

1.1.1 Stomatal movements

The guard cell turgor is dynamically adapted to environmental and hormonal signals to promote proper CO_2 uptake and avoid excessive water loss. Stomatal opening and closure occur when guard cells swell or shrink, respectively. This process is ensured by the accumulation and release of osmotically active ions in guard cells, and thereby, the regulation of stomatal movements depends on the activity of different ion channels, transporters, and pumps in the guard cell membranes (Daszkowska-Golec and Szarejko, 2013).

Plasma membrane proton pumps serve a central role in modulating stomatal movements. Both processes, stomatal opening, and closure depend on the activity regulations of proton pumps. To initiate stomatal opening, plasma membrane (PM) proton pumps need to be activated. However, it is critical to inversely inhibit their work to close stomata. Throughout the active state, proton pumps generate a gradient of protons by pumping protons from inside to the outside of the guard cells. The resulting electrochemical gradient activates many secondary transporters essential for the uptake of ions and other metabolites. Due to ion accumulation in guard cells, the turgor pressure increases. This, in turn, decreases guard cell water potential, which provides a driving force for water transport into the guard cells (Pandey et al., 2007). In contrast, during stomatal closure, solutes outflow results in low osmotic and turgor pressure. Stomatal movements are discussed in detail in the following subsections.

1.1.2 Stomatal opening

The stomatal opening can be triggered by sufficient illumination in the environment. Stomata respond by opening due to the perception of increasing intensities of blue and red light. Blue

light serves as a signal, while red light serves as both a signal and a source of energy for photosynthesis (Shimazaki et al., 2007). The stomatal response is predominantly induced by blue light (400-500 nm), but weak red (680 nm) and far-red (730 nm) background lights enhance the impulse (Ogawa et al., 1978; Mao et al., 2005). Another example of a positive regulator of stomatal opening is low $CO_{2.}$ concentrations. When the CO_2 levels inside the leaves drop, stomata need to be opened to let the required CO_2 in for proper photosynthesis. The opening of stomata is led by pumping H^+ from the guard cell cytoplasm to the apoplast by plasma membrane proton pumps (Figure 2A). Maintaining proton pumps active requires a high amount of energy and hence is an ATP-dependent process (Palmgren et al., 2011). For this reason, plant proton pumps are also designated as H^+-ATPases. The activation of proton pumps in guard cells is positively regulated by all the same stimuli triggering stomatal opening, adding growth hormone auxin to the list (Kinoshita and Shimazaki, 2001; Hager et al., 1991; Elmore and Coaker, 2011).

Proton outflow provokes plasma membrane hyperpolarization, which drives the influx of cations, predominantly K^+ (Schroeder et al., 1987). K^+ are pumped into guard cells through polarization-dependent K^+ inward rectifying channels KAT1, KAT2 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1; 2), AKT1 and AKT2 (ARABIDOPSIS THALIANA K^+ TRANSPORTER 1; 2) (Schachtman et al., 1992; Pilot et al., 2001; Szyroki et al., 2001). Also, apoplastic acidification promotes the activation of referred K^+ channels (Taiz and Zeiger, 2010; Daszkowska-Golec and Szarejko, 2013).To obtain ion balance in the guard cells after K^+ uptake, Cl^- and NO_3^- are taken up from the apoplast via cotransporters (Pandey et al., 2007). While NO^{-3} has been demonstrated to be delivered to guard cells via nitrate transporter AtNRT1.1 (CHL1) [NITRATE TRANSPORTER 1 (CHLORINA 1)], then no specific transporter has been discovered for Cl^- so far (Guo et al., 2003; Pandey et al., 2007).

Alongside inorganic charged molecules, organic malate^{2-} and neutral compound sucrose contribute to the increase of osmotic pressure in guard cells. An ABC-class transporter, ABCB14 (ATP-BINDING CASSETTE B14), has been identified as a malate importer (Lee et al., 2008). The amount of free malate in the apoplast is limited, and therefore, malate^{2-} is generated from starch breakdown and glycolysis (Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005). Also, there is evidence about sucrose being taken up from the apoplast during the stomatal opening, presumably by the H^+ antiporter (Reddy and Das, 1986; Ritte et al., 1999). The ion influx results in water uptake, increase in turgor pressure, and eventually guard cell swelling, which leads to the opening of stomatal pores.

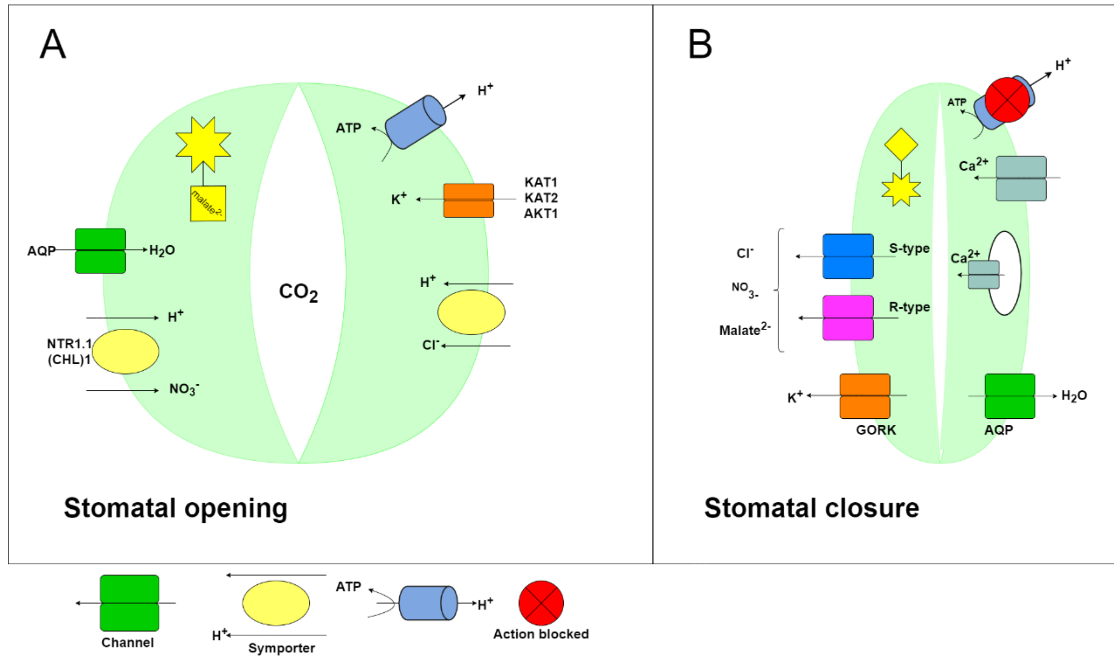

Figure 2. Stomatal opening and closure. During stomatal opening (A), the proton pump uses ATP to pump hydrogen molecules to the apoplast, which leads to membrane hyperpolarization and subsequent activation of polarization-dependent K^+ inward rectifying channels. For ion balance, Cl^- and NO_3^- enter cells from the apoplast via cotransporters. When stomata open, CO_2 is uptaken from the atmosphere. Starch is converted into malate during stomatal opening. During stomatal closure (B), proton pump is deactivated, which leads to membrane depolarization, and malate^{2-}, Cl^-, and NO^{-3} are released through rapid- and slow-type o anion channels. For K^+ release, outwardly rectifying K^+ channel, GORK is activated water leaves the guard cells via aquaporins.

1.1.3 Stomatal closure

The inactivation of proton pumps and the activation of guard cell anion efflux channels result in depolarization of the membrane leading to stomatal closure. Exposure to plant hormone abscisic acid (ABA), elevated Ca^{2+}, darkness, and high CO_2 concentration can trigger the inactivation of H^+-ATPases (Hayashi et al., 2011). However, to understand how environmental signals regulate proton pump shut-off, further research is required (Falhof et al., 2016).

When environmental stimuli trigger stomatal closure, it leads to the deactivation of proton pump and activation anions outflow through special channels (Schroeder et al., 1993)(Figure 2B). Malate^{2-}, Cl^-, and NO^{-3} are released through rapid- and slow-type of anion channels (Schroeder and Keller, 1992; Roelfsema et al., 2004; Roelfsema and Hedrich, 2005). The rapid type (R-type) anion channel is voltage-dependent and activates rapidly (activation time 5-50 ms), whereas S-type channels have minimal correlation with voltage and the activation time is slow (activation time 5-50 s) (Roelfsema and Hedrich, 2005).

While R-type channels need further investigation, studies with S-type anion channels revealed more information (Hoekenga et al., 2006; Meyer et al., 2010; Vahisalu et al., 2008; Negi et al., 2008). The R-type channel is encoded by a member of the ALMT (ALUMINUM-ACTIVATED MALATE TRANSPORTERS) gene family (Hoekenga et al., 2006). QUAC1 (QUICK ACTIVATING ANION CHANNEL 1) is the only member of its family which is located in the guard cell plasma membrane and considered forming an R-type anion channel (Meyer et al., 2010; Sasaki et al., 2010). QUAC1 knockdown mutants expressed partially impaired stomatal closure as a response to ABA, elevated CO_2, and darkness (Meyer et al., 2010). One of the S-type anion channels in the guard cell plasma membrane is SLAC1 (SLOW ANION CHANNEL ASSOCIATED 1). The significant importance for stomatal closure regulations of SLAC1 was discovered when mutants lacking functional SLAC1 demonstrated impaired response almost to every stimulus, together with ABA, CO_2, and O_3, affecting stomatal movements (Vahisalu et al., 2008). In addition to SLAC1, there is another anion channel represented in guard cells – SLAH3 (SLAC1 HOMOLOGUE 3) (Geiger et al., 2011). SLAC1 is demonstrated to conduct Cl^- and NO_3^-, while SLAH3 is mostly permeable to NO_3^- (Geiger et al., 2011; Negi et al., 2008; Vahisalu et al., 2008).

As a next step in the signaling pathway of stomatal closure, K^+ is released from the guard cells. Outwardly rectifying K^+ channel GORK (GATED OUTWARDLY RECTIFYING K^+ CHANNEL) are activated due to anion efflux that causes the depolarization of guard cell plasma membrane (Ache et al., 2000; Hosy et al., 2003). As an outcome of anion outflow, water is released from guard cells via aquaporins (Maurel et al., 2008). The ion and water efflux lead to guard cell shrinkage resulting in decreased cell volume and, thus, in the diminished aperture of the stomatal pore.

1.2 Plasma membrane proton pumps in *Arabidopsis*

The plasma membrane P-type ATPases form a large ubiquitous family of proteins that mainly function as cation and phospholipid pumps (Møller et al., 1996; Palmgren and Harper, 1999). Members of the P-type ATPase superfamily have specific conformations, different transport characteristics and are considered to be essential for living (Palmgren et al., 2011). These pumps are grouped into five major subfamily branches according to their specificity, which is further divided into small subgroups (Axelsen and Palmgren, 1998). In plants, the most highly expressed pumps belong to the P3A-type subfamily and operate as ATP hydrolysis-energized transporters for hydrogen ions (protons) (Gaxiola et al., 2007).

Plasma membrane H^+-ATPases do not exist in the animal kingdom, but they are structurally and functionally similar to the Na^+/K^- pumps required for bioenergetic systems in animals (Palmgren, 2001; Lefebvre et al., 2003). H^+-ATPases are encoded by the P-type ATPase families of 9-12 gene members in higher plants (Arango et al., 2003). Altogether, 11 AHAs have been discovered in *Arabidopsis* (AHA1-AHA11) that can all be detected in various plant tissues (Ueno et al., 2005; Arango et al., 2003).

The high expression levels of proton pumps in every plant tissue refer to their importance in plants – they are indispensable for plant growth and development. Although the main role of H^{+-}ATPases is to create an electrochemical gradient across the plasma membrane, previous experiments have indicated additional impacts of AHAs on different plant developmental aspects. For example, AHA10 is involved in the development of seed cover. Furthermore, *aha10* mutant failed in producing flavonoid proanthocyanidin, which is an essential class of compounds participating in the regulation of plant defense mechanisms (Baxter et al., 2005).

There are two known mechanisms responsible for H^+-ATPase regulation: phosphorylation with the ability to inhibit or activate proton pump, and 14-3-3 proteins binding as positive regulators (Palmgren and Christensen, 1993; Ekberg et al., 2010; Svennelid et al., 1999; Emi et al., 2001). Additionally, it was discovered that blue light photoreceptors (PHOTOTROPIN1) and PHOT2 (PHOTOTROPIN2) are able to activate proton pumps (Takemiya et al., 2013). However, the details and other mechanisms modulating the activity of H^+-ATPases need further elucidation.

1.2.1 AHAs in stomata

There are several cell types in plants where AHAs are more abundant due to their specialization in transporting different solutes. One such example is stomata, where all the AHAs are evidently expressed, although in variable concentrations (Ueno et al., 2005). Transcriptome analysis of 11 AHAs showed that three isoforms are the most expressed in guard cell protoplasts – AHA1, AHA2, and AHA5 (Ueno et al., 2005; Haruta et al., 2010). Also, phylogenetic analysis revealed that AHA1 and AHA2 are closely related and might share the most recent common ancestor (Haruta et al., 2010).

Initially, most of the information about AHA1 role in stomatal regulation was based on research conducted by Merlot et al. (2002; 2007). Later a series of experiments were conducted by Haruta et al. (2010) to understand the importance of AHA1 and AHA2 proteins in plant

development. *aha1* and *aha2* mutants were used to assess the growth alterations in *Arabidopsis*. First, plants lacking AHA1 or AHA2 did not show any growth deformities under standard conditions. AHA1 and AHA2 seem to perform overlapping functions since the loss of one functional gene does not cause a specific phenotype in plants. Second, transgenic lines disrupted in both AHAs were generated. However, the germinating seeds were not viable, suggesting that insertional sequences in double mutants could have affected either gametogenesis or embryogenesis. Later, the analysis showed that mutations did not block gametogenesis, and the early developmental lethality of these mutants was rather caused by disruptions in embryogenesis. (Haruta et al., 2010)

Most of the experiments investigating proton pump functions are conducted on the roots level. While AHA5 is negligibly expressed in roots, it is highly expressed in guard cells. Thus, there is not much information available about AHA5 (Ueno et al., 2005). Moreover, previous experiments with stomata indicated that *aha5* mutants do not show any specific phenotype similarly to *aha1* and *aha2* single mutants (Yamamuchi et al., 2016). Furthermore, Yamauchi et al. (2016) revealed that *AHA1* is essential for the blue light-induced stomatal response while the other two abundant AHAs are not accountable for this process. *aha2* and *aha5* single mutants still show response to blue light. In contrast, *aha1* mutant demonstrated impaired response to blue light when *AHA1* had an amino acid mutation which resulted in decreased activation of H^{+-}ATPase. Subsequently, it was suggested that AHA1 is important for stomatal opening by blue light, and this discovery indicates that still, all the AHAs have differing functions, even though some of them may overlap.

It can be concluded that AHAs can replace each other without a functional loss when one is absent (Haruta et al., 2010). More information about AHA2 is revealed from research conducted with roots. Nevertheless, AHA1 remains the most important on the stomatal level as distinctive *ost2* mutants were found (Merlot et al., 2007). The next chapter will provide more details about mentioned mutants.

1.2.2 AHA1 role in stomatal regulation

As mentioned above, the activation of PM H^+ATPases stimulates stomatal opening (Kinoshita et al., 2001). In the experiment conducted by Merlot et al. in 2002, mutants with cooler leaf phenotypes in dehydrated conditions were selected using infrared imaging. Cooler phenotype refers to plants with lower leaf temperature due to excessive water loss via transpiration. Further experiments showed that two of the identified mutants with cooler

leaf phenotype carried dominant mutations in H^+-ATPase AHA1 and were named as *open stomata 2-1D* and *2-2D* (*ost2-1D* and *ost2-2D*) (Merlot et al., 2002; 2007). *ost2-1D* and *ost2-2D* were isolated from different natural accessions, from *Arabidopsis* plants Landsberg *erecta* and Col-0, *respectively* (Merlot et al., 2002). Mutants vary between each other in the amino acid substitutions causing their phenotypical difference. Using yeast complementation analysis, it was revealed that *ost2-1D* carries alteration P68S and *ost2-2D* allele carries G867S mutation. Present understanding supports the fact of constitutive activity of the AHA1 pump in these *ost2* mutants (Merlot et al., 2007).

According to phenotypic analysis, *ost2* mutants tend to wilt even in normal water growth conditions, are delayed in growth, and have lower fertility. At first, the basal stomatal aperture of *ost2-2D* was claimed to be similar to wild-type, but *ost2-1D* stomata to be 1.5-fold more open. According to Nuhkat (2013), experiments stomata of both *ost2* mutants are characteristically more open. Constantly open stomata in mutants result in higher water loss and, consequently, display lower leaf temperatures compared to their respective wild-type lines (Merlot et al., 2007).

Merlot et al. (2007) experiments were conducted on epidermal peels, and both *ost2* mutants demonstrated the inability to close their stomata in response to ABA treatment. However, a later study on leaves from intact rosettes showed that *ost2* mutants are sensitive to ABA treatment and demonstrated a clear response to ABA by closing stomata (Nuhkat, 2013; Pantin et al., 2013). The results suggest that there could be another ABA signaling pathway in leaf internal tissue as in contrast to ABA mutants, *ost2* mutants are ABA insensitive only at the level of stomata (Pantin et al., 2013). Additionally, *ost2-1D* and *ost2-2D* from Merlot et al. (2007) experiment display wild-type-like response to elevated CO_2 and shift from light to darkness, slight stomatal response to H_2O_2 and no response to Ca^{2+} -treatment. However, later study with intact plants indicated impaired response to air pollutant ozone, high CO_2 concentrations, and to darkness, whereas the responses ABA and low air humidity were unaffected (Nuhkat et al., 2013).

1.3 amiRNA-mediated gene silencing in plants

As the plant genomic data are abruptly increasing due to well-refined whole-genome sequencing technologies, more elaborate approaches are needed to alleviate the lag in functional genomics (Zhang et al., 2019). Over the last few decades, mostly loss-of-function

mutants have been created through mutagenesis, as well as efficient CRISPR/Cas9 gene editing has been used to study the role of specific genes in plants (Bortesi and Fischer, 2015). However, these approaches are limited in performance. For example, generating lines disrupted in vitally important genes will cause plant lethality or tissue differential knockouts are unachievable. (Budziszewski et al., 2001; Dai et al., 2015; Zhang et al., 2018).

Recently a powerful tool for characterizing gene function has been devised that can avoid the restrictions of other frequently used methods. Artificial micro RNA (amiRNA) technology represents an RNA interference strategy designed to silence single or multiple genes under interest. Synthetic amiRNA technology uses natural miRNA precursors as a backbone that post-transcriptionally repress gene expression in plants (Chapman and Carrington, 2007; Martínez de Alba et al., 2013). miRNAs are structured of imperfect self-complementary foldback structures forged by DICER-LIKE1 enzyme (DCL1) from premature transcripts (Axtell et al., 2006; Peragine et al., 2004). Completed miRNAs are fused to RNA-induced silencing complex (RISC) in which miRNAs direct the recognition of complementary mRNAs that are degraded by another member of the RISC complex Argonaute 1 (AGO1) (Llave et al., 2002; Rhoades et al., 2002). In amiRNAs, a fragment of endogenous unprocessed premature miRNA is replaced with a carefully selected sequence compatible with the desired target gene(s) (Ossowski et al., 2008; Park et al., 2009).

The ability to target specifically several even closely related genes and minor estimated off-target effects are significant benefits of amiRNAs (Ossowski et al., 2008; Molnar et al., 2009). It has also been proposed that amiRNAs cause less environmental and biosafety issues in agriculture and have long-term silencing activity that lasts for many generations (Molnar et al., 2009; Duan et al., 2008; Liu and Chen, 2010).

1.3.1 Designing amiRNAs

The mature amiRNA is usually 21-22 nucleotides long and complementary to the target gene (Mallory et al., 2004; Schwab et al., 2006) (Fig. 3). Therefore, the first step in designing amiRNAs is choosing proper sequences complementary to desired genes. To be able to silence the expression fully in plants, near-perfect complementation needs to be induced between the amiRNA sequence and its mRNA targets (Tang et al., 2003). There are several bioinformatics tools created to design amiRNAs, such as the WMD (Web MicroRNA Designer) platform, amiRNA Designer (Schwab et al., 2006; Mickiewicz et al., 2016).

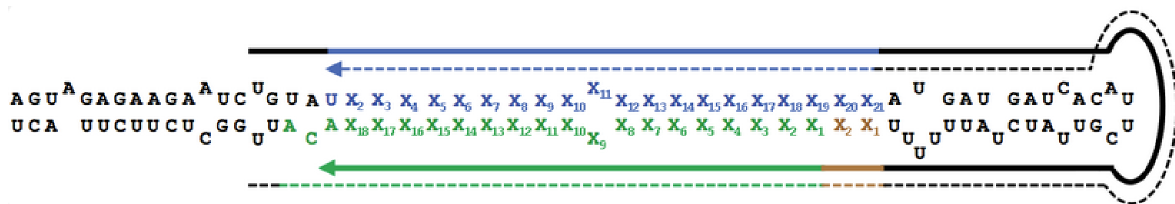

Figure 3. AtMIR390a precursor used in the silencing of genes. The figure demonstrates miRNA foldback sequence (black), amiRNA guide strand (blue), amiRNA strand (green) strand, and precursor foldback secondary structure (red). The dashed line illustrates reverse oligonucleotides, and the solid line represents forward oligonucleotides. The figure is obtained from P-SAMS amiRNA Designer (http://p-sams.carringtonlab.org/).

An improved method for amiRNA generation was introduced by Carbonell et al. (2014). The approach allows to skip several steps in the amiRNA cloning and make gene interference faster and cost-efficient. Sequencing analysis demonstrated the precision of the method, and molecular and phenotype tests showed the implications of gene silencing. The *MIR390a* precursor was selected as a backbone for vectors due to its beneficial features. The advantages of chosen precursor are shorter distal stem loop compared to previously used skeletons, and as the MIR90 family is highly conserved in plants, it increases the chance of accurate amiRNA generation (Axtell et al., 2006; Cuperus et al., 2011). The *AtMIR390a-B/c* cloning vectors consist of insert developed by annealing two overlapping and partially complementary oligonucleotides covering the amiRNA sequence. Type II *BsaI* restriction sites are introduced to the vector that, after digestion, produce non-complementary ends that prevent self-ligation of the plasmid (Schwab et al., 2006; Molnar et al., 2009). amiRNAs can be directionally cloned to vectors and then introduced to bacteria via transformation.

1.3.2 Example of amiRNA application in stomata

The list of amiRNA manipulated species, where both dicotyledonous and monocotyledonous plants are exhibited, has vastly expanded within a decade (Alvarez et al., 2006; Li et al., 2014). This suggests that amiRNA technology can be utilized in a diverse set of plant species all over the plant kingdom. However, *Arabidopsis* has remained a pioneer to question the limits and improve the method. For example, genes related to the regulation of pollen development or circadian rhythm have been studied in *Arabidopsis* (Pereiera et al., 2005; Kim and Somers, 2010).

In addition, an example of successful amiRNA application in stomata of *Arabidopsis* has been demonstrated (Hõrak et al., 2016; Tõldsepp et al., 2018). Previous findings suggested that MPK4 (MITOGEN-ACTIVATED PROTEIN KINASE 4) together with very similar MPK12 (MITOGEN-ACTIVATED PROTEIN KINASE 12) could be essential regulators

of CO_2-induced stomatal movements (Marten et al., 2008; Jakobson et al., 2016). However, *mpk4* mutants display a severe dwarfed phenotype due to MPK4 importance also in defense signaling, and hence the mutants are problematic to study (Petersen et al., 2000). Guard cell-specific silencing of *MPK4* was performed to defeat the growth impairment of *mpk4* mutants and to be able to examine the protein function in guard cell CO_2 signaling (Hõrak et al., 2016). The T1 plants of constructed *mpk12 mpk4GC* double mutants showed clear unresponsiveness to high and low CO_2 concentrations. Later, stable homozygous T4 generation plants of *mpk* double mutants were generated (Tõldsepp et al., 2018).

2 THE AIMS OF THE THESIS

Arabidopsis thaliana plasma membrane H^+-ATPases belong to a gene family of 11 members (Axelsen and Palmgren, 2001). AHA1 and AHA2 are assessed as the most abundant isoforms in *Arabidopsis* tissues. The same applies to stomata (Baxter et al., 2003). In addition to these two major proteins, another H^+-ATPase, AHA5, is highly expressed in stomata compared to other gene family members (Ueno et al., 2005). Due to potential functional overlapping of the AHA family members, there is no stomatal phenotype emerging in single *aha* loss-of-function mutants. Also, *aha1aha2* double mutants were proved to be embryo lethal, rendering them impossible to test (Merlot et al., 2007; Haruta et al., 2010). Because of these reasons, the functional characterization of AHAs in stomata has remained challenging.

The aim of this thesis is to overcome the lethality of AHA double mutants to make studying stomatal effects of the main disrupted AHAs possible. Three mentioned most abundant AHAs are used in the study to generate transgenic lines by combining T-DNA-based gene disruption and guard cell-specific microRNA-based gene silencing methods. The specific aims of the thesis are following:

- To design suitable constructs for AHA silencing in guard cells
- To generate transgenic T-DNA/amiRNA::*aha* lines with impaired AHA function in guard cells;
- Describe the phenotypes of transgenic T-DNA/amiRNA::*aha* T1 plants
- Select the best suitable T-DNA/amiRNA::*aha* lines for further studies

3 EXPERIMENTAL PART

3.1 Materials and Methods:

3.1.1. Plant lines

To generate transgenic lines, *Arabidopsis thaliana* accession Col-0 was used. Also, AHA1 and AHA2 knockdown mutants, *aha1-9* and *aha2-4* were used in the studies as additional backgrounds for creating intended transgenic lines (Haruta et al., 2010) Wild-type Col-0 seeds were included as a negative control in the selection process of transgenic lines. Col-0 seeds were gained from the Plant Signal Research Group collection, *aha2-4* seeds were provided by Dr. Rainer Waadt and *aha1-9* from Prof. Toshinori Kinoshita.

All generated transgenic lines in this study were designated in a similar way to Tõldsepp et al., (2018) The first part of the name represents the T-DNA knockdown mutant used as a background or wild-type accession. The other segment symbolizes the target gene(s) which expression is aimed to be specifically suppressed in the guard cells (referred as *GC* in the end). The two-name components have been separated with a slash. All used plants are indicated in Table 1.

Table. 1 Arabidopsis lines used in this study.

Line	**Description**	**Background**	**Gene ID**	**Alteration**	**Reference**
Col-0	Wild-type				
aha2-4	Knock-down AHA2 mutant	Col-0	AT4G30190	T-DNA insertion	Haruta et al., 2010; SALK022010

aha1-9	Knockout AHA1 mutant	Col-0	AT2G18960	T-DNA insertion	Yamauchi et al., 2016; SAIL_1285_D12
Col-0/*aha1aha2GC*	Guard Cell specifically silenced AHA1 and AHA2 mutant	Col-0	AT2G18960; AT4G30190	T-DNA insertions	Current research
aha1/aha2GC	Knock-down AHA1 mutant/Silenced AHA2 mutant	*aha1-9*	AT2G18960; AT4G30190;	T-DNA insertions	Current research
aha1/aha2aha5GC	Knock-down AHA1 mutant/Guard Cell specifically silenced AHA2 and AHA5 mutant	*aha1-9*	AT2G18960; AT4G30190; AT2G24520;	T-DNA insertions	Current research

aha2/aha1	Knock-down AHA2 mutant/Guard Cell specifically silenced AHA1	aha2-5	AT2G18960; AT4G30190	T-DNA insertions	Current research
aha2/aha1aha5GC	Knock-down AHA2 mutant/Guard Cells specifically silenced AHA1 and AHA5	*aha2-5*	AT2G18960; AT4G30190; AT2G24520;	T-DNA insertions	Current research

3.1.2. Plant growth conditions

To promote and even the seed germination, the *A. thaliana* seeds were prior to sowing placed in distilled water at 4°C for 2-3 days. Subsequently, seeds were sown into regular medium pots with standard dimensions of 8.5x8.5x6.5 cm, each pot containing approximately 170 g of soil mixture. The soil mixture was prepared beforehand as follows: consisting of 2 volumes of peat (Kekkilä), 4 volumes of vermiculite, and 3 volumes of water. Vermiculite was applied to the soil to prevent excessive soil drying and thus, avoid soil compacting. The seeds were sown into the soil using a pipette. The trays with plants in pots were covered with translucent plastic casings to maintain high air humidity underneath it until shoots were starting to develop. A week after sprouting, the covers were removed, and the seedlings were thinned out to leave five plants to grow per each pot. The pots were bottom-watered every three to four days.

Plants were first cultivated in short-day growth room for 2 weeks. The growing conditions were set in the room to 22-24°C in the day and 18-20°C during the night with, light intensity of 90-150 µmol m^{-2} s^{-1} and the day-night cycle 12-h light/12-h dark. Entomopathogenic nematode (Koppert Biological Systems) treatment was done in every 2 weeks on a regular basis to all plants. Pots were showered from the top with water where nematode stock was dissolved (0.3 g per 1 l of).

To induce rapid flowering, plants were transferred to a long-day growth room. The growing conditions were similar to short-day room, except day-night cycle was set to 16h light/8h dark. As large number of flowers were needed for further treatment, the first 3-5 cm long bolts were cut off, which would promote development of greater number of bolts. To minimize the number of untransformed seeds, all formed siliques were removed. Plants were used for transformation when they started to bloom again after ten days. Two months after transformation, seeds were dried and collected.

Transformed T1 seeds were first plated on square Petri dishes (see chapter 3.1.6) and then 10 days old seedlings were individually transplanted to special expanding peat pellets (Jiffy Products International AS), Pellets were irrigated after every 3 days. 3 weeks on peat pellets grown plants were used in porometer measurements.

3.1.3. Vector generation

The method for amiRNA-mediated silencing described in Carbonell et al. (2014) was used to design amiRNA constructs for AHA1, AHA2 and AHA5 silencing. Guard cell specific MPK12 promoter was used in the study similarly to Hõrak et al., 2016. pMKP12-*AtMIR390a-B/c* vector was provided by PhD student K. Koolmeister and used for plasmid construction in plant transformation.

Firstly, NOS terminator was replaced in the initial background vector with HSP18.2 (HEAT SHOCK PROTEIN 18.2) terminator. HSP18.2 terminator was cleaved from pGGE-tHSP18.2 vector using SacI and EcoRI restriction enzymes (Thermo Scientific). The cleavage mixture was then subjected to gel electrophoresis to separate the cleaved DNA bands on a 1% agarose (Fisher Bioreagents) gel with ethidium bromide (0.5 µg / ml) at 100 V. Expected product sizes of the cut vector were ~11.5 kb and 257 bp, the latter indicating the required terminator sequence. 257 bp long band was cut out from the gel with a scalpel, and DNA was purified using Favorprep Gel/PCR purification mini kit (Favorgen). pMPK12-*AtMIR390a-B/c* vector was also cut with SacI and EcoRI restriction enzymes. The HSP18.2

terminator was then ligated (1 hour at room temperature) into the vector using T4 DNA ligase (Thermo Scientific). The instructions provided by manufacturers for DNA ligase and restriction endonucleases were followed (New England Biolabs and Thermo Fisher Scientific). Subsequently, the vector was transformed to *E.coli* DB3.1 strain.

P-SAMS amiRNA Designer web app (http://p-sams.carringtonlab.org/) was used to design oligos for amiRNA cloning indicated in Table 2. The app is specially created to utilize *MIR390a* foldback in *BsaI-ccdB* vectors and presents the most promising sequences of amiRNAs together with the sequences required for the cloning oligonucleotides. Multiple results were provided, and the optimal forward and reverse oligos were selected based on the specificity score. 50 µl of annealing mixture for each amiRNA oligonucleotides was prepared. Annealing mixtures consisted of 10 µl of each oligonucleotide, 5µl of buffer O (Thermo Scientific), and 25 µl of mQ water. amiRNA oligonucleotides were annealed by using heat block (Biosan, TDB-120 dry block thermostat) where tubes with oligonucleotides were incubated at 95 °C for 5 min. The tubes were left to cool on a switched-off bench for at least 2 hours to reach room temperature. For oligonucleotide cloning, pMPK12-*AtMIR390a*-tHSP18.2 vector was cut with *BsaI* endonuclease. 1.5 µl of annealing mixture was used to ligate oligonucleotides to vector. Purified plasmids were then introduced to *E. coli* DH10β strain.

Table 2. **List of primers used for cloning and sequencing in this study.**

Name	**Sequences[1] (5′→3′)**	**Target region**	
pMDC32 S seq-for2	TTCACTATCTCTCTATAATCGG	amiRNA region, HSP18.2 terminator	
pMDC32 S seq-rev1	CGGAGCCTGCTTTTTTGTAC	MPK12 promoter; amiRNA region	
tHSP18.2 _seq_R	CACAAATTCATAACACAACAAG	HSP18.2 terminator	
SIL_AHA2_Fw	TGTATCAACACGACAGCTTTGTCCCATGATGATCACATTCGTTATCTATTTTTGGGACAAAGCGGTCGTGTTGA	amiRNA oligo for AHA2	
SIL_AHA2_Rw	AATGTCAACACGACCGCTTTGTCCCAAAAATAGATAACGAATGTGATCATCATGGGACAAAGCTGTCGTGTTGA		

SIL_AHA1_Fw	TGTATCCTAAGGTCAGGCCTGGCAAATGATGATCACATTCGTTATCTATTTTTTTGCCAGGCCGGACCTTAGGA	amiRNA for AHA1	
SIL_AHA1_Rw	AATGTCCTAAGGTCCGGCCTGGCAAAAAAATAGATAACGAATGTGATCATCATTTGCCAGGCCTGACCTTAGGA		
SIL_AHA1AHA5_Fw	TGTATTAACTTGTAGATACACACCTATGATGATCACATTCGTTATCTATTTTTAGGTGTGTATATACAAGTTAA	amiRNA for AHA1 and AHA5	
SIL_AHA1AHA5_Rw	AATGTTAACTTGTATATACACACCTAAAAAATAGATAACGAATGTGATCATCATAGGTGTGTATCTACAAGTTAA		
SIL_AHA2AHA5_Fw	TGTATCTGTATAGCCAGATCACCCGATGATGATCACATTCGTTATCTATTTTTCGGGTGATCTTGCTATACAGA	amiRNA for AHA2 and AHA5	
SIL_AHA2AHA5_Rw	AATGTCTGTATAGCAAGATCACCCGAAAAAATAGATAACGAATGTGATCATCATCGGGTGATCTGGCTATACAGA		
SIL_AHA1AHA2_Fw	TGTATTAACTTGTAGATACACACCTATGATGATCACATTCGTTATCTATTTTTAGGTGTGTATATACAAGTTAA	amiRNA for AHA1 and AHA2	
SIL_AHA1AHA2_Rw	AATGTTAACTTGTATATACACACCTAAAAAATAGATAACGAATGTGATCATCATAGGTGTGTATCTACAAGTTAA		

3.1.4. *Escherichia coli* transformation

For terminator and amiRNA cloning, two different bacteria strains were needed, *E. coli* strain DB3.1 and DH10β, respectively. The competent cells were prepared by the lab technician M. Välbe. Bacterial transformation was performed for terminator using heat shock method. First, competent cells were taken from -80°C refrigerator and left to thaw on ice. 100 µl of liquid competent cells were added to ligation mixture and kept on ice for 10 minutes. Then, tubes were transferred to water bath at 42°C. After 90 s of heating, tubes were returned to ice for couple of minutes. 800 µl of LB medium was added to the cell mixtures and incubated at 37°C on a shaker (New Brunswick Scientific) for half an hour. After tubes were briefly centrifuged, collected cells were resuspended in a small volume of LB and plated on Petri dishes. Plates with agarised medium (Duchefa Biochemie, 1.5% agarose) contained 50 µg/ml of kanamycin and 25 µg/ml of chloramphenicol. The existence of a replaced terminator in growing colonies was checked via PCR using primers indicated in Table 2.

Another protocol on recommendation of Dr. H. Hõrak was used for cloning to DH10β strain, where 1.5 µl of annealing mixture, 78.5 µl of mQ water, 20 µl of 5x KCM (0.5 M KCl, 0.15 M $CaCl_2$ and 0.25 M $MgCl_2$) and 120 µl of chemically competent cells were mixed. The rest of the cloning protocol remained the same as previously described. Subsequently, colonies were selected from plates and inoculated into 5 ml of liquid LB medium (containing only 50 µg/ml of kanamycin) and incubated at 37°C overnight. Plasmid was isolated with suitable kit and following manufacture protocol Favorprep Gel/PCR purification mini kit (Favorgen)

3.1.4 Agrobacterium tumefaciens transformation

After sequencing,the *pMPK12-AtMIR390a:aha-tHSP18.2* construct was transformed to *A. tumefaciens* GV3101 strain by using chemical/heat shock transformation. 5 µl of a plasmid was added to Eppendorf tube containing 20 µl of Ca^{2+} treated competent *Agrobacterium.* Tubes were kept on ice for 10 min, next placed into water bath at 37°C for 3 minutes and then quickly transferred back to ice for couple of minutes. Content of the tubes was mixed with 600 µl of LB medium and incubated at 28°C on a shaker for 2 hours. After incubation, cells were centrifuged at 4000 rpm for 3-4 minutes and transferred to agarized LB plates containing selective kanamycin (50 µg/ml). Colony PCR was performed to check success of transformation. 5 ml of LB and 5 µl of kanamycin was mixed and 2 colonies from each plate were added to the mixture. The constructs were sequenced using primers listed in Table 2.

3.1.5 Floral dip method

Arabidopsis aha1-9, *aha2-4*, Col-0 plants were infected with *Agrobacterium* cells carrying different AHA silencing constructs. For producing transgenic plants, the fine-tuned floral dip protocol described by Zhang et al. (2006) was used. As a first step, test tubes with *Agrobacterium* starter suspensions were prepared. For each starter culture, single colony from a plate was selected and inoculated into 5 ml liquid LB medium containing 50 µg/ml selective kanamycin. Bacterial suspensions were incubated at 28°C for 2 days on a shaker. Subsequently, 1 ml of preculture was diluted to 100 ml LB broth with kanamycin added. Flasks with suspensions were then incubated overnight following similar parameters as previously applied to starters. The *Agrobacterium* cells were pelleted in 50 ml Falcon tubes by centrifugation at 4000 rpm (Eppendorf Centrifuge 5810 R) for 10 minutes. Supernatant was discarded and *Agrobacterium* pellet was resuspended in 50 ml of 5% sucrose solution with 0.05% surfactant (Silvet L-77, Momentive). Adding surfactant increases the transformation frequency by enhancing the entry of *Agrobacterium* into the flower interior

(Clough and Bent, 1998). Next, flowering bolts were dipped into the mixture with gentle agitation for 30 s to ensure the soaking of every flower. Pots with dipped *Arabidopsis* plants were placed on the sides into the opaque trays covered with another identical tray to avoid direct sunlight. Wet wipes were placed at the bottom of the trays to maintain humidity. On the next day, covers were removed, plants were lifted upward and moved back to long-day growth-room for further seeds development.

3.1.6 Selection of transgenic plants

T1 seeds were collected from transformed plants and were plated to square 12x12 cm Petri dishes to select viable resistant seedlings on selective medium. The seeds were surface-sterilized before sowing to prevent the spreading of microbial contaminants which can disturb the seed germination and seedling growth on the plates. A mixture of 70% ethanol and 0.1% Triton X-100 (Amreco) was used as sterilization solution. Seeds were treated with sterilization solution by inverting the tubes for 15 min on a shake (brand) for 15 min at 1500 rpm^{-s}. The sterilized seeds were washed 3 times in mQ water and then left in the tubes containing water for stratification at 4°C for 2-3 days in darkness. Subsequently, seeds were transferred onto phyto agar (Duchefa) containing MS medium (Murashige and Skoog basal medium, Sigma-Aldrich). Hygromycin and cefotaxime in concentrations of 25 µg/ml and 100 ng/ml were added to the medium.
Approximately 1 ml of seeds were plated under the laminar to Petri dishes per each line. The dishes were sealed with Nanopore tape (3M) and transferred to an incubator (Sanyo versatile environmental test chamber MLR-351), where conditions were set to 21°C and 16 hours light/8 hours dark cycle. After 10 days, the seedling with developed roots were transplanted to expanding peat pellets.

3.1.7 Microscopy

Transmitted light microscope Compound Variable Laboratory Microscope OBF-1 (KERN) with 40x objective (Plan 40x/0.65, KERN) was used for imaging of plant leaves. Leaves from 4 weeks old plants were collected and incubated abaxial side upwards under light (~150 µmol m^{-2} s^{-1}) in imaging buffer (5 mM KCl, 50 µM $CaCl_2$, 10 mM MES-Tris, pH 5.6).

3.1.8 Porometer experiments

The stomatal conductance was measured using portable Li-600 porometer (LI-COR Biosciences). The leaf of three weeks old plant was placed to cuvetteabaxial side directed to detector. The environment conditions in room during experiment was following, 22-24°C

with light intensity of 90-150 µmol m^{-2} s^{-1}. All measurements were conducted within half an hour to avoid excessive accumulation of CO_2 in the growth room.

3.2 RESULTS AND DISCUSSION

3.2.1 Limitations and unanswered questions related to AHAs

H^+-ATPases have a pivotal role in many physiological processes that affect plant growth and development, including stomatal movements (overviewed in Palmgren, 2001). Stomatal opening and closure both depend on the activity regulations of H^+-ATPases. AHAs can be activated, for example, in response to blue light and inhibited by high CO_2 concentrations, stress hormone ABA, high ozone level, and darkness (Shimazak et al., 2007).

The essential importance of AHAs for plants was confirmed by studies with the loss-of-function mutants of the most expressed AHA isoforms (Haruta et al., 2010). These experiments demonstrated that *aha1aha2* double mutants resulted in embryonic lethality and are impossible to test. Also, the redundancy of gene function has been proved between AHA family members as under laboratory growth conditions no specific phenotype is displayed in single *aha1* or *aha2* mutants. Thus, it has been suggested that the loss of a single gene can be compensated by the work of other AHAs, making gene function analysis difficult.

In addition, stomata-related *ost2* mutants with hyperactive AHA1 were discovered in 2007. Mutants had a cooler leaf phenotype in dehydrated conditions and characteristically more open stomata compared to wild-type. Nuhkat (2013) demonstrated that intact *ost2* plants showed impaired response to elevated CO_2, ozone treatment, and transition to darkness. However, the mutants clearly closed their stomata in response to ABA and low humidity treatment. Another research conducted on *aha1*, *aha2,* and *aha5* mutants revealed AHA1 importance in blue light-induced stomatal opening and established that other AHAs are less important in this process (Yamauchi et al., 2016). According to these experiments, there is still a lack of information regarding proton pump signaling pathways and the distinctive functions of different AHAs. The question arises whether and how the responses to stimuli of hyperactive AHA mutants would differ from mutants disrupted in several AHA genes expressed in stomata.

Moreover, what is known, H^+-ATPases are post-transcriptionally activated or inhibited by phosphorylation of different amino acid residues and/or subsequent binding of 14-3-3 proteins (Kinoshita and Shimazaki, 1999; overviewed in Falhof et al., 2016). However, the

kinases inducing phosphorylations and how the activity of these kinases is regulated have not been yet described (Falhof et al., 2016).

3.2.2 The importance of AHA amiRNA mutants

Although the role of H^+-ATPases in the stomatal opening was hypothesized long ago (Zeiger, 1983), the regulatory mechanism of proton pumps in stomatal movements remains challenging to identify. Therefore, a novel approach is needed to study H^+-ATPases in stomata. In this study, a method with amiRNA-mediated guard cell-specific gene silencing of AHAs was exploited. The advantage of amiRNA method is the ability to target several related genes (Chapman and Carrington, 2007; Martínez de Alba et al., 2013). Therefore, technology could be useful to downregulate the expression of multiple AHAs as they belong to the same gene family and have requisite similarity to design oligonucleotides specific to several genes (Palmgren, 2001; Ueno et al., 2005). Also, the method allows targeting genes in a cell-specific manner that has been used to analyze gene functions associated with mutant lethality (Zhang et al., 2017; 2018). As the *aha1aha2* double mutant cannot survive, using amiRNAs can be an effective tool to employ cell-specific promoters and to avoid the lethality of the mutant. To encapsulate, the downregulation of several AHAs allows us to test their function, overcome the lethality of double mutants and identify the possible importance of AHAs in the regulation of stomatal movements.

3.2.3 Expression of AHAs in guard cells

There are altogether 11 H^+-ATPases represented in *Arabidopsis* (Palmgren, 2001). Based on Ueno et al. (2005), three of the AHA isoforms have significantly higher expression levels in guard cells. Here, *Arabidopsis eFP* bioinformatics browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) was used to confirm and specify this knowledge for the current thesis (Fig.4). As expected, AHA1, AHA2, and AHA5 were enriched in guard cells, and the other AHAs showed almost non or minor expression levels. AHA1 expression is valued to be more than 3 times higher compared to AHA2 that has the second-highest expression of AHAs in guard cells. It indicates that AHA1 could play a central role in stomata, and the discovery of ost2 mutants supports this statement. AHA1 and AHA2 have high sequence similarity and have shown to perform overlapping functions, but their specific roles in the regulation of stomatal function have not been described (Haruta et al., 2010). Also, the role of AHA5 in stomatal regulations has not been studied. Therefore, all 3 highly expressed AHAs in stomata were selected for amiRNA transgenic line generation

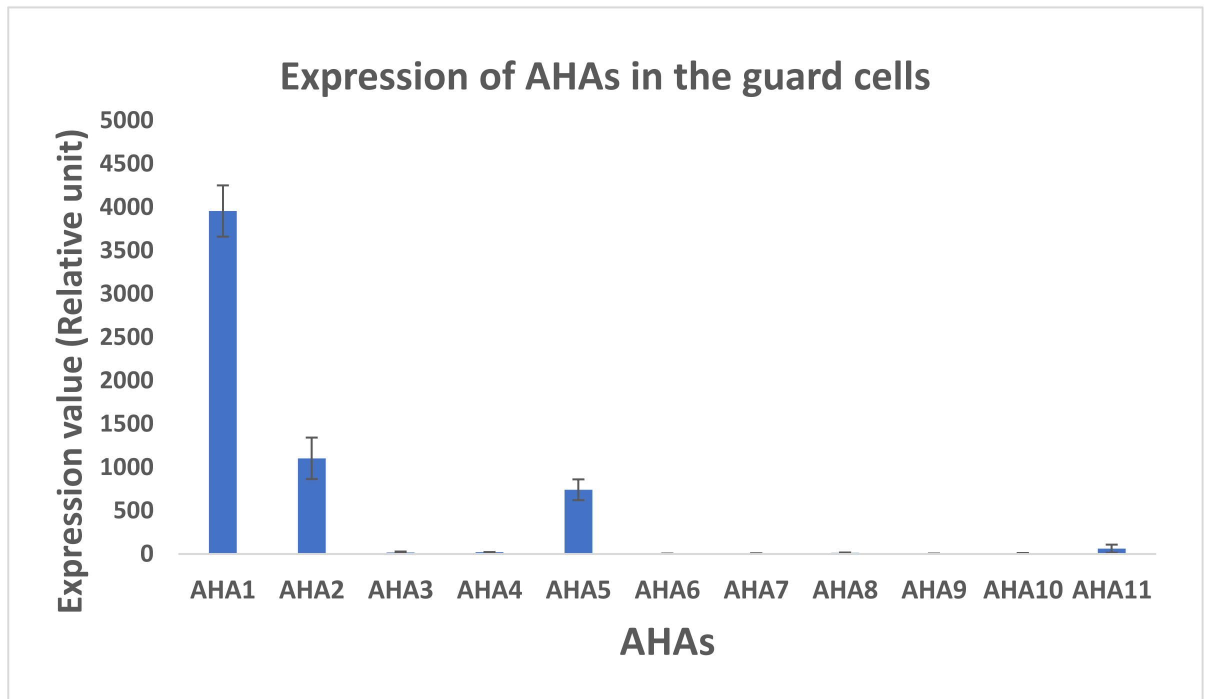

Figure 4. Comparison of expression levels of different AHAs in guard cells. The data is obtained from *Arabidopsis eFP browser* (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The epidermal peels of 5-week-old wild-type Col-0 plants were used to measure expressions of AHAs.

3.2.4 Generation of cloning vectors

amiRNA silencing method introduced in Carbonell et al. (2014) was used to generate transgenic lines, and Hõrak et al. (2016) approach was set as an example to silence target genes in stomata. *pMPK12-AtMIR390a-B/c* vector was selected as a precursor for cloning and was modified according to the targets (Figure 5). Antibiotic resistance genes were used as selection markers, kanamycin and chloramphenicol at the cloning stage, and hygromycin in selecting plants with correct amiRNA insertions. *pMPK12* was used as a promoter, and the initial *NOS* terminator in the precursor vector was replaced with *HSP18.2* terminator. *Bsa*I restriction enzyme was used to cut the vector, and annealed amiRNA oligonucleotides were inserted.

The oligonucleotides for amiRNAs were designed to silence *AHA1* and *AHA2* individually, *AHA1* and *AHA2* together, and *AHA1* or *AHA2* together with *AHA5*, attempting to include double-silenced AHAs into the study. It was not possible to silence three *AHAs* at the same time since there are no specific oligonucleotides for all three genes, according to P-SAMS amiRNA Designer. The grown colonies on the kanamycin plates were screened by using PCR to select correct genetic constructs. All of the colonies contained the required plasmid,

confirming the success of transformation (Fig. 6). The constructed vectors were also sequenced to avoid unwanted mutations.

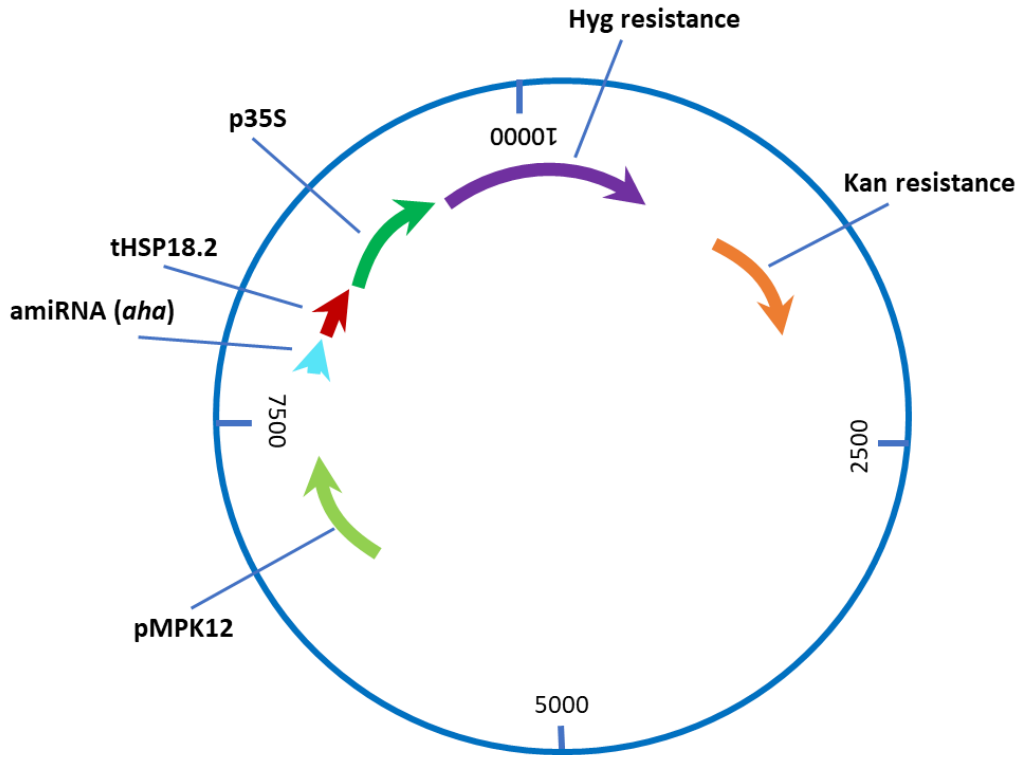

Figure 5. ***pMPK12-AtMIR390a:aha-tHSP18.2*** **vector generated in current study.** amiRNA silencing method described in Carbonell et al. (2014) was used to design amiRNA constructs for *AHA1*, *AHA2*, and *AHA5* silencing that were inserted into designated amiRNA::*aha*. amiRNA sequences were placed under the control of *pMPK12* promoter and *HSP18.2* terminator was used. Final vector contained kanamycin (Kan) and hygromycin (Hyg) recistance genes. Constructed vector is ~11.5 kb in size.

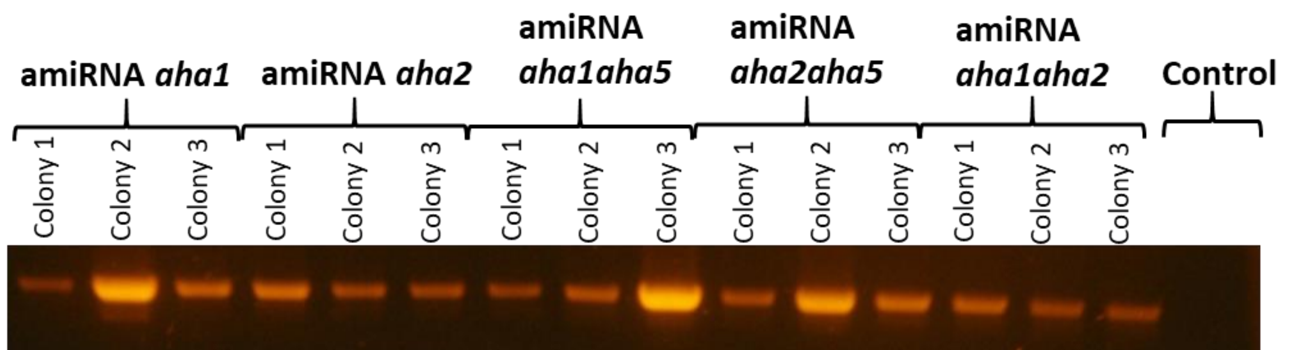

Figure 6. ***E. coli*** **colony PCR. Expected** amiRNA insertion along with *HSP18.s* terminator was found in all checked colonies. All amplified PCR fragments were correct sizes in accordance to the DNA ladder (not shown).

3.2.5 Selection of promoter and terminator

The advantage of amiRNA technology is high specificity which allows to silence of genes only in specific cells or tissues. Here, we aimed to downregulate the expression of AHAs by placing amiRNA::*aha*s under a guard cell-preferential promoter. AHAs are involved in a wide range of physiological processes in *Arabidopsis,* and they are abundantly expressed in many tissues (Palmgren, 2001). Thus, a native promoter of AHA is not reasonable to use as it could cause irreversible plant lethality. Hõrak et al. (2016) used HT1 and MPK12 promoters to silence genes in guard cells, but they concluded that the guard cell specificity of these promoters is not complete. Therefore, several other candidates for promoters were considered as choosing a suitable promoter for the generation of AHA trangenic plants is essentially important. Promoters *GC1*, *SLAC1* and *MYB60* have been selected in studies to produce proteins at the guard cell level (Vahisalu et al., 2008; Yang et al., 2008; Cominelli et al., 2011). p*SLAC1* has been shown to be more specific than *pGC1* (Manjur, 2016). However, based on information obtained from Genevestigator expression database (https://genevestigator.com/), *pMPK12* was proved to be the most specific to stomata. Thereby, MPK12 natural promoter was used in the study over others as it is predominantly guard cell-specific.

As AHAs are highly expressed in stomata, the silencing efficiency was important to increase to enhance the expression level of amiRNAs. Since the MPK12 promoter is not very strong, the terminator was used to elevate the expression of the amiRNAs. Subsequently, *NOS* terminator was replaced with *HSP18.2* terminator. *tHSP18.2* has shown to increase the gene expression 2 times compared to *NOS* terminator (Nagaya et al., 2016).

3.2.6 Background lines of transgenic lines

Different backgrounds were used in the study to generate and identify the best AHA transgenic lines for further research. Developing useful amiRNA lines can be complicated since finding the potent sequence to maximal gene silencing is technically challenging (Zhang et al., 2017; 2018). For this reason, three *Arabidopsis* lines were used as a background for plant transformation – wild-type Col-0, *aha1-9*, *aha2-4*. The higher the number of genes targeted for silencing, the more likely the amiRNA sequence is not specific enough to completely abolish the expression of target genes. Here, AHA1 and AHA2 knockdown mutants were exploited to enhance the probability for accurate gene silencing and to generate double or triple AHA mutants. Both *aha1-9* and *aha2-4* have T-DNA insertion in AHA1 and AHA2, (Haruta et al.,2010; Yamauchi et al., 2016). *aha2-4* mutant was obtained from the SALK

and *aha1-9* from the SAIL collection. The insertion in *aha2-4* occurs in the tenth intron, while *aha1-9* has a T-DNA insertion in the third exon. *aha1-9* is a confirmed null allele mutant. However, all described *aha2* mutants T-DNA insertions are located in introns, and these plants still display low expression of the protein (Haruta et al., 2010; Yamauchi et al., 2016). It should be noted that also *aha2-4* retains some expression of AHA2 but being reduced by 90-95% compared to wild-type plants (Haruta et al., 2010). Also, both mutants do not express elevated levels of other *AHA* in the absence of *AHA1* or *AHA2* on transcriptional and translational levels (Haruta et al., 2010). Later, generated *aha2* knockout mutant *GABI_209D04* could be described to use as a better background for amiRNA transgenic plants (Rosso et al., 2003). It has a T-DNA insertion in the seventh exon and could potentially show lower protein expression than *aha2-4* mutants (Zheng, 2018).

3.2.7 Selection of transgenic lines

To assess whether plant transformation was successful, harvested T1 seeds were transferred to the MS plates supplemented with hygromycin. Suitable transgenic lines were expected to grow on hygromycin as plasmids introduced to plants contained the selective resistance gene against the chemical. Before plating, seeds were sterilized to prevent microbial contamination. Nearly all plated seeds were able to germinate. Wild-type *Arabidopsis* Col-0 and *aha1-9* seeds were chosen as control. The control seeds and untransformed seeds failed to develop roots longer than a few millimeters (Figure 7). However, on every plate where transgenic lines were sowed, there were plenty of seeds that were able to develop longer roots and grow larger leaves, confirming the transformation successfulness. After 1.5 weeks, viable transgenic seedlings were planted into expanding peat pellets. Visually, plant lines transformed with amiRNAs targeting two AHAs looked smaller than single amiRNA-targeted mutants.

Figure 7. Seed selection. The seeds were planted to MS plates containing hygromycin. Col-0 wild-type seeds were chosen as control. After 10 days, transformed seeds containing resistance to required antibiotics developed roots and could grow bigger (some encircled), while control plants failed to grow further.

3.2.8 Expected stomatal conductance of AHA transgenic plants

The stomatal density, the degree of stomatal complex openness, and size can be assessed by measuring stomatal conductance. Stomatal conductance values are determined by water loss through stomata and are usually measured in mmol m^{-2} s^{-1} (Zeigler et al., 1987). *ost2* mutants with constitutively active AHA1 have demonstrated ~2.5-fold higher basal stomatal conductance values than their corresponding wild-type plants (Nuhkat, 2013). This knowledge correlates with the fact that the stomata of *ost2* mutants are steadily more open and plants can exchange gases more extensively with the atmosphere (Merlot et al., 2007; Nuhkat et al., 2013). The opposite phenomenon could be expected when the work of proton pumps is impaired.

The stomatal conductance and aperture of a*ha1* and *aha2* single mutants under normal growth conditions have not been shown to be significantly different the wild-type plants (Haruta et al., 2010; Yamauchi et al., 2016; knowledge within the study group). This might emerge due to overlapping functions of AHAs in stomata (Haruta et al., 2010). When all AHAs responsible for the stomatal movements are disrupted, the stomatal apertures should be decreased. Hypothetically, in this situation, the electrochemical potential could not reach the required level to activate the anion channels necessary for stomatal opening. Therefore, the expected stomatal conductance values should be lower than those of wild-type plants.

To test this idea, three weeks on peat pellets grown transgenic, background, and wild-type plants were selected for stomatal conductance measurements since *AtMIR390a*-based approach was demonstrated to efficiently silence genes and show the corresponding phenotype also in T1 generation plants (Carbonell et al., 2014; Hõrak et al., 2016). Due to limited time resources, collected data will be analyzed later.

3.2.9 Visual phenotypes of transgenic lines

After 3 weeks of growth on peat pellets, transgenic lines were visually evaluated for presence of specific phenotype (Fig. 8). All background plants of transgenic lines, Col-0 and two mu-tants with T-DNA insertion aha1-9 and aha2-4, grew normally and no specific phenotype was observed. Similarly, transgenic plants with single AHA amiRNA-targeted constructs displayed normal phenotype. However, aha1/aha2aha5GC and aha2/aha1aha5GC plants showed specific dwarfed growth. This observation suggests the importance of AHA5 in stomatal movements

Figure 8. **Visual assessment of plant phenotype.** Representative plants of each generated transgenic and background plants were selected.

The reason for such stunted phenotype could rely on completely closed stomata in these lines. For clarity, stomata of transgenic lines were observed under the microscope (Fig. 9). Indeed, the stomata of *aha1/aha2aha5GC* and *aha2/aha1aha5GC* mutants appeared to be

tightly shut and very small compared to other lines. No visual differences of stomatal aperture and size between wild-type Col-0, single AHA-targeted amiRNA plants and plants transformed with construct containing *amiRNA::aha1aha2* could be detected. *aha1-9* and *aha2-4* stomata were also visually similar to those of Col-0.

In *aha1/aha2aha5GC* and *aha2/aha1aha5GC* mutants, all AHAs possible responsible for stomatal opening can be dysfunctional. The near-completely closed stomata could lead to inability to uptake CO_2. Consequently, plants are not able to photosynthesize properly and grow biomass. These preliminary results imply that *AHA5* could be important in stomatal movements. However, further investigation is needed to elucidate these questions. It would be interesting to additionally have *AHA5*-silenced plants in the background of *aha1-9* and *aha2-4*.

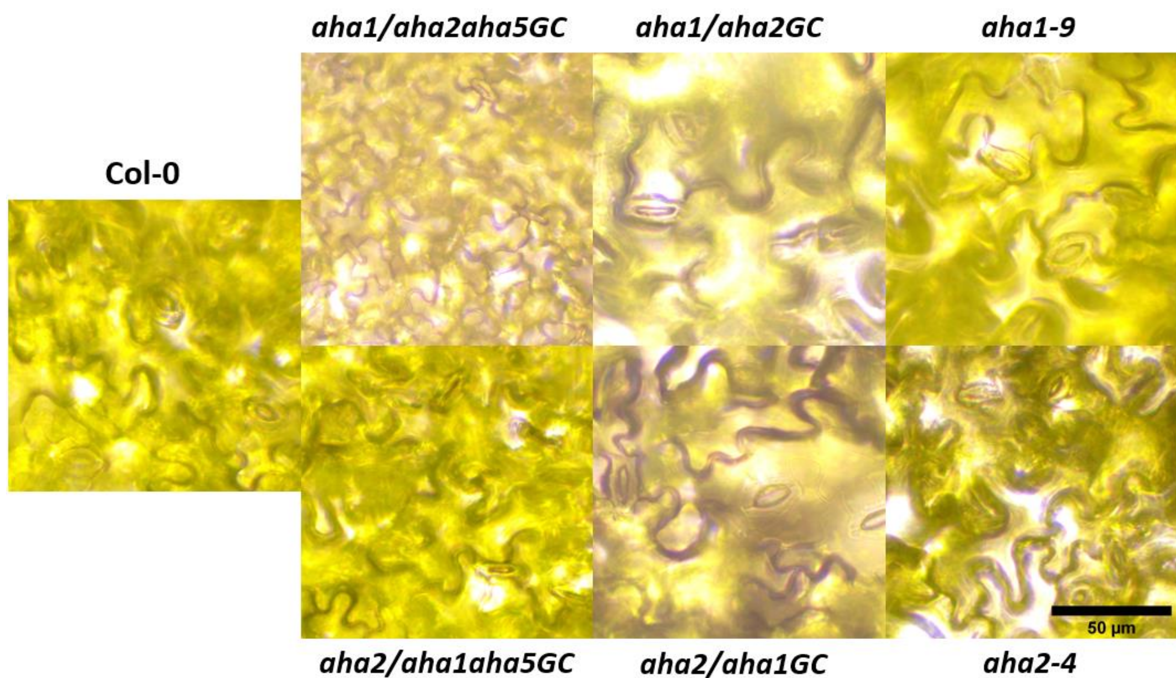

Figure 9. Plants leaves under the light microscope. Images of transgenic lines epidermises from 4 weeks old plants. Before imaging, plants were incubated abaxial side upwards under the lights in imaging buffer

3.2.10 Perspectives

To avoid *AHA2* remained expression in *aha2-4* line, a different line will be used for future research, *GABI_209D04*. The seeds were already ordered from Nottingham Arabidopsis Stock Centre collection (http://arabidopsis.info/) and for following studies homozygous seeds will be selected. The main advantage of *GABI* line is T-DNA insertion in exon which should result in lower expression of *AHA2* compared to *aha2-4*.

The generated transgenic line will be verified for successful transformation using PCR. T1 plants containing correct AHA silencing sequences will be left to self-pollinate, and seeds will be collected from those plants. T2 generation seeds will be planted again to obtain homozygous plants and check the number of insertions in the line. Research only with 1 T-DNA insertional plants will be continued. T3 generation with 100% resistance to selective antibiotic will be chosen and then planted again to obtain final stable T4 seeds. The transcripts level of AHAs in stomata in T4 generation of transgenic lines will be measured to confirm the silencing effect of generated constructs. Stomata of transformed plants should be characterized in accordance to their shape, size and basal aperture.

In case of success, gas exchange experiment with special custom-made will be performed to compare the response of intact *ost2* and *amiRNA::aha* plants to different environmental stimuli (Kollist et al., 2007). Subsequently, transgenic lines can be also crossed with lines carrying genetically encoded biosensors to get a better understanding of the AHA signalling pathways. (Waadt et al., 2020).

3.2.11 Conclusions

Three most abundant AHA isoforms in guard cells were studied in this thesis. The high expression of AHA1, AHA2 and AHA5 in guard cells was confirmed using *Arabidopsis* eFP bioinformatics browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). *amiRNA::aha* vectors were successfully constructed for cloning. *pMPK12-AtMIR390a-B/c* vector was modified for and guard cell-specific promoter *MPK12* was inserted into the vector to increase gene silencing specificity. Additionally, *NOS* terminator was replaced with *HSP18.2* termintaor to increase transgene expression.

Throughout the study, five different transgenic lines were generated with impaired AHA function in guard cells. The phenotypical analysis of transgenic lines revealed growth restriction in mutants with silenced *AHA5* which could indicate yet to be described function of AHA5 in plant development. Double mutants experienced normal growth and had no specific phenotype showing the ability to overcome presumed loss of function OF THIS AND THAT AHA. Subsequently, gas exchange experiment will be performed with transformed plants and corresponding AHA1 mutants with constitutively active AHA1 to check the response to different environmental stimuli. Additionally, T4 generation will be crossed with biosensors to judge the role of AHAs in the regulation of guard cell Ca2+ and ROS homeostasis for the stomatal functioning in various growth conditions.

SUMMARY

Stomata are essential for plant living. They mediate the gas exchange between the plant and its environment. Each stoma is surrounded by two guard cells. The guard cell turgor is dynamically adapted to environmental and hormonal signals to promote proper CO_2 uptake and avoid excessive water loss. Stomatal opening is triggered by the activated plasma membrane proton pumps that induce electrochemical gradient across the membrane, activating subsequent necessary signalling. Plasma membrane H^+-ATPases are encoded by the P-type ATPase families of 9-12 gene members in higher plants. There are 11 plasma membrane proton pumps (AHAs) in *Arabidopsis* (AHA1-AHA11), with three isoforms being the most abundant in guard cells, AHA1, AHA2, and AHA5.

In the current study, transgenic lines with AHA-targeting constructs were were generated, attempting to silence the AHAs with the highest expression in the guard cells. AHA-silencing approach was used to overcome the lethality of *aha* double mutants. Since the AHAs seems to have overlapping functions, specific double, or triple mutants of AHAs in the guard cells could give us details about the importance and functions of these proton pumps. The visual assessment was conducted on first generation of transformed lines. Transgenic line appeared visually similar to wild-type Col-0 plants, except for potential triple mutants. These potential triple mutants also with *AHA5* silencing constructs showed limited growth and unusual phenotype compared to other transformed plants in current study. Moreover, the plants showed completely closed under the microscope and had visually smaller stomata. This result suggests the possible AHA5 involvement in the regulation of stomata. The ability of expected double mutants to grow normally in the absence of most essential AHAs could demonstrate the success of an attempt to overcome embryo lethality. However, subsequent studies need to be performed to confirm the accuracy of generated lines. After verification, the lines with the lowest stomatal conductance will be selected and subjected to gas exchange experiments.

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