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The roles for EARLY RESPONSIVE TO DEHYDRATION 15 and EARLY RESPONSIVE TO DEHYDRATION 15-LIKE in guard cells functioning

Bachelor's Thesis (12 ECTS)

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The roles for EARLY RESPONSIVE TO DEHYDRATION 15 and EARLY RESPONSIVE TO DEHYDRATION 15-LIKE in guard cells functioning

Abstract:

Availability of water is important for plants as it is frequently a limiting stress factor for plants. Drought is one of the most common environmental stresses that soil plants are exposed to and it distresses the growth and development of plants through interchanges in absorption and gene expression. Abscisic acid (ABA) regulates many aspects of plant adaptation to stress conditions, including closure of stomatal pores, located on leaf surfaces and regulating water loss and CO2 uptake for photosynthesis. It is known from previous studies that overexpression of ERD15 (EARLY RESPONSIVE TO DEHYDRATION 15) in Arabidopsis plants results in an impaired response to ABA and enhanced sensitivity to drought. Our previous studies also showed that one of the ERD15 overexpressor lines had a secondary mutation in TREHALOSE-6-PHOSPHATE PHOSPHATASE (TPP) B, resulting in reduced stomatal response to ABA and darkness. To verify this notion, we characterized stomatal behavior of the novel double mutants carrying ERD15 overexpression and knockout of TPPB and TPPG. We also used BiFC (Bimolecular fluorescence complementation) to study protein-protein interactions of potential partners for ERD15 and ERD15-like, showing high similarity to ERD15. Our results show that phenotype in the double mutants had unaffected stomatal responses to ABA and darkness, while PAB4 (Poly(A)-binding protein 4) strongly interact with both ERD15 and ED15-like.

Keywords:

Abscisic Acid, Stomata, Protein Synthesis, ERD15, ERD15-like

CERCS:

B310 Physiology of Vascular plants

Geenide ERD15 (early responsive to dehydration 15) ja ERD15-sarnane (early responsive to dehydration 15-like) roll sulgrakkude funktsioneerimises

Lühikokkuvõte:

Vee kättesaadavus taimedele on oluline, kuna see on sageli limiteerivaks stressifaktoriks taimede kasvule ja arengule. Põud on üks sagedasemaid keskonnast tingitud stressistiimuleid taimedele, häirides nende kasvu ja arengut absorptsiooni ja geenieksprессiooni vaheliste

muutuste kaudu. Abstsiishape (ABA) reguleerib taimede adapteerumist stressitingimustes, muuhulgas õhulõhede sulgumist, mis asuvad lehepindadel ning reguleerivad vee kadu ning CO2 neeldumist fotosünteesiprotsessis. Eelnevatest uuringutest on teada, et ERD15 üleekspressioon Arabidopsis'es põhjustab häirunud ABA vastust ning suurenenud tundulikkust põuale. Meie varasemad uuringud on näidanud, et üks ERD15 üleekspresseerivatest liinidest omas sekundaarset mutatsiooni trehaloos-6-fosfaat fosfataas (TPP) B geenis, põhjustades vähenenud õhulõhede vastust ABA-le ning pimedusele. Selle kinnitamiseks iseloomustasime uute topeltmutantide, mis ekspresseeruvad üle ERD15 geeni ning kannavad TPPB ja TPPG KO (knockout), õhulõhede käitumist. Oma töös kasutasin bimolekulaarse fluorestsentsi komplementatsiooni (BiFC - biomolecular fluorescence complementation) uurimaks ERD15 ja ERD15-sarnase (omab kõrget sarnasust ERD15-ga) geenide potentsiaalseid interaktsioonipartnereid. Meie tulemused näitasid, et topeltmutantide fenotüüpidel puudusid muutused õhulõhede vastustes ABA-le ja pimedusele, samal ajal kui PAB4 (poly(A)-seonduv valk 4) omas tugevat interaktsiooni ERD15 ja ERD15-sarnase valkudega.

Võtmesõnad:

Abstsiishape, õhulõhed, valgusüntees, ERD15, ERD15-sarnane

CERCS:

B310 Soontaimede füsioloogia

TABLE OF CONTENTS

ABBREVIATIONS

PAB - Poly (A) Binding Protein

PAB 2 - Polyadenylate-binding protein 2

PAB 4 - Polyadenylate-binding protein 4

PAB 8 - Polyadenylate-binding protein 8

MPK6 - Mitogen-activated protein kinase 6

ABA - Abscisic acid

ERD 15 – Early responsive to dehydration 15

PIC - Pre-initiation complex

P-site - Peptidyl site

ATPases - Adenosine triphosphatase

H^+-ATPase - ATP dependent H^+-channel

K^+_{in} - K^+ inward channel

K^+_{out} - K^+ outward channel

RRM - RNA recognition motif

tRNAi - Initiator transfer RNA

eIF - Translation initiation factor

elF4G - Eukaryotic translation initiation factor 4 G

GEF - Guanine nucleotide exchange factor

Met - Methionine

mRNA - Messenger RNA

PCR - Polymerase chain reaction

INTRODUCTION

Plants are essential for ecosystem as energy assimilated directly from the sunlight. They also produce oxygen and absorb carbon dioxide during photosynthesis. Oxygen is essential for cellular respiration for all aerobic organisms. It also maintains the ozone layer that helps protect Earth's life from damaging UV radiation (Fernando 2012).

Stomata, the microscopic pores formed by guard cells, are important for plant gas-exchange between plants and the atmosphere. Stomata regulate water loss from plant tissues and nutrient transportation with water flow though transpiration. CO_2 fixation comes at the cost of water loss. Stomata are typically found in plant leaves but can also be found in some stems (M.B. Kirkham et al, 2014). Abscisic acid (ABA) regulates many aspects of plant adaptation to stress conditions, including closure of stomatal pores, located on leaf surfaces and regulating water loss and CO_2 uptake for photosynthesis. It was previously shown that ABA is produced only in the vascular system of plants and carried to other plant organs by specific transporters and xylem. However, it was demonstrated that guard cells themselves have the biosynthesis pathway for ABA synthesis (Bauer et al., 2013). Plant response to ABA is regulated by many proteins, including EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15) that has been reported to act as a negative regulator of ABA signaling (Kariola et al. 2006).

In this study, we aimed to study roles for ERD15 in stomatal functioning. We studied stomatal responses to ABA and darkness in the double mutants carrying ERD15 overexpression and knockout of TREHALOSE-6-PHOSPHATE PHOSPHATASEs B or G. We also investigated protein-protein interactions of ERD15 and its analogue ERD15-like with poly-A binding proteins (PABs) and MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) by using bimolecular fluorescence complementation (BiFC).

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

1. LITERATURE OVERVIEW

1.1. *Arabidopsis thaliana*

Plants separated from animals around 1.5 billion years ago resulting in development of different mechanisms of living as multicellular organisms. Arabidopsis is an Angiosperm / flowering plant, which has used as the primary experimental organism for the study of all aspects of plant biology (Somerville and Koornneef 2002). The main reasons for using Arabidopsis as a model for plant molecular genetics include short-generation time (8 weeks from seed to seed), easy growing of plants at a high density in a glasshouse or a growth-room, has a diploid genome, making analysis of retreating mutations easy, self-pollination allowing researchers to propagate individual plants without a need to cross pollinate them, and high productivity (a single plant yields hundreds or thousands of seeds) (Koornneef and Meinke 2010).

1.2. Roles of stomata in plant life

Water is one of the main factors which drives plant growth. It is the key element of plants, comprising up to 95% of its structure in some species (Neil E. Robbins et al, 2018). Water is mainly up taken into plants through roots and then carried to the different tissues, distributing nutrients and hormones all over plant organs. Plants fixate carbon from the ambient carbon dioxide (CO_2) during photosynthesis. To accomplish photosynthesis, plants require six molecules of water and six molecules of CO_2 to produce sugar and oxygen (Daszkowska-Golec 2013). Stomata drive water transport through the pulling force resulting from transpiration to the atmosphere. Stomata have two main roles, specifically for gas exchange, acting as an entrance for CO_2 and releasing oxygen (O_2). The other function of stomata is regulating water movement through transpiration. Stomata differ in shape and size.

Some environmental factors, for instance, night period, low air humidity, high soil salinity as well as pathogen attack induce stomatal closure. High CO_2 also induces stomatal closure to avoid unwanted water loss when substrates for photosynthesis are in excess. In ideal conditions, stomata are wide open, allowing gas exchange with the atmosphere.

The fast closure of stomata in response to overflowing of the intercellular spaces with water proposes that in a normally functioning leaf, water may be confined to the interstitial spaces of the cell walls rather than filling the intercellular spaces (Kirkham, M. B. 2014) (Fig-1). Mesophyll cells are a type of ground tissue found in the plant's leaves. Mesophyll cells are specialized for photosynthesis. Transpiration occurs through stomata, cuticle, guard cells and mesophyll cells where water is lost in the form of vapor (Bovi et al., 2016; Veraverbeke et al.,

2003) (Fig-1). Thus, stomata play an essential role in water loss and CO_2 assimilation by plants, consequently ensuring optimal conditions for photosynthesis.

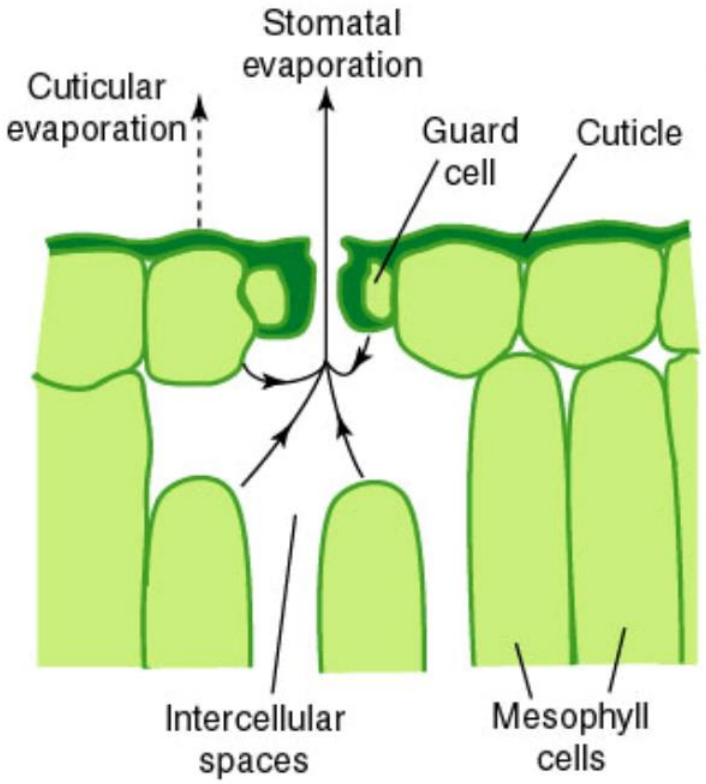


Fig-1: The pathway of the water evaporation through stomata (John A. Dutton e-Education Institute, Pennsylvania State University, 1983).

1.3. Stomatal movements

Stomata are formed by pairs of guard cells which dynamically regulate cellular concentration of potassium ions (K^+) and anions such as Cl^-. Guard cells can regulate their turgor pressure. K^+ and Cl^- are released during stomatal closure. This makes water in guard cells to move osmotically from areas of low solute concentration to areas of high solute concentration. Stomatal pore opening or closure is controlled by a variety of signals: CO_2, air humidity, red and blue light, some plant hormones, including ABA (Abscisic acid). ABA is a plant hormone which triggers a signaling cascade in guard cells that results in stomatal closure and inhibits stomatal opening.

1.3.1. Stomatal closure

During stomatal closure, the plasma membrane gets depolarized by activating anion channels through inhibition of H^+-ATPases (adenosine triphosphatase). Electrophysiological measurements indicate that ABA treatments result in a depolarization of the plasma membrane.

ABA activates cytosolic calcium ($[Ca_2^+]_{cyt}$) increases (McAinsh et al., 1990; Fig-2A). $[Ca_2^+]_{cyt}$ elevation stimulates two different types of anion channels: slow sustained (S-type) and rapid transient (R-type;) anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990). Both types of channels intermediate anion release from guard cells, causing depolarization (Fig-2A).

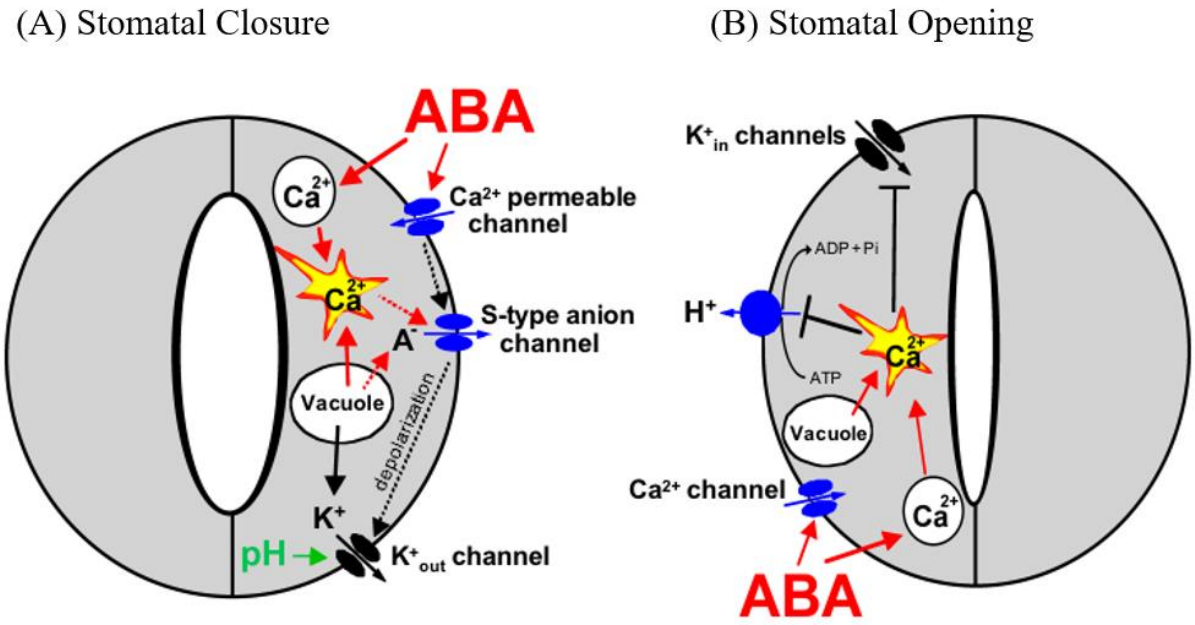


Fig-2: Stomatal opening and closing are mainly regulated by plasma membrane ion channels, proton pumps and transporters. During stomatal closure (A) plasma membrane is depolarized. Potassium ions move out of the vacuole and out of the cells. Water moves out of the vacuoles, following potassium ions. The guard cells shrink in size resulting in stomatal closure. During stomatal opening (B) the membrane is hyperpolarized because H^+-ATPase pumps H^+ from the guard cells. This results K^+ inward rectifying channels activated. Provided ions into the guard cells and water which is carried via aquaporins produce the turgor what keeps the stomata open (Schroeder and Waner 2001).

Depolarization activates the slow (S-type) anion channel, the S-type channel responsible for the slow but constant efflux of anions over a wide range of voltage, in addition to inhibition of the inward-directed potassium channel. ABA causes an alkalization of the guard cell cytosol (Blatt and Armstrong, 1993) which directly enhances K^+ out channel activity (Blatt and Armstrong, 1993; Ilan et al., 1994; Miedema and Assmann, 1996) and down-regulates the transient R-type anion channels (Schulz-Lessdorf et al., 1996). The sustained efflux of both anions and K^+ from guard cells via anion and K^+ out channels contributes to loss of guard cell turgor, which leads to stomatal closure. Basically, depolarization provides the driving force for potassium efflux through voltage-activated K^+ channels. Levels of K^+ (and malate) drop out of the cell or to intracellular compartments, which results in loss of water and closure of the pore (Fig-2A).

1.3.2. Stomatal opening

The elementary mechanism underlying stomatal opening in light, in most cases, is supposed to be connected to the uptake of K^+ by guard cells in amounts enough to lower significantly the solute potential. Opening of the stomata depend on blue and red light. Stomata open in feedback to weak blue light and the opening is improved by background red light. During stomatal opening, blue light activates an enzyme (plasma membrane H^+-ATPase). The H^+-ATPase pump drives the efflux of H^+ from the guard cells. (Daszkowska-Golec and Szarejko 2013). H^+-ATPases drives K^+ uptake via K^+ in channels (Fig- 2B) (Kwak et al., 2001). Due to an influx of K^+ and anions such as Cl^- from apoplastic space, intensification in osmotic pressure of guard cell vacuoles happens, which results stomatal opening. Organic solutes, such as sucrose and malate, also contribute to the rise in osmotic pressure. Accumulation of these solutes in vacuole results in an influx of water and an increase of cellular volume. Cytosolic Ca^{2+} elevations in guard cells down-regulate both K^+ in channels (Schroeder and Hagiwara, 1989) and plasma membrane H^+-ATPases (Kinoshita et al., 1995), providing a mechanistic basis for ABA and Ca^{2+} inhibition of K^+ uptake during stomatal opening (Fig-2B). The structure of guard cells is such that swelling out of the thin outer cellular wall pull the thick inner wall (bordering the pore), leading to opening of the pore (Kim et al., 2010).

1.4. Importance of ABA regarding plant functioning

ABA (Abscisic acid) is a phytohormone that is involved in stomatal closure. It regulates plant growth, stomatal apertures, seed maturation, dormancy as well as inhibition of seed germination (Finkelstein and Gibson, 2002) and plant adaptation to environmental stresses. ABA plays a vital role to regulate the transpirational water loss (Leung and Giraudat, 1998; Schroeder et al., 2001). It controls RNA processing, phosphorylation and metabolism of second messengers (Finkelstein et al., 2002). Many ABA-responsive have cis regulatory ABRE sequences (ABA-responsive element), the binding sites for the basic-domain Leu zipper-class transcription factors, AREBs (ABRE-binding proteins), or ABFs (ABRE-binding factors; Yamaguchi-Shinozaki and Shinozaki, 2005). Activation of these transcription factors depends on phosphorylation (Yamaguchi-Shinozaki and Shinozaki, 2005, 2006). ABA also regulates protein phosphorylation through PP2Cs (type 2C protein phosphates) and SnRKs (Sucrose Non-Fermentable related kinases). When ABA is absent, inhibition of the protein kinases happens by activation of PP2Cs which act as a negative regulator closure in ABA signaling (Kollist, Nuhkat, and Roelfsema 2014).

In addition to the roles ABA is playing for abiotic stress, It has been shown that ABA also reacts to biotic stress responses and might enhance with signaling that is delimited by the more ‘‘traditional’’ hormones of pathogen defense: SA, jasmonic acid (JA), and ethylene (ET; Mauch-Mani and Mauch, 2005).

1.5. Regulation of protein synthesis in plants

Studies of transcript levels have been supplemented with proteome studies that started to establish a connection between transcriptional and the corresponding translational changes. The translation factors are significant factors controlling plant performance and adaptation. Translation needs several types of RNA: a messenger ribonucleic acid (mRNA) transcript, the transfer RNAs (tRNAs) and the ribosomal RNAs (rRNAs) as well as multiple translation factors which act as individual proteins or multi-subunit complexes. Regulation of mRNA stability is one of important regulatory mechanisms in gene expression and it is an indispensable molecular machinery used by plants to achieve remarkable growth flexibility (Ferrando et al. 2018). mRNA levels are determined by a balance between their synthesis and degradation. Stability of individual mRNAs can be different in response to environmental stimuli including carbon sources, viral infection, and developmental transitions. Regulation of RNA stability is required for cell growth in both prokaryotes and eukaryotes, highlighting the importance of this process (Sydney Brenner and Jefferey H. Miller 2001). Components of the complex apparatus controlling RNA stability are recognizable in plant, fungal and animal kingdoms. Some studies showed that in Arabidopsis, translation mRNAs react extensively to stresses as well as environmental and growth signals (Tiruneh 2013).

1.5.1. Poly(A) Binding Proteins

Plants have a large family of genes encoding PABs (Poly(A) Binding Proteins) with considerable protein sequence diversity. PABs bind to the 3’ poly(A) tail of mRNAs and interacts with eIF4G (Eukaryotic translation initiation factor 4 G) and eIF4B (Eukaryotic translation initiation factor 4 B) (Park et al., 2011). In mammals, PABs have great roles in mRNA processing, translation, and degradation (reviewed in Goss and Kleiman, 2013). In higher eukaryotes, there are multiple genes for PABs which regulate translation (Gorgoni et al., 2011). In plants, PABs plays very important roles. PABs in Arabidopsis and other plants have four RRM (RNA recognition motif) domains that contains 2 α-helices and 4 anti-parallel β-sheets. C-terminal domain PABC contains 4 to 5 α-helices and a motif for protein-protein interaction (Siddiqui et al., 2007). A. thaliana genome contains genes of eight PABs that function in a tissue-specific manner (Le and Gallie 2000; Belostotsky, 2003). This is well

known that PABs bind poly(A) tails of mRNA at any given time, so resulting in a diversity of potential PAB molecules on one transcript.

The presence of eIF4G or eIF4B enhances PABs RNA binding activity as proven by the biochemical studies of wheat PAPBs, where the presence of PAB can increase the affinity of the eIF4F advanced for the cap (reviewed in Gallie, 2014; Luo and Goss, 2001; Khan and Goss, 2005; Cheng and Gallie, 2010). lately, it has been proved that plant eIF4G has a PAB binding domain which binds eIF4B in a competitive and reciprocally exclusive manner (Cheng and Gallie, 2010; Cheng and Gallie, 2013). PABs are additionally involved in infective agent replication (Smith and gray, 2010) and plant PABs were shown to move with the polymerase of turnip mosaic virus (Dufresne et al., 2008).

1.6. Functions of the PAM2 (PAB-interacting motif 2) Proteins

It has been demonstrated that PAM2 domain interact with the MLLE (Mademoiselle) domain (also known as PABC) present in poly(A)-binding proteins (PABs) in many species, with a HECT (Homologous to the E6-AP Carboxyl Terminus)-family ubiquitin and with the MATERNALLY EXPRESSED PAB C-TERMINAL (MPC) (Tiwari et al. 2008). The PAM2 motif is one of two known PAB interacting domains (PAM1 and PAM2) and has been recognized in various eukaryotic proteins as an important binding site for the PABC domain (G. Roy, G. De Crescenzo, 2002). A. thaliana PABC/PAM2 is shown to have multiple binding partners, many of that interfere with *in vitro* translation or are involved in RNA metabolism. N-terminus PAM2 motif consists of a 12 amino acid core sequence. The PAM2 motif has been discovered in several other eukaryote's protein including humans.

PAM2-contaning protein family is phylogenetically characterized in Arabidopsis, soybean and rice. 19 with PAM2-domain were found in the Arabidopsis genome, whereas 31 and 32 members were found in soybean and rice, respectively (Belostotsky 2003). The PAM2-like family was divided in seven subfamilies (A-G), based on sequence conservation and structure was characterized extensively, regarding its phylogenesis, dehydration responsiveness and cellular perform (Belostotsky 2003).

1.7. Discovery of EARLY-RESPONSIVE TO DEHYDRATION 15 proteins

Plants are immobile and therefore, must cope with environmental stresses through biological and physiological adaptations. Dehydration is one of the most common environmental stresses that plants are exposed to and that distresses the growth and development of plants through changes in interest and gene expression (Leopold AC, Mansfield TA 1990). A. thaliana plants were exposed to dehydration stress, they lost water gradually, reaching up to 90% water loss

after 10 h of dehydration stress (Kiyosue, Yamaguchi-Shinozaki, and Shinozaki 1994). ABA concentration started to increase after 1 h of dehydration stress, and stressed plants accumulated ABA 4 times higher than unstressed plants. One-h dehydration treatment revealed 26 up-regulated Arabidopsis mRNAs (Takahashi T, (Kiyosue, Yamaguchi-Shinozaki 1994). The cDNA clones were classified into 16 groups based on Southern blot hybridization and named ERD (Early-responsive to dehydration). ERD15 (Early-responsive to dehydration 15) is a member of the dehydration stress-induced proteins. ERD15 mRNA level was higher in dehydrated plants within 1 h of stress onset and remains higher for up to 24 h (Kiyosue, Yamaguchi-Shinozaki, and Shinozaki 1994). Another study showed that one of light stress-regulated) clones was the same as drought-inducible ERD15 (Dunaeva and Adamska 2001).

Phylogenetically, ERD15 proteins can be divided into several groups based on the presence of distinct ERD15 paralogs reflecting some diversifications in function. In cases where the number of paralogs is more than three (e.g. in *Populus trichocarpa*, *Medicago truncatula*, *Glycine max*, *Manihot esculenta* and *Zea mays*) duplication events can be detected from the sequences (Aalto et al. 2012). ERD15 proteins are 120–170 aa in size and have PAM2 and PAE1 (Pectin acetylesterase 1) motifs in the N-terminus and C-terminal (Albrecht and Lengauer 2004).

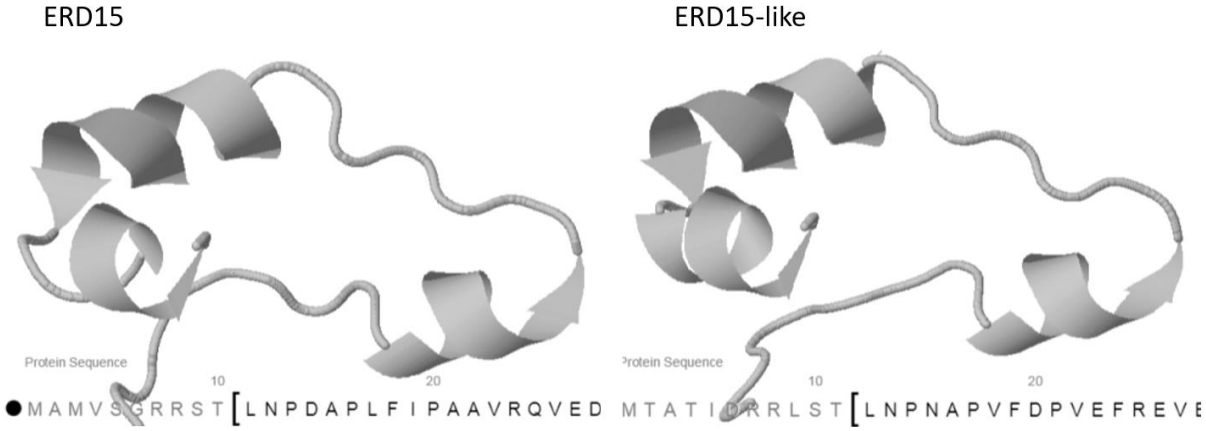


Fig-3: Structure of ERD15 and ERD15-like protein. This figure was taken from public data base.

The Arabidopsis thaliana genome has two genes sharing high similarity: At2g41430 (ERD15) and At4g14270 (ERD15-like) (Aalto et al. 2012) (Fig-3). ERD15 and ERD15-like have a homologous fragment (67% of similarity). Interestingly, phosphorylation of the S1 (Serine1) amino acid in the PAM2 domain confirmed by an unidentified kinase in ERD15 and ERD15-like (Reiland et al. 2009).

1.7.1. The role of ERD15 in plant functioning and its expression in different environmental conditions

Characterization of ERD15 (Early-responsive to dehydration 15) expression pattern showed that, in addition to pathogen elicitors and dehydration, the gene was also rapidly induced after the bacterial necrotroph *Erwinia carotovora* (Ecc) infection (Kariola et al., 2003; Li et al., 2004; Kariola et al., 2005). The stress-responsiveness of ERD15 genes has been found in several microarray studies. For instance, in rice (*Oryza sativa*), induction of ERD15 happened under both drought and cold, while wheat ERD15 genes were induced by dehydration, pathogen, low and high temperature. Additionally, maize genes and orthologs of ERD15 were induced by pathogen inoculation (T. Hruz, O. Laule. 2008).

To achieve better understanding of roles for ERD15 in plants, researchers generated Arabidopsis Columbia-0 (Col-0) lines harboring ERD15 overexpression (OE) or silencing by RNA interference (RNAi) constructs (Kariola et al. 2006). ERD15 protein in ERD15 OE plants was in a much higher level than in Col-0 plants, while expression of ERD15 protein in RNAi silenced plants were hardly noticeable (Kariola et al. 2006). ERD15 overexpression plants resulted in some morphological differences, namely, more narrow leaves. To assess the drought tolerance phenotype of the transgenic plants, drought tolerance of ERD15 RNAi, ERD15 OE and wild-type (Col-0) were tested by keeping them under 50% humidity for 2 weeks without watering (kariola et al, 2006). The majority (72%) of ERD15 OE plants were dead, whereas a significant fraction of Col-0 plants were still alive while only 14% of the RNAi silenced plants were dead.

ERD15 acts as a transcription factor in some plant species. In 2017, one study showed that, ERD15 from mulberry drove a high level of transcription of reporter genes in yeast indicating that it acted as a functional transcription factor (B., and Khurana, P.2017). Elucidation of roles of ERD15 in details and recognizing specific transcripts regulated by this protein would be of great interest for future studies and would give new insights into plant ABA signaling (Heino et al. 2006). Microbial induced systemic tolerance to drought was detected in Arabidopsis plants primed with *Paenibacillus polymyxa* because of initiation of drought stress responsive gene, ERD15 (Early Response to Dehydration 15) (Kaushal, M. 2019).

1.7.2. Different responses to ABA in ERD15

It is described in Kariola 2006 that ERD15 OE reduced the ABA sensitivity of Arabidopsis (Kariola et al, 2006). To understand the possible roles of ERD15, the authors generated two transgenic lines: ERD15 OE21 and ERD15 OE52. In our laboratory, it was found that ERD15

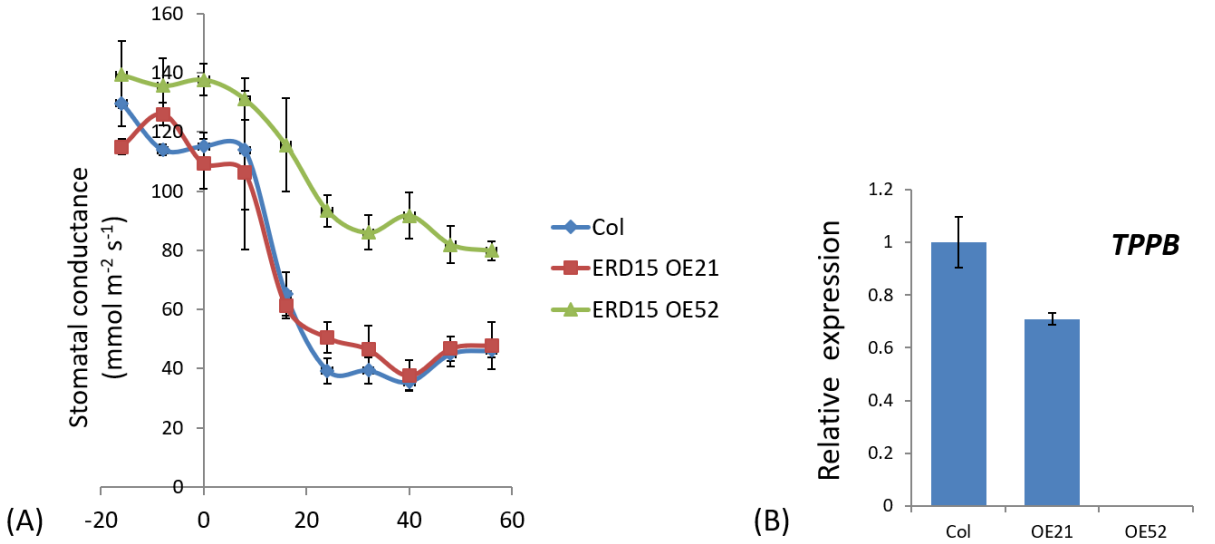


Fig-4: Different response to ABA in ERD15 overexpressors. This figure was provided by Dmitry Yarmolinsky. Comparison of ERD15 OE lines described in Kariola et al. 2006. (A) Different responses to ABA in ERD15 overexpressors. (B) Location of T-DNA insertion in OE52. ERD15 OE52 did not respond to ABA due to a different location of T-DNA insertion while ERD15 OE21 reacted as wild type Col.

OE52 did not respond to ABA while ERD15 OE21 reacted to ABA as wild type Col (Fig-4A). The further studies showed that OE52 had the insertion of its T-DNA in the promoter region of *TREHALOSE-6-PHOSPHATE PHOSPHATASE B* (Fig-4B). Quantitative PCR showed that *TPPB* was not expressed in ERD15 OE52 in contract to OE21.

2. EXPERIMENTAL WORK

2.1. Main Goals

In order to explore the possible roles of ERD15 in stomatal functioning, stomatal reactions in ERD15 OE plants have been studied in our lab. In this work, we studied stomatal responses to ABA and darkness in the double mutants carrying ERD15 OE and knockout of *TPPB* or *TPPG*. We also investigated protein-protein interactions of ERD15 and ERD15-like with PABs and MPK6 by using bimolecular fluorescence complementation (BiFC).

Specific goals:

A. Cloning;

B. Gas Exchange Experiments to characterize the double mutants ERD15 OE x *tppb* and ERD15 OE x *tppg*;

C. Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions with PABs and MPK6;

D. Western-Blot to verify expression of proteins in BiFC assays.

2.2. MATERIAL AND METHODS

2.2.1. Plant lines

T-DNA insertion lines *tppb* (salk 037324) and *tppg* (salk 078443) were obtained from European Arabidopsis Stock Centre (Alonso et al., 2003). ERD15 OE52 line was described by Kariola et al (2006). New ERD15 OE lines (lines 62-8 and 62-14) and the double mutants *tppb* x 62-8, *tppb* x 62-14 and *tppg* x 62-8 and *tppg* x 62-14 were generated by Dmitry Yarmolinsky. All lines were in the Col-0 genetic background.

2.2.2. Gas exchange experiments

Plastic pots for gas-exchange experiments were filled with 250 g of soil (peat: vermiculite: water mixed as 4:3:3, respectively) and covered with a glass plate (10 x 10 cm, 0.25 cm thick) having a drilled conical hole (Ø 0.3 cm) in the middle (Kollist et al. 2007). The Arabidopsis seeds were incubated in water at +4^{o}C for 48h and sowed into the hole on top of the substrate using a pipette. The hole was covered with a little petri dish to ensure high air humidity. A week after germination, the Petri dish was removed. The plants were thinned so only one plant was left to grow in each pot. Plants were grown in growth chambers with LED lamps 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 darkness. Plants were illuminated with photosynthetic photon flux density at 150 µmol m^{-2} s^{-1}. The pots were watered by bottom-watering at least once per week to keep the weight of pots around 340-350 g. In 3 days, before experiments, the hole in the glass was closed by wax. This was done to avoid humidity from the soil to affect assays of stomatal conductance.

The custom-made 8-chamber gas-exchange system (Fig-5A) which enables monitoring of whole-plant rapid-response gas exchange was used to measure stomatal conductance (Kollist et al., 2007). The cylinder-shaped 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 below a chamber and pressed against the metal walls by a spring forming a sealed off cuvette. Data about the air difference entering and exiting the chambers were collected every 1 minutes. The standard conditions were as following:

ambient CO_2 (~400 ppm), light 150 µmol m^{-2} s^{-1} and relative air humidity at ~70%. A gas analyzer (LI-Cor) was used to collect data about the gas content in the chamber. (Fig-5B).

Two treatments were applied: darkness and sprays with 5 µM ABA solution containing silvet L77. To apply darkness, each chamber was covered to shade the plants. To study ABA effect, the plants were sprayed from a distance of approx. 30 cm. Changes in stomatal conductance were monitored during 40 min.

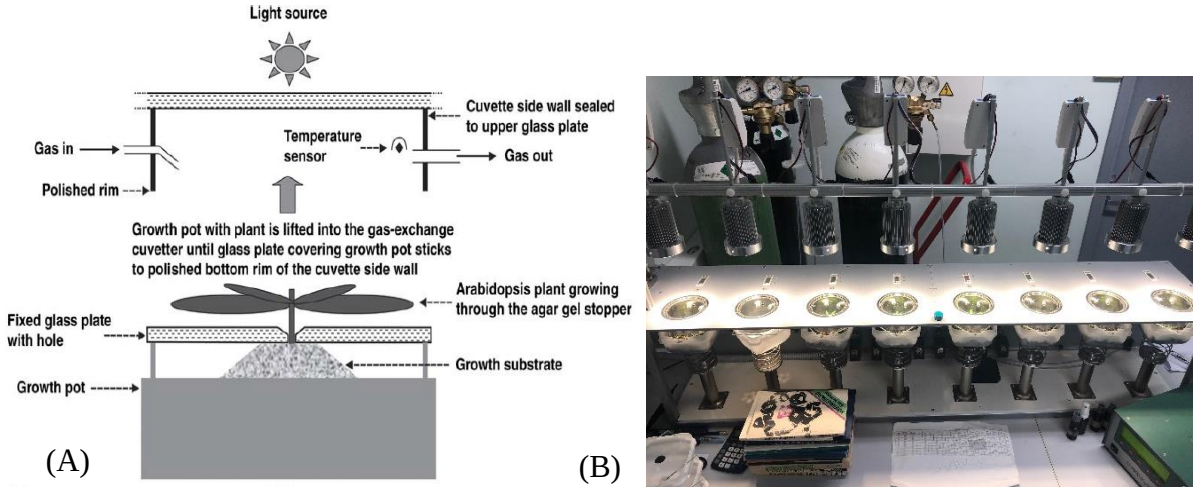


Fig-5: The design shows the gas-exchange system defined in Kollist et al. (2007) (A). Growth chamber for gas exchange experiments (B). Arabidopsis thaliana plants were grown through a hole in a polished glass plate to separate the rosette for gas-exchange measurements in the cuvette.

Stomatal conductance was calculated using a custom program based on the data gathered by the system and the projective leaf area (Kollist et al., 2007). Pictures of each plant were taken by a digital camera. The leaf area was estimated by ImageJ. A ruler was used for scaling the pictures.

2.2.3. Cloning

Constructs for BiFC were based on the pDOE9 vector (Double ORF Expression) (Gookin and Assmann et al, 2014). This plasmid has two *UBIQUITIN 10* promoters (UBQ10) connected to the Omega translation enhancer (Ω) to drive expression of N- and C-parts of mVenus (NmVen210 and CmVen210, respectively; Fig-6). Sequences of the studied proteins were amplified by using Phusion DNA polymerase (Thermo Fisher), complementary DNA synthesized from Arabidopsis total RNA, and appropriate primer pairs (see Table 1).

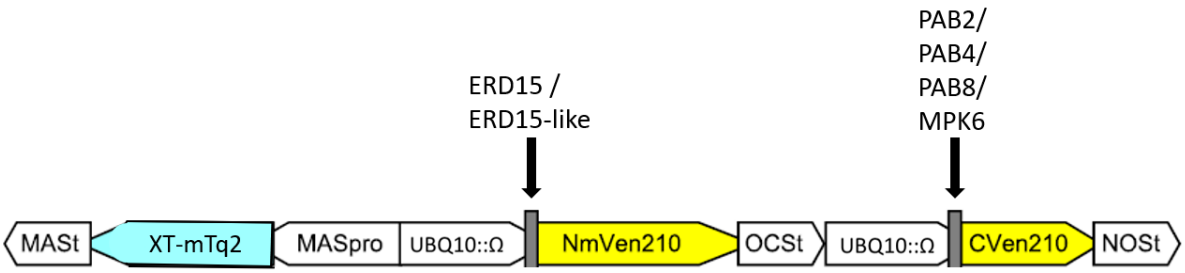


Fig-6: The scheme for the pDOE-09 vectors. The UBIQUITIN10 promoters and ERD15/ERD15-Like::NmVen210–PAB2/PAB4/PAB8/MPK6::CVen210 are shown (from Gookin and Assmann 2014 with modifications).

PCR products and plasmids were cut with the appropriate restriction enzymes to generate sticky ends. ERD15 and ERD15-like were cloned upstream NmVen210 and the potential interaction partners (PAB2, PAB4, PAB8, MPK6) were cloned upstream CVen210 (Fig-6). The XT-mTurquoise2 (mTq2) fluorescent protein was expressed under the MAS promoter in all the plasmids to provide the inner control for transformation of plant cells.

2.2.4. Transformation of agrobacteria

The generated genetic constructs for BiFC were introduced to *Agrobacterium tumefaciens strain GV3101*. To obtain competent cells, A. *tumefaciens* GV3101 cells were grown on Luria-Bertani (LB; #L3022, Sigma) medium plates containing gentamycin (15 µmg/ mL) at 28ºC. 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 µmg/ 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_{600} 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 $CaCl_2$. The competent cells were put into 1.5-mL tubes and frozen at -80°C.

The transformation was done by using heat shock. Competent A. tumefaciens cells (100 µl) were gently mixed with approx. 1 µg plasmid and incubated on ice for 20-30 minutes. Then, the cells were incubated at 37°C for 3 minutes to induce a heat shock. 1 mL of LB liquid medium was added; then, the cells were incubated at 28°C in a shaker at 200-250 rpm for 2 hours. To collect cells, the tubes were centrifuged for 2 minutes at 7000 rpm. Most of the liquid medium was removed. The cells were spread on LB plates with 25 µg/ml gentamicin and 50 µg/ml kanamycin as selective markers for the plasmids. The plates were incubated at 28°C for

2-3 days before the colonies appeared. The strains of agrobacteria were verified by colony PCR to carry the required plasmids.

2.2.5. Bimolecular fluorescence complementation (BiFC) Experiment

In a BiFC assay, two proteins of interest are fused with different parts of a fluorescent protein and are expressed in plant cells. If the studied proteins physically interact, the fluorescent protein is restored, and a fluorescent signal can be detected in plant cell (Fig-7).

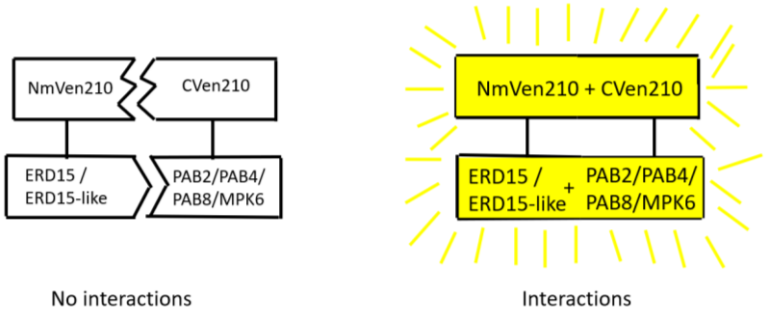


Fig-7: Bimolecular fluorescence complementation (BiFC). BiFC is a protein complementation methodology that assesses protein–protein interactions in live cells.

Agrobacterium strains were grown in 5 mL of LB medium containing 50 µmg/ml kanamycin and 15 µmg/ mL gentamycin for 16 hours at 28°C on a shaker. The optical density at 600 nm (OD_{600}) was measured by using the Ultrospec Cell density meter. The agrobacterial cells were taken to have OD_{600} to be 0.05 in 1 ml. The cells were precipitated at 4000 rpm for 3 min and resuspended in 1 ml infiltration buffer containing 10mM MES, 10mM $MgCl_2$ and pH of 5.7 adjusted with KOH. 1µL of 100 mM Acetosyringone was added to the mixture. The agrobacterial cells were incubated at room temperature for 2h.

Tobacco (*Nicotiana benthamiana*) plants were grown under 25°C with photoperiod of 16 h light (120 µmol m−2 s−1) and 8 h darkness. The leaves of 3-weeks-old plants were injected with the prepared agrobacterial cell from abaxial side with a needleless 1-ml syringe. The tip of the syringe was pressed against the leaf and a gentle pressure was applied on the plunge to inject the agrobacterial suspension into the leaf. The borders of the infiltrated suspension were marked with a permanent marker on the leaves. Infiltrations were performed, at least, on three plants in an experiment.

The BiFC signals were detected in 72 h after the infiltrations. Confocal microscopy was accomplished using a Zeiss LSM 510 META laser scanning microscope (European Molecular Biology Laboratory (EMBL), Heidelberg GERMANY). Fluorescence of mTq2 and mVenus was excited with the 458 nm and 488 nm lasers and detected in the ranges of 480-520 nm and

500-550 nm, respectively. Chlorophyll autofluorescence was also detected by using a 650-710 nm bandpass filter. Image acquisition parameters (e.g. laser power, pinhole, detector gain, etc.) and sampling time post-infiltration were held constant within all experiments. The pictures of the individual channels were exported from the Zen software. The ratio between mVenus and mTq2 signals was calculated by using the ImageJ software.

2.2.6. Protein Extraction and Western-Blot Analyses

Injected leaf samples were cut and stored with glass beads in a 1.5mL Eppendorf tube in -80^{o}C. The samples which were used for the western blot were kept in liquid Nitrogen. Samples were crushed with a bead-beater for 7 seconds in 3 cycles. Protein extraction was performed using lysis buffer which is mixture of 100mM Tris (pH 7.5), 150mM NaCl, 5mM EDTA (ETHYLENEDIAMINETETRAACETIC ACID), 10% glycerol (v/v), 1% triton (v/v), 2mM PMSF (PHENYLMETHYLSULFONYL FLUORIDE). Protein concentration was measured according to the standard Bradford Assay (Bradford Reagent)-Bio-Rad. Samples were boiled in SDS-loading buffer for 5 min at 90 ^{0}C. 20 µg of protein was loaded onto 8% SDS-PAGE (SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS) gel. Starting of the gel run was 80V, later it was increased to 120V. Nitrocellulose membrane was used for blotting the protein from SDS-PAGE gel. The blot was blocked for 1h in 5% non-fat milk-TBST ((Tris-buffered saline (TBS) and tween 20) mixture of 20mM Tris, 150mM NaCl, 0.1% Tween 20), rinsed twice and primary antibody was incubated over-night in 3% BSA-TBST. Anti-FLAG (rabbit) Abcam antibody was used in 1:5,000 dilution. Detection of the proteins was made using “Clarity™ Western ECL Substrate”. CCD camera-based imager was used to make the picture of the bands.

2.2.7. Statistical analysis

Tukey-Kramer HSD test was performed by using the statistical software JMP, version 8. The differences considered to be statistically significant at $P<0.05$.

Table 1. The primers used in this study

Primer Name	Sequence	Restriction sites	Purpose
ERD15-NcoI-Fw	AAAACCATGGCGATGGTATCAGGAAGA	NcoI	Cloning
ERD15-SpeI-Rw	AAAACTAGTGCGAGGCTGGTGGATGTTTC	SpeI	Cloning
ERD15like-AscI-Fw	AAAAGGCGCGCCACGGCGACGATAGATCGGAG	AscI	Cloning
ERD15like-SpeI-Rw	AAAACTAGTCCGTGGCTGATAGATATGATG	SpeI	Cloning
PAB2-RsrII-Fw	AATGCGGTCCGCGCAGGTTCAACTTCAGGGTCA	RsrII	Cloning
PAB2-BiFC-Rw	AAAACACGTGAGAGAGGTTCAAGGAAGCGA	PmlI	Cloning
PAB4-RsrII-Fw	AATGCGGTCCGCTCAGGTTCAAGCTCCTTCTTC	RsrII	Cloning
PAB4-BiFC-Rw	AAAACACGTGTAAATGATCATTGATGGAAAGT	PmlI	Cloning
PAB8-RsrII-Fw	AATGCGGTCCGCTCAGATTCAGCATCAGGGTCA	RsrII	Cloning
PAB8-BiFC-Rw	AAAACACGTGAGGTACGATGTTGTCTCCAA	PmlI	Cloning
pab2seq1-Rw	ATTCCCCCAGGACGTCTTCC		Sequencing
pab2seq2-Rw	TCTCTGACTTCTTCTGGGCTCTACC		Sequencing
pab2seq3-Rw	TCTTGGATCGCTCTTGCAGC		Sequencing
pab4seq1-Rw	GGACCGTCAGGCATGTTTCTAC		Sequencing
pab4seq2-Rw	GGTGACGGTATCATCAAGGTTCTTA		Sequencing
pab4seq3-Rw	TCCCAACCCCACTTCTACGG		Sequencing
pab8seq1-Rw	CGATACATTCGACCTCTTGGATG		Sequencing
pab8seq2-Rw	GACGCTCTCATCCAAGTTCTTAACA		Sequencing
pab8seq3-Rw	CCGCTCTTACGGAGACTTGGAT		Sequencing
MPK6-RsrII -Fw	AATGCGGTCCGACGGTGGTTCAGGTCAA	RsrII	Cloning
MPK6-PmlI-Rw	AAAACACGTGTTGCTGATATTCTGGATTG	PmlI	Cloning
MPK6-seq-Fw	TCCGTGGATTGAAATACAT		Sequencing
MPK6-seq-Rw	GCGTTCAGGAGGAGATTACTTGG		Sequencing
Nos-term-Rw	CGCAAGACCGGCAACAGGAT		Colony PCR

2.3. RESULTS AND DISCUSSION

2.3.1. Stomatal phenotypes of double ERD15 OE x *tppb* and ERD15 OE x *tppg* mutants

The phenotyping of plant lines is a technique used to describe observable characteristics, such as height, biomass, leaf shape etc. More specifically, the term phenotype is used to describe the collective expression of the genotype in combination with the environment on a plant's noticeable features (Horovitz 1996). When a genetic interaction happens, a double mutant (tppb x ERD15 OE and tppg x ERD15 OE) would show a phenotype that is not simply explained by independent actions of two alleles. In our study, we used a wild-type line (Col-0), single mutants (*tppb*, *tppg*, ERD15 OE), and the corresponding double mutants (ERD15 OE lines: 62-8 and 62-14).

In gas-exchange experiments, stomatal conductance was monitored. Previously charecterized ERD15 OE52 line with an T-DNA insertion in the promoter region of *TPPB* showed partially impaired stomatal closure when the plants were treated with ABA (Fig-8).

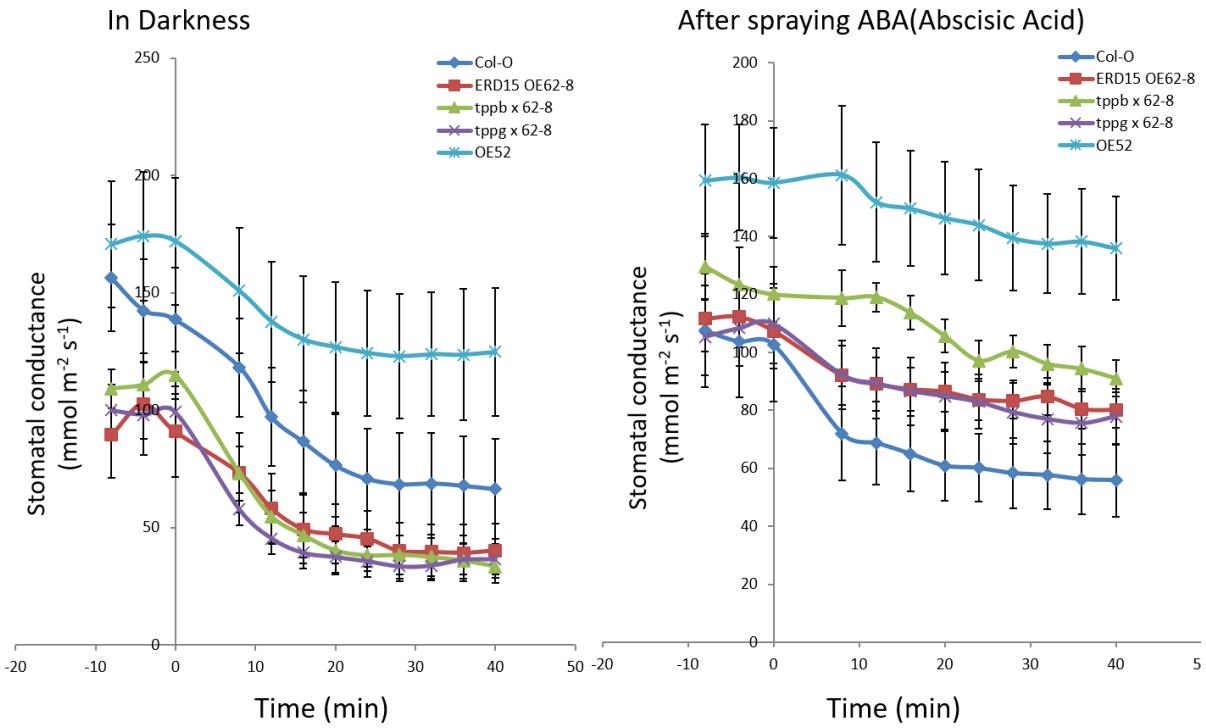


Fig-8: The graphical representation shows the results obtained from gas exchange experiment. As described in the Material and methods sections, two independent experiment occurred to distinguish the stomatal behavior of the phenotype in single and double mutant plants. Phenotype in double mutant of plants were applied in 4 transgenic lines (tppb x 62-8 & tppb x 62-14, tppg x 62-8 and tppg x 62-14). In

this figure 2 transgenic lines (tppb x 62-8 and tppg x 62-8) are shown. The data on stomatal conductance is presented as average±SE.

Stomatal conductance was about 110 mmol $m^{-2}s^{-1}$ in Col-0 before the experiments. Upon onset of darkness, stomatal conductance dropped. The same response was observed after spraying plants with a solution of 5 µM ABA. The double mutant plants (*tppb* x 62-8 and *tppg* x 62-8) hardly responded to ABA (Fig-8). Single mutants (ERD15 OE62-8 and ERD15 OE62-14) showed weak response to ABA compared to wild type Col-0 (Fig-8,9).

From Fig-9, it is seen that the stomatal conductance was around 165 mmol $m^{-2}s^{-1}$ in double mutant plant (*tppb* x 62-14). Upon beginning of darkness period, stomatal conductance dropped. Similar response was observed after spraying ABA. Other transgenic line (*tppg* x 62-14) of the double mutant plant also showed very low response to ABA but its response in darkness was not affected (Fig-9).

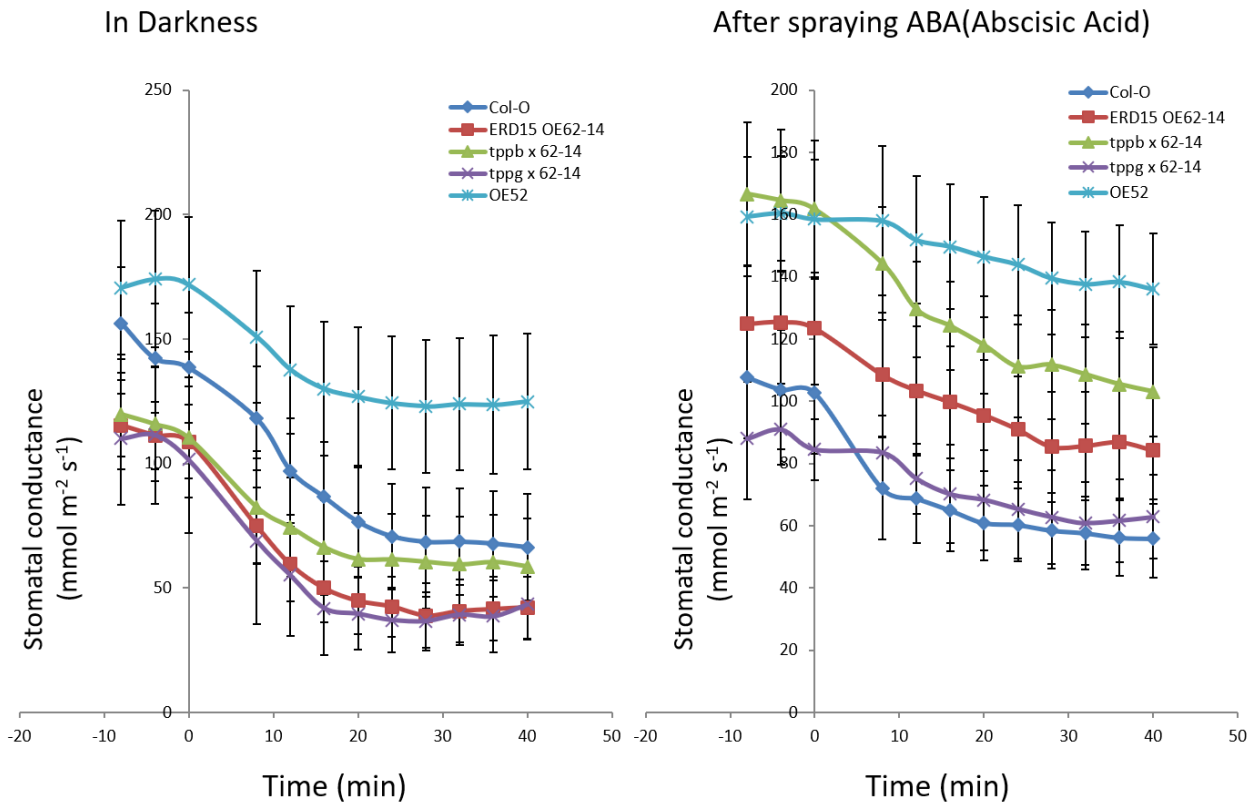


Fig-9: Other transgenic lines (tppb x 62-14 and tppg x 62-14) are shown here.

In the earlier experiment by Dmitry Yarmolinsky, ERD15 OE lines (OE21, 62-8, and 62-14, bit not OE52) reacted similarly to the wild type Col-0. Our new results contradict the previous experiments, as both lines ERD15 OE62-8 and ERD15 OE62-14 didn't have strong response to ABA (Fig-8 and 9). A possible reason could be different growth conditions (for example, LED lamps vs. fluorescent lamps which were used in the different experiments).

Figure 10 shows that single mutants *tppb* and *tppg* mostly responded to ABA in the same rate as the wild type Col-0. Furthermore, in darkness, wild type Col-0 stomatal conductance was reduced by almost 60 mmol m^{-2} s^{1} whereas single mutant plants *tppb* and *tppg* stomatal

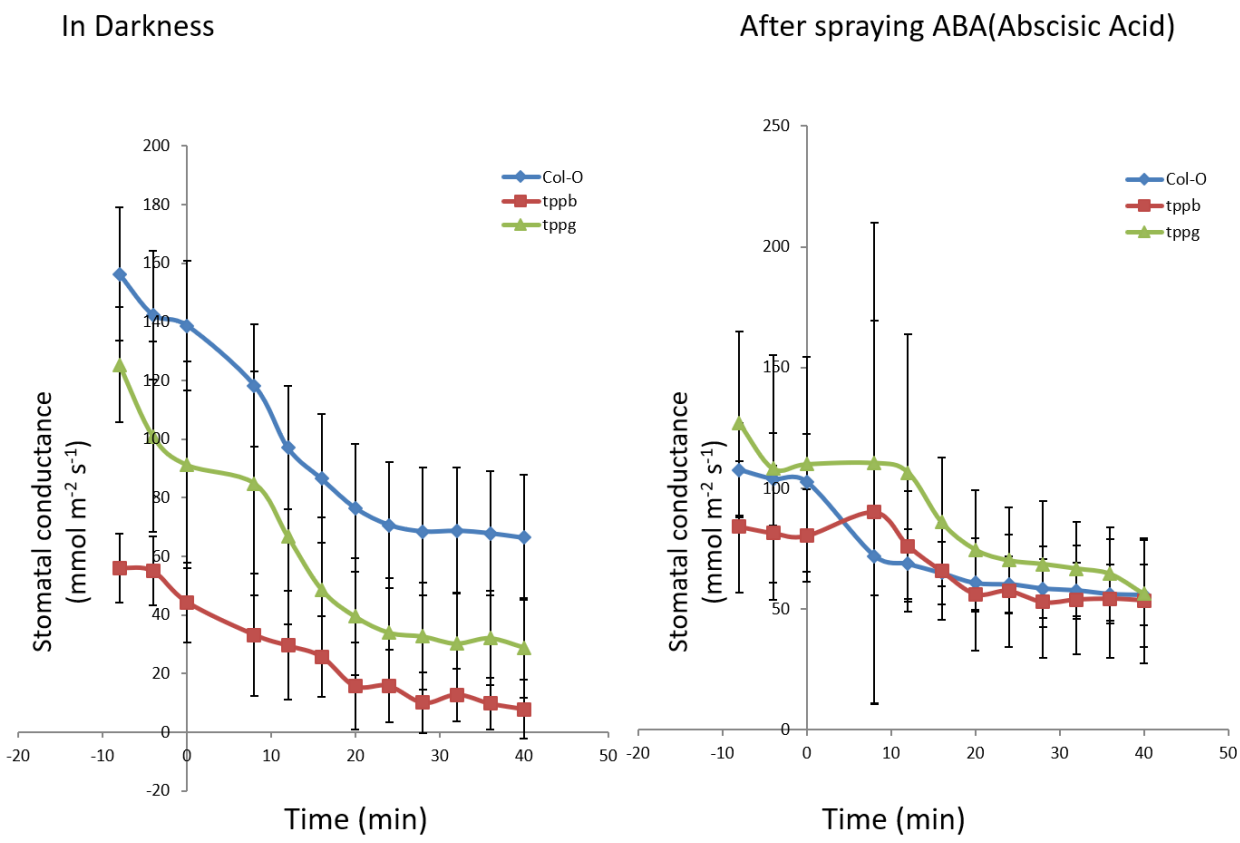


Fig-10: For the phenotype in single mutant, tppb and tppg T-DNA insertion lines were implemented.

conductance was reduced by almost 30 mmol m^{-2} s^{1} and 60 mmol m^{-2} s^{1} respectively. The response of the single mutant plants (*tppb* and *tppg*) to ABA were not clear. The stomatal conductance was around 90 mmol $m^{-2}s^{-1}$ when single mutant plant (*tppb*) stomates were open (fig-10) and in darkness, it drops but after spraying ABA, *tppb* didn't show strong response to ABA. Other single mutant (*tppg*) plant also showed low response to ABA. Single mutant plants response in darkness were not affected (Fig-10).

We used Tukey-Kramer HSD test to estimate differences between the studied line in stomatal closure which was calculated as the difference between stomatal conductance in 40 min of a treatment and before the treatment. From figure 11 we can see that the statistically calculated data were all the same, meaning there were no specific difference for stomatal closure in different lines. Based on the results what we have obtained, we can conclude that there was no clear effect of ABA and darkness on stomata in the double mutants (ERD15 OE x *tppb* and

ERD15 OE x *tppg*) and single mutants (*tppb* and *tppg*) plants. Thus, our results indicate that we should optimize plant growth conditions to reduce variability in plant responses, increase the number of analyzed plants. We also should verify levels of protein/transcript expression for ERD15, TPPB, and TPPG to confirm ERD15 over-expression and TPPB and TPPG knockout. It is possible that we would have to generate new sets of double mutants.

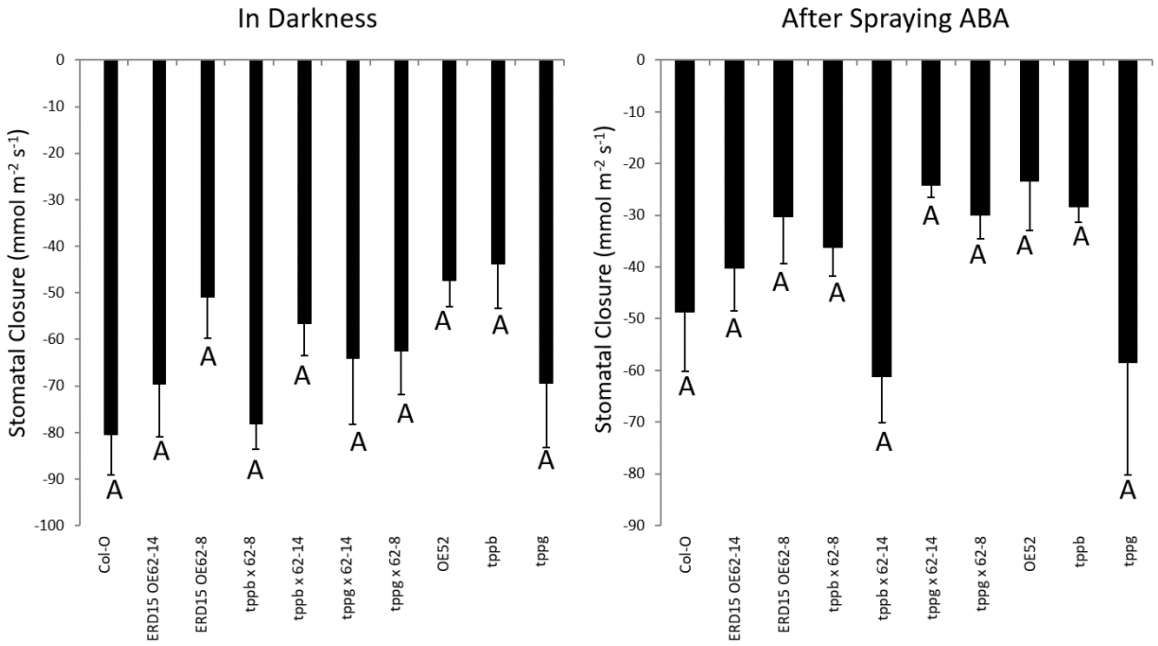


Fig – 11: Rates of stomatal closure are presented as average±SE. The values which are not connected by the same letters are statistically different conferring to Tukey-Kramer HSD.

2.3.2. ERD15 interacts with PAB4, PAB8, MPK6 while ERD15-like interacts with PAB4

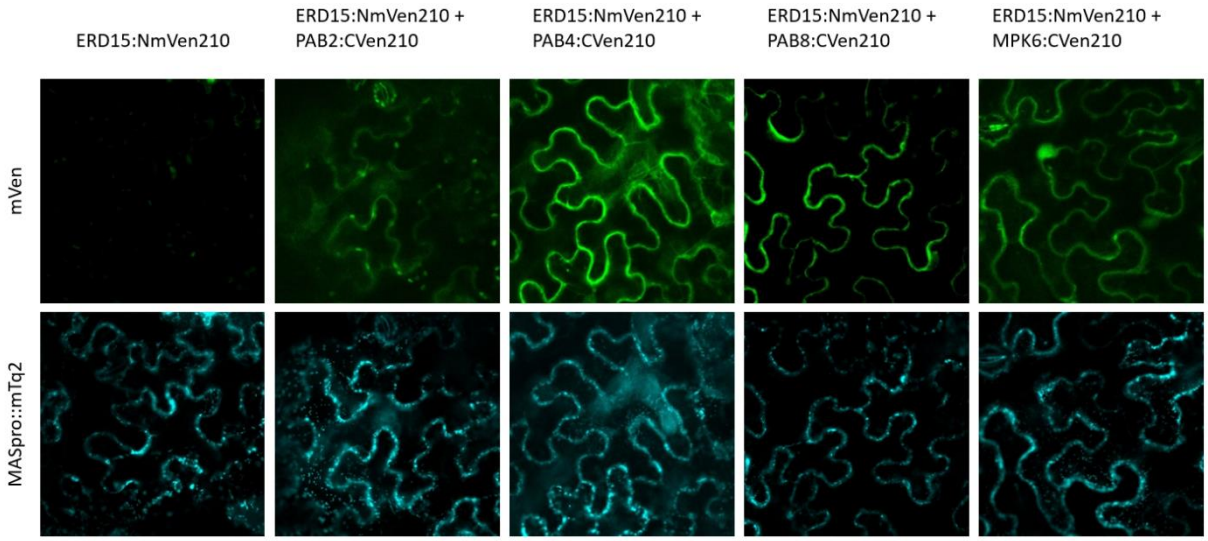
Protein-protein interaction is important for a plethora of biochemical reactions within cells. Signals from the outside of a cell can be transduced to the inside of that cell by protein-protein interactions. There are plenty of methods available to study protein-protein interactions. Yeast two-hybrid system is a relatively robust way to study protein-protein interactions. This method is scalable to screen possible interactions among many proteins (Rao, V. S., Srinivas, K., Sujini, G. N., & Kumar, G. N. S. 2014). But on the other hand, this method can have some limitations such as elevated numbers of false positives & negatives and may show false protein-interaction due to overexpression of fusion proteins (P. Braun and A. C. Gingras 2012).

An alternative method, the bimolecular Fluorescence Complementation (BiFC) assay is a technique which visualizes protein-protein interaction *in situ* using imaging in alive cells.

It measures spatial protein-protein interactions. In these experiments, we used BiFC because it is well-known assessment to study protein-protein interactions in plants (Lai and Chiang 2013).

pDOE9 vector (Double ORF Expression) based construct was made for BiFC (Gookin and Assmann et al, 2014). This plasmid has two *UBIQUITIN 10* promoters (UBQ10) connected to the Omega translation enhancer (Ω) to drive expression of N- and C-parts of mVenus (NmVen201 and CmVen210, respectively; as described in material and methods, Fig-6). Two independent BiFC experiments were done.

Experiment 1



Experiment 2

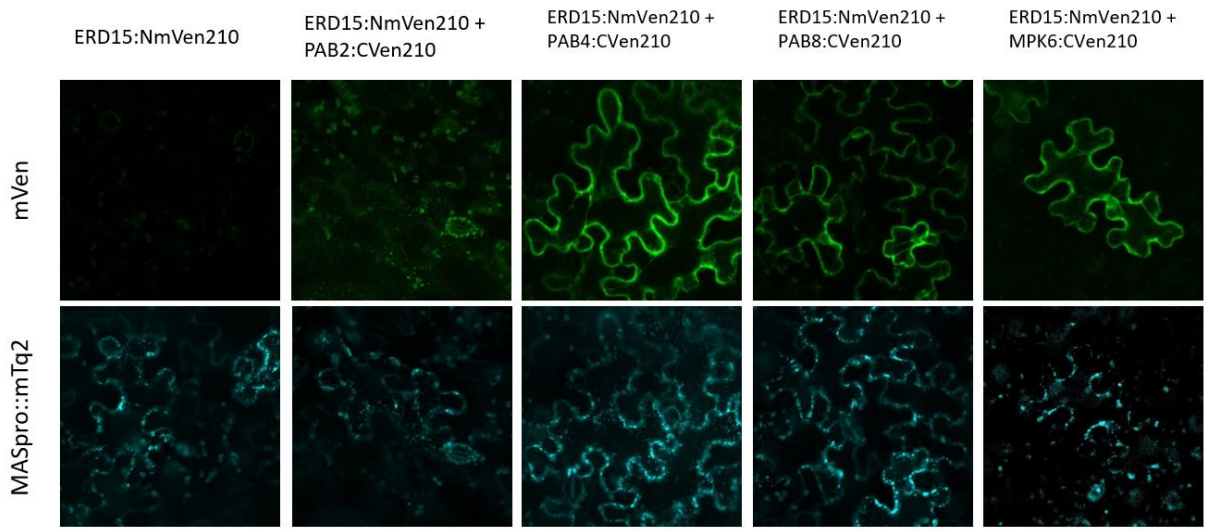
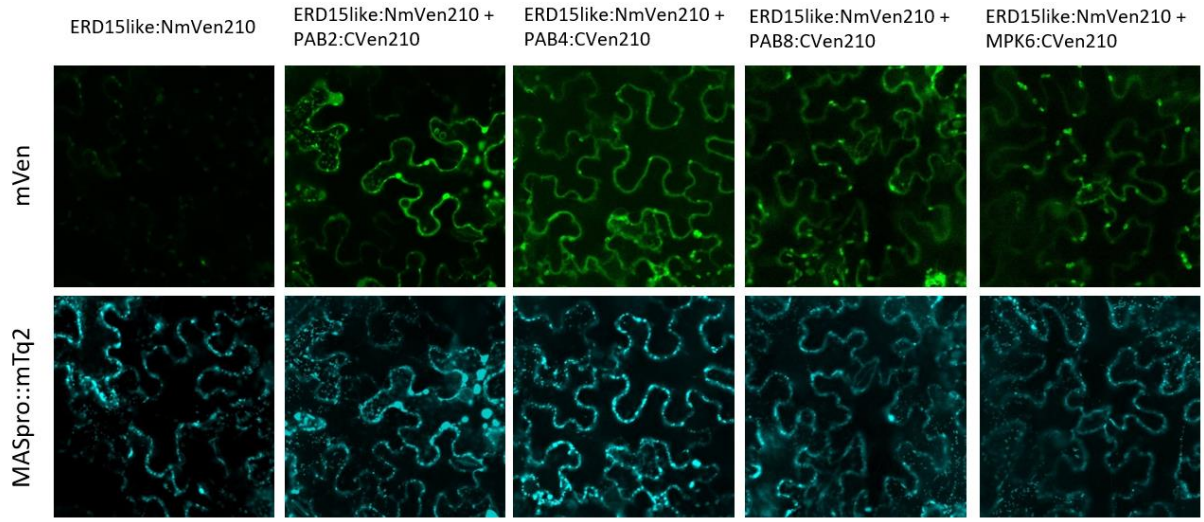


Fig - 12: BiFC studies of ERD15 in experiment 1 and 2. Leaves of 3 weeks old tobacco plants injected with agrobacterial strains and studied by using a Zeiss LSM 510 META laser scanning microscope as described in the Material and Methods section. Venus and Turquoise are abbreviated as "Ven" and "Tq" and a former "m" indicates that the sequence harbors the A206K mutation for fluoroprotein

monomerization. The signal of mTq2 shows successful transformation events and the mVen fluorescence represents interactions between proteins.

Infiltration of tobacco leaves with only ERD15:NmVen210 and ERD15-like:NmVen210 did not result in any mVenus fluorescence (Fig- 12,13 experiment 1 and 2). PAB4:CVen210 showed a strong signal in interaction with ERD15:NmVen210 and ERD15-like:NmVen210 in both experiments (Fig-12 and 13). MPK6 (Mitogen-activated protein kinase 6) showed different results in interaction with ERD15 and ERD15-like. The interaction between ERD15:NmVen210 and MPK6:CVen210 was quite visible (Fig-12), however, an interaction between ERD15-like:NmVen210 and MPK6:Cven210 was almost absent (Fig-13). We also

Experiment 1



Experiment 2

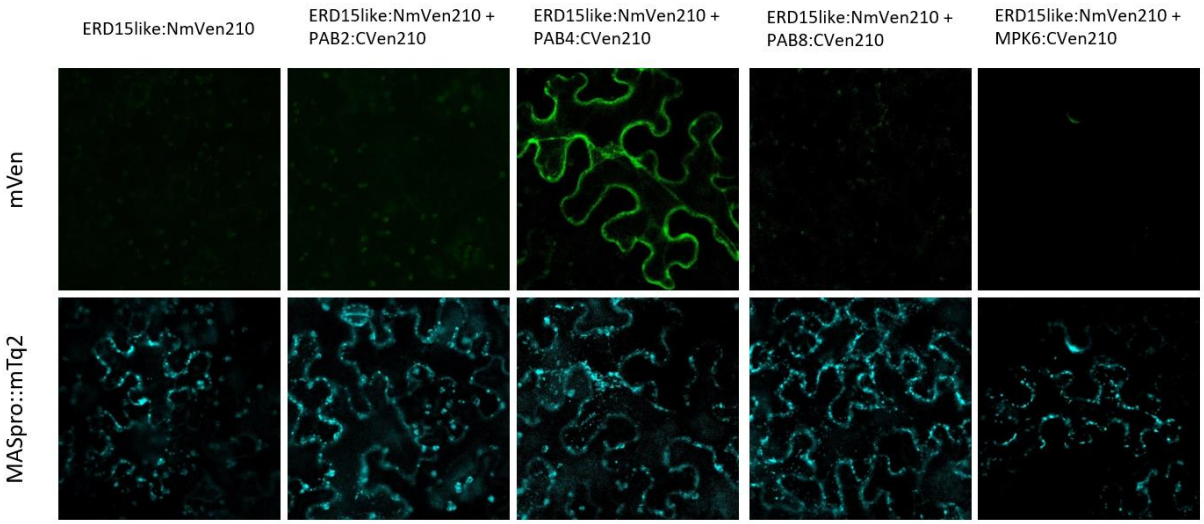
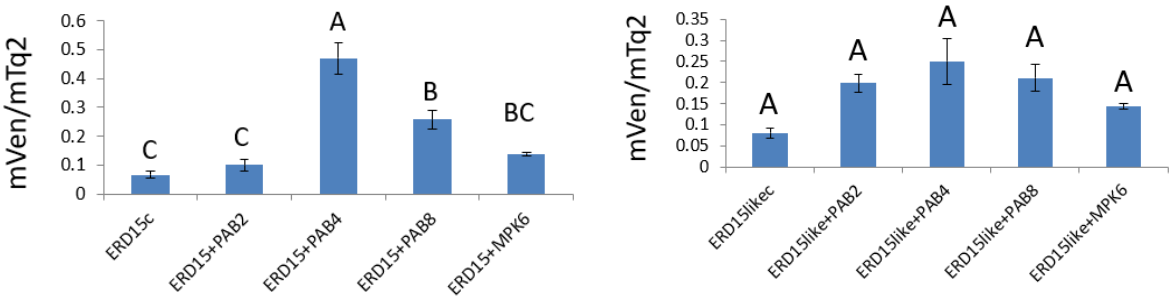


Fig - 13: BiFC studies of ERD15-like in experiment 1 and 2.

observed that ERD15 interacted with PAB8 (ERD15:NmVen210 + PAB8:CVen210) (Fig-12) but ERD15-like interaction was ambiguous (Fig13). Moreover, it is seen that both ERD15 and ERD15-like did not produce any signal or produce a weak signal in the combinations with PAB2, although experiment 2 (Fig-13) showed that ERD15-like:NmVen210 and PAB2:CVen210 resulted in a mVenus signal.

We used Tukey-Kramer HSD test to evaluate the ratios between mVenus and mTq2 in the BiFC experiments to evaluate the protein-protein interactions. From Fig. 14, it is seen that ERD15

Experiment 1



Experiment 2

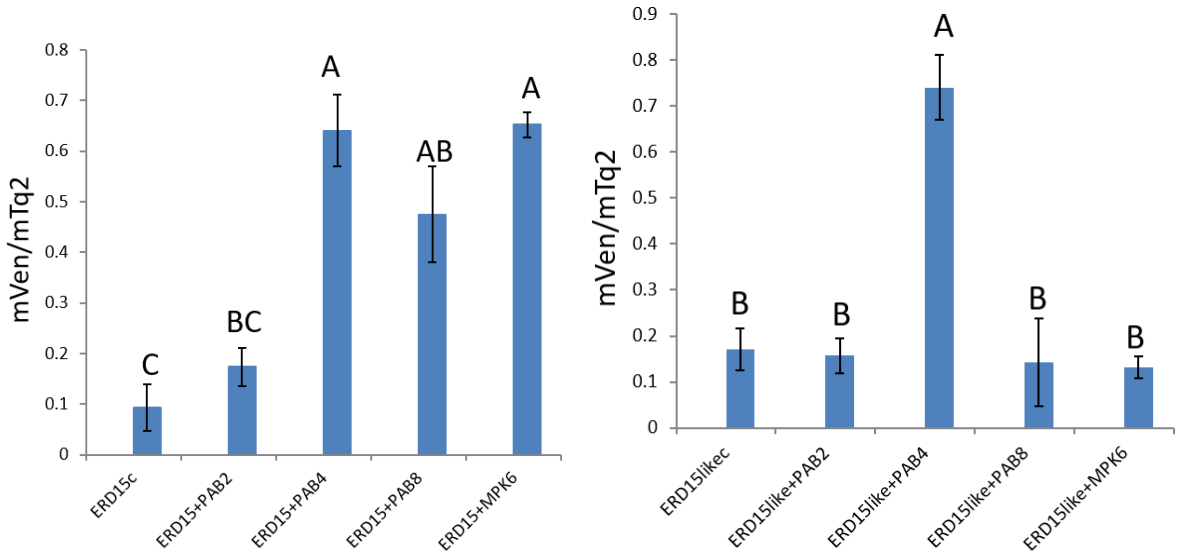


Fig- 14: The histogram shows average±SE and the values which are not connected by the same letters are statistically different by Turkey-Kramer HSD.

interacted with PAB4, PAB8, MPK6 in both experiments. On the other hand, the interaction of ERD15-like was not clear in experiment 1, however, the differences in experiment 2 were clear. PAB2 showed unclear signal in interaction with both ERD15 and ERD15-like. Since there were differences between two experiments, it is possible that conditions for the

experiments should be optimized such as growth conditions, plant age, level of injected leaves, etc.

2.3.3. Confirmation of protein expression in BiFC experiments

Western blot is a technique that is very useful for protein detection as it allows researchers to quantify protein expression. Western blotting allows protein detection by comparison of the molecular weight of the marker and a positive control weight & signal (Mahmood and Yang 2012). In this experiment, we intended to verify that all potential interaction partners (PAB2/PAB4/PAB8/MPK6) were expressed in BiFC experiments. The expected molecular weights of the target proteins were PAB2 - 76.6 kD, PAB4 - 79.6 kD, PAB8 - 80.6 kD, MPK6 - 52.6 kD, respectively. CVen210 has the FLAG tag and it allowed us to use anti-FLAG antibody to detect PAB2/PAB4/PAB8/MPK6 in the BiFC experiments.

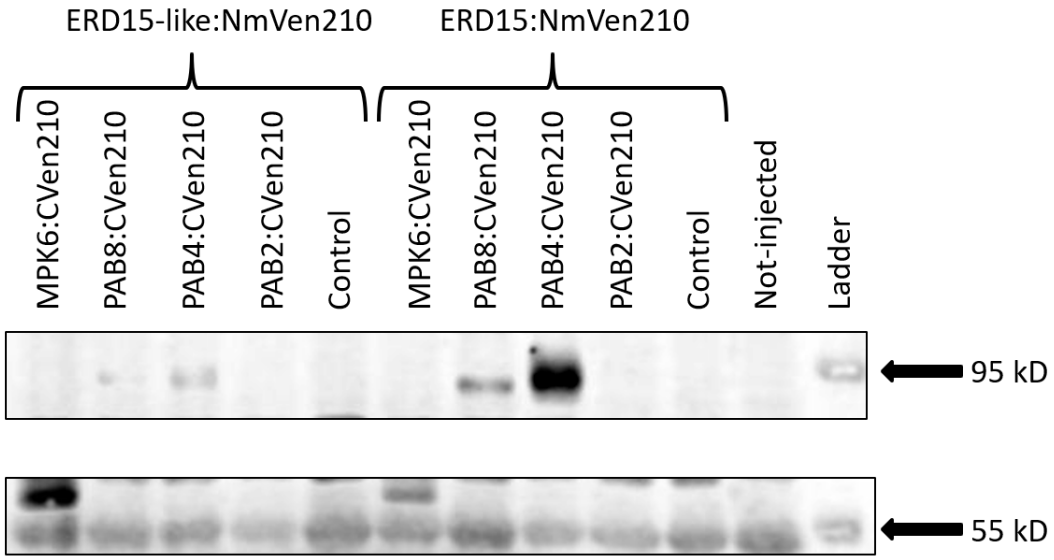


Fig-15: Protein expression in ERD15 and ERD15-like. The Western blot was probed with the primary antibody Anti-FLAG (rabbit) then anti-rabbit secondary antibody was added. Recognition was made using "Clarity™ Western ECL Substrate". In the figure, 95 kD & 55 kD are the PageRuler Prestained protein ladder weight.

Expression of PAB8:CVen210 protein was clear in ERD15:NmVen210 but it showed weak expression in ERD15-like:NmVen210. Similarly, PAB4:CVen210 protein was expressed in ERD15:NmVen210 but the expression was weak in ERD15-like:NmVen210 compared to the control and not injected leaves. Additionally, expression of the protein MPK6:CVen210 was clear in both ERD15:NmVen210 and ERD15-like:NmVen210. PAB2:CVen210 protein wasn't expressed in neither ERD15:NmVen210 nor ERD15-like:NmVen210. (Fig-15).

2.3.4. Roles for ERD15 and ERD15-like in plant signalling

As shown by Kariola et al. (2006), ERD15 overexpression plants had impaired response to ABA (Kariola et al. 2006). Our results from gas exchange experiment demonstrate that double mutants (ERD15 OE x tppb and ERD15 OE x tppbg) do not have strong response to ABA, which was similar to that in ERD15 OE 62-8 and 62-14. However, it is still possible that ERD15 influences stomatal movements under certain conditions and/or in combination with unknown factors. Therefore, additional research is needed to elucidate roles for ERD15 in stomatal functioning.

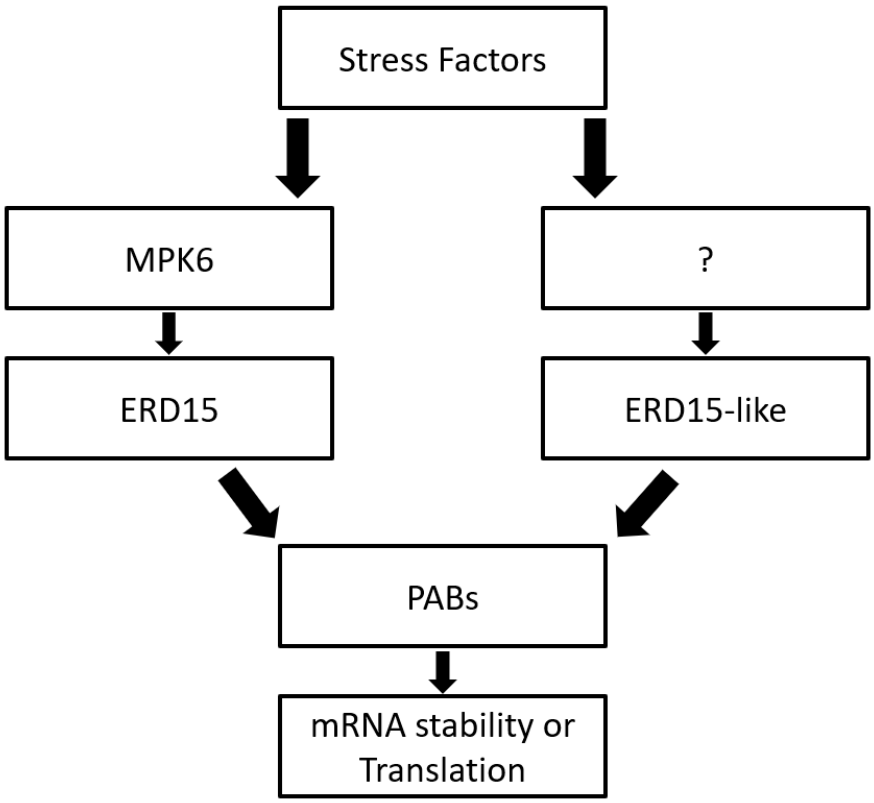


Fig-16: A hypothetical model which indicates MPK6 may interact with ERD15 in planta.

Based on our results, we can conclude that MPK6 that plays a prominent role in plant adaptation to stress conditions likely activates or inhibits ERD15 in response to some stress factors. The factors activating or inhibiting ERD15-like are still not known. Both ERD15 and ERD15-like interact with PABs, however this interaction is specific to different members of the PAB family. PAB4 and PAB8 interact with ERD15, while PAB4 interacts with ERD15-like. Interaction with PAB2 is still not clear. We can speculate that interaction of ERD15 and ERD15-like with PAB modulates processes which are controlled by PABs: mRNA stability and/or translation (Fig-16).

SUMMARY

Though previously it had been shown by Kariola et al (2006) that ERD15 overexpression (OE) plants have impaired response to ABA, in our laboratory, stomatal response to ABA differed in the individual ERD15 OE lines described by Kariola et al (2006). To elaborate stomatal functioning in ERD15 OE lines, in this work, we studied stomatal responses to ABA and darkness in the double mutants carrying ERD15 OE and knockout of TPPB or TPPG. Our results demonstrate that there was no clear difference in ABA and darkness responses of stomata in the double mutants (ERD15 OE x *tppb* and ERD15 OE x *tppg*) and single mutants (*tppb* and *tppg*) plants. Investigation of protein-protein interactions of ERD15 and ERD15-like with PABs and MPK6 was the second main goal of this study. To study protein-protein interactions, Bimolecular fluorescence complementation (BiFC) technique was used. Our results show that ERD15 interacts with PAB4, PAB8, MPK6 while ERD15-like interacts with PAB4 but does not interact with PAB8 and MPK6. Interaction of PAB2 with ERD15 and ERD15-like is still not clear. Thus, our BiFC data indicate that MPK6 can phosphorylate ERD15 and modulate its interactions with PAB4 and PAB8. Both ERD15 and ERD15-like bind individual members of the PAB family and can regulate functions of PABs, although details of this mechanism remain unresolved. Even though ERD15 and ERD15-like have been studied before, their roles in guard cells functioning remain obscure and new research is needed in the future.

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