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Elucidating the role of receptor like kinases ERf in plant development

Pawel Zbigniew Kosentka
University of Tennessee, pkosentk@vols.utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Pawel Zbigniew Kosentka entitled "Elucidating the role of receptor like kinases ERf in plant development." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

Elena D. Shpak, Major Professor

We have read this dissertation and recommend its acceptance:

Barry D. Bruce, Karen W. Hughes, Bruce D. Mckee, Albrecht G. von Arnim

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Elucidating the role of receptor like kinases ERf in plant development

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DEDICATION

I dedicate this work to my parents Ryszard and Anna and brother Jakub.

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ABSTRACT

Intercellular communication is indispensable for development of complex multicellular organisms. Cell to cell communication in plants is heavily reliant on receptor-like-kinases (RLKs) located on the surface of cells. ERECTA (ER) and its two paralogs ERECTA-like 1 (ERL1) and ERL2 are leucine-rich repeats RLKs that regulate multiple developmental processes. Ligands of the ERf receptors are small secreted peptides known as Epidermal Patterning Factor-Like (*EPFL*). In *Arabidopsis,* the *EPFL* family is made of 11 genes, several of which remain to be characterized. Results presented in this work include:

1) The use of structure function analysis found that juxtamembrane domain and kinase activity is essential for ERECTA signaling activity while the carboxy-terminal tail is not. Analysis of the activation loop in the kinase domain revealed the importance of phosphorylation sites that modulate the signaling of ERECTA. Lastly, not all developmental processes regulated by the ERECTA family require kinase activity suggesting that there are different mechanisms for stomata development and regulation of organ growth.

2) Ectopic expression of *ERECTA* in specified regions of the shoot apical meristem (SAM) releveled that central zone expression was sufficient to rescue the meristem size and leaf initiation defects of *er erl1 erl2* mutant. Transcriptional reporter lines identified the putative *ER* family ligands that were expressed near the SAM. A genetics approach reveled *EPFL1, EPFL2, EPFL4* and *EPFL6* to redundantly regulate meristem size and rate of leaf initiation. Lastly, ectopic expression of *EPFL1* in the peripheral zone of the SAM rescued SAM phenotypes of the *epfl1 epfl2 epfl4 eplf6* mutant. These results suggest that the ERECTA family signaling pathway mediates communication between the peripheral zone and central zone of the SAM.

This work expands our knowledge of ERECTA family signaling and its implementation in the role of SAM regulation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

General background

The fundamental goal of developmental biology is to understand how a single zygotic cell becomes a complex multicellular organism. At the heart of the developmental process lies intercellular communication. Cell to cell communication allows for coordinated differentiation and thus formation of specialized tissues. This communication is of particular importance in plants because cells develop in a position dependent manner rather than the lineage dependent method seen in animals. Although plants cells are able to communicate via channels known as plasmodesmata, they also heavily rely on signals sent through the extracellular space. Extracellular signaling has the advantage that it covers larger distances at faster rates but requires cell surface receptors to perceive the signals. Plants make use of several different types of cell surface receptors; the largest and most diverse group of plant receptors is the receptor-like kinase (RLKs) family (Shiu and Bleecker 2001).

With 610 members, the RLK gene family is one of the largest gene families in *Arabidopsis thaliana,* accounting for roughly 2.5% of protein coding genes (Shiu and Bleecker 2001). Sharing a common ancestor with animal receptor tyrosine kinases (RTKs), plant RLKs diversified to encompass a wide array of different functions from hormone perception to recognizing foreign proteins such as bacterial pathogens (Shiu and Bleecker 2001). Structurally the RLKs are composed of three major parts: an extracellular domain that recognizes the signal, a cytoplasmic kinase domain, and a single pass transmembrane domain that connects the two together, figure 1.1. Unlike animals, which use tyrosine kinases to signal, plants predominantly use serine/threonine kinases. The largest subfamily of the RLKs is named after the leucine rich repeats found in the extracellular domain of the receptor (LRR-RLK). The *Arabidopsis* genome contains 223 LRR-RLK genes and only 60 have been linked to a biological function, but even those are not fully understood and new roles are being discovered and further studied (Wu, Xun et al. 2016).

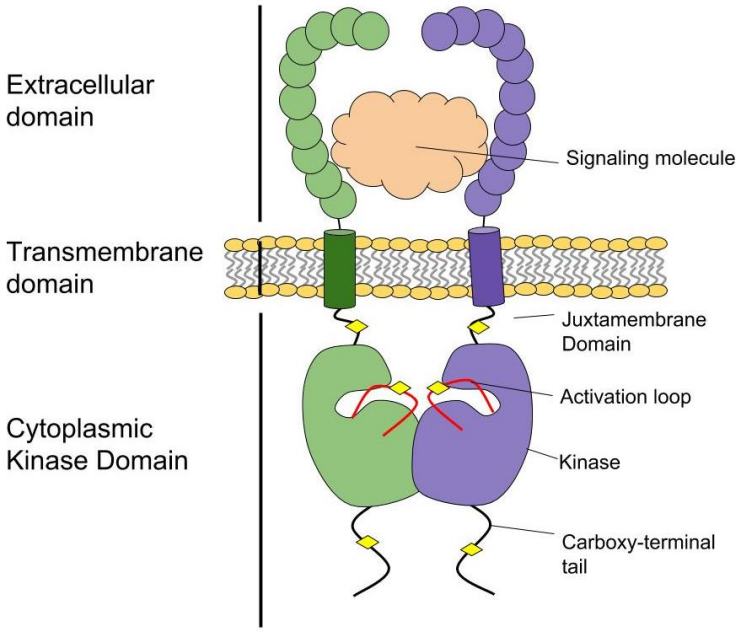

Figure 1.1. Cartoon depiction of a generalized Receptor Like Kinase heterodimer. Two receptor proteins (one green, the other purple) become associated with each other by the signaling molecule (peach). Major domain names are labeled on the right and specific components are labeled on the left. Regulatory phosphorylation sites are labeled with yellow diamonds.

Studies on the plant LRR-RLKs, BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED KINASE 1 (BAK1) have found that activation, regulation, and signaling occur in a manner similar to mammalian RTKs (Wang, Goshe et al. 2005, Wang, Li et al. 2005). BRI1 and BAK1 function as co-receptors for sensing the plant hormone brassinosteroid, which regulates several aspects of plant growth (Belkhadir and Jaillais 2015). Brassinosteroid binds to the LRR regions of BRI1 and BAK1 creating a molecular bridge between receptors allowing for the formation of a receptor heterodimer (Santiago, Henzler et al. 2013, Sun, Han et al. 2013). Ligand induced dimerization brings the kinase domains of BRI1 and BAK1 within proximity allowing for sequential transphosphorylation of residues in a ping-pong manner that increases kinase activity at each step (Wang, Kota et al. 2008). The cytoplasmic domain of LRR-

RLKs can be further divided into the juxtamembrane domain, kinase domain and C-terminal tail, all three of which contain regulatory phosphorylation sites. Kinase domain phosphorylation occurs in the activation loop and this is a frequent mechanism for kinase activation (Adams 2003). The juxtamembrane domain (JMD) and C-terminal tail show little sequence conservation among different receptors and thus have different regulatory roles such as kinase inhibition or a docking site for phosphorylation substrate (Pawson 2002). The BRI1 receptor contains phosphorylation sites in both the juxtamembrane domain and the c-terminal tail which have a negative impact on kinase activity when unphosphorylated (Wang, Kota et al. 2008). Once the BRI1-BAK1 heterodimer has been fully activated the kinase of BRI1 initates a signaling cascade that ultimately alters gene expression (Belkhadir and Chory 2006).

ERECTA family receptors

The *ERECTA* gene family (*ERf*) is composed of LRR-RLKs that first appeared in early land plants (Villagarcia, Morin et al. 2012). The *erecta* phenotype has been known since 1957 when it was isolated from X-ray irradiated *Arabidopsis* seeds giving rise to a short, compact, erect plant known as Landsberg *erecta,* figure 1.2 (Redei 1992). Originally, this gene was linked to plant architecture due to the phenotype of compact inflorescences (flower clusters), short siliques (seed pods), and short pedicels (organs attaching flowers to the main stem), but a more complete picture emerged when the paralogs of *ERECTA* were discovered (Shpak, Berthiaume et al. 2004). All angiosperms that have had their genome sequenced contain at least two members in the *ERf* gene family. In *Arabidopsis* there are three member: *ERECTA (ER), ERECTA LIKE 1 (ERL1)* and *ERECTA LIKE 2 (ERL2)* (Shpak, Berthiaume et al. 2004)*. erl1* and *erl2* single mutants along with the *erl1 erl2* double mutant lack any noticeable phenotype but double mutants of *er erl1* and *er erl2* display an enhancement of the *er* phenotype (Torii, Mitsukawa et al. 1996, Shpak, Berthiaume et al. 2004). The *er erl1 erl2* triple mutant displays the strongest phenotype with extreme

dwarfism, figure 1.2, which is believed to be caused by ERf receptors influencing the length of the cell cycle and thus cell proliferation (Shpak, Berthiaume et al. 2004, Bundy, Thompson et al. 2012). The most famous phenotype of the *er erl1 erl2* mutant is stomata clustering (Shpak, McAbee et al. 2005). Stomata, located on the epidermis, are pores composed of two cells which regulate gas exchange and transpiration (Han and Torii 2016). Under normal conditions, stomata only form interspersed between two pavement cells, never clustered together. The ERf receptors have been found to suppress the asymmetric cell divisions that lead to stomata (Han and Torii 2016). The stomata clustering phenotype of the *er erl1 erl2* mutant has been particularly useful because it is easy to identify and is unique to this pathway thus leading to the discovery of many other components in the ERf signaling pathway (Tameshige, Ikematsu et al. 2017). The *ERf* genes also play other regulatory roles in reproductive processes such as ovule development, early anther development, and floral organ identity that cause the *er erl1 erl2* triple mutant to be infertile (Pillitteri, Bemis et al. 2007, Hord, Suna et al. 2008, Bemis, Lee et al. 2013). Lastly, the most recently discovered regulatory role of the *ERf* genes is in the vegetative shoot apical meristem (SAM) where they regulate meristem size, leaf initiation, and phyllotaxy (Chen, Wilson et al. 2013, Uchida, Shimada et al. 2013, Tameshige, Okamoto et al. 2016).

Of all the developmental processes that ERf receptors regulate, their role in the SAM is least understood. Located at the top of the main stem, the SAM houses the stem cells fror *erecta* ıll aboveground organs will form, figure 1.3. The slowly dividing stem cells are confined in the central zone (CZ) and are displaced into surrounding zones, peripheral zone (PZ) or underlying rib zone (RZ), where they will differentiate into organs such as leaves, flowers or the stem (Poethig 1987). Stem cell homeostasis and differentiation must be concurrently regulated to prevent depletion of stem cells that would terminate the SAM or an over proliferation of cells that would alter the architecture of the plant. The vegetative SAM of the *er erl1 erl2* mutant is greatly increased in width, roughly

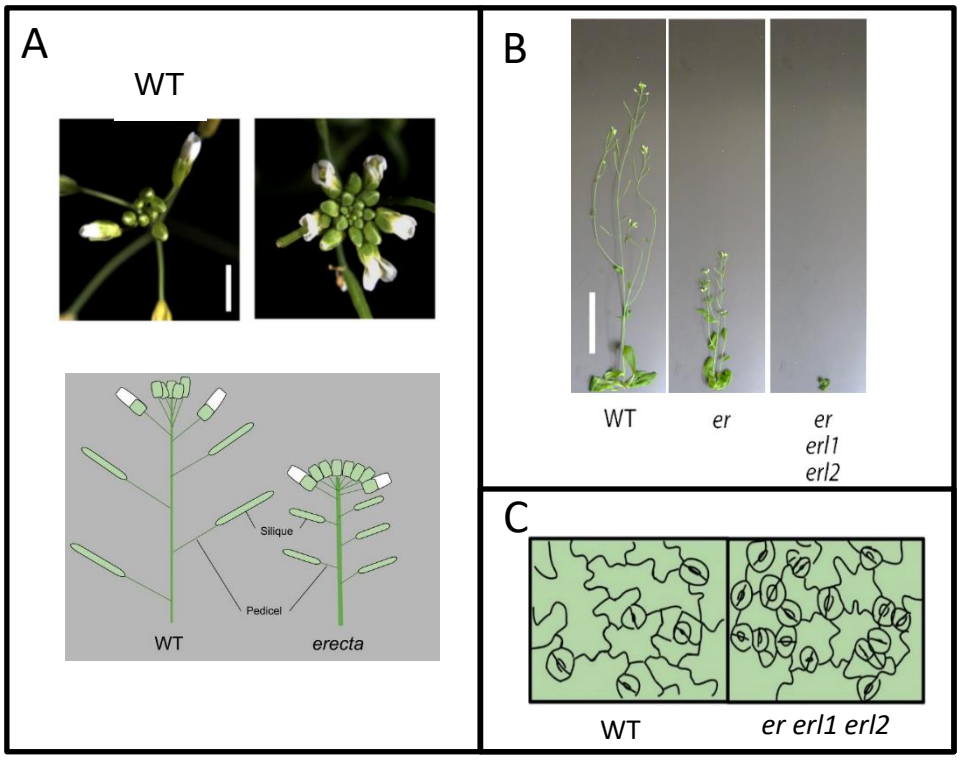

Figure 1.2. Major phenotypes of *erecta* and *erecta* family mutants. (A) Compact inflorescence architecture of erecta single mutant and cartoon cross section depiction. Short pedicels of erecta cause the floral buds to cluster closer together. (B) Plant height phenotype of *erecta* and *erf* mutants, picture taken at 30 days post germination. (C) Stomata clustering phenotype of erf mutant, in WT stomata are interspersed in between puzzle shaped pavement cells, in *erf* mutant stomata form in abnormal clusters, adapted from (Shpak, McAbee et al. 2005).

twice as large as the SAM in wildtype (Chen, Wilson et al. 2013, Uchida, Shimada et al. 2013). Increased SAM size can be caused by a number of different reasons; one possibility is that stem cells in the CZ are dividing too rapidly causing the CZ to swell in size (Reddy and Meyerowitz 2005). Another possibility for SAM enlargement is that cells in the PZ are not differentiating and exiting the PZ fast enough, causing a buildup of stem cells in the CZ (Reddy and Meyerowitz 2005). One group has presented evidence that the ERf receptors regulate stem cell homeostasis by modulating cytokinin signaling (Uchida, Shimada et al. 2013). The plant hormone cytokinin is known to regulate stem cell

homeostasis in the SAM by influencing the WUSHEL-CLAVATAs (WUS-CLV3) signaling pathway (Leibfried, To et al. 2005, Gordon, Chickarmane et al. 2009, Chickarmane, Gordon et al. 2012). The SAM of *er erl1 erl2* mutant is more sensitive to cytokinin and there do exist genetic interactions between the *ERf* genes and both *WUS* and *CLV3* but these interactions fail to address the leaf initiation phenotype or explain how intercellular communication is incorporated into this mechanism.

Reduced organ formation and abnormal patterning is another phenotype of the *er erl1 erl2* mutant (Chen, Wilson et al. 2013). To ensure efficient capture of sunlight and to accommodate for environmental conditions herbivory, plants regulate the rate of production and patterning of leaves in the PZ of the SAM (Reinhardt, Pesce et al. 2003). Leaf initiation is controlled by the plant hormone auxin which is transported throughout the outermost layer of the SAM to form patterns of gradients and maxima that mark the site of leaf initiation (Bayer, Smith et al. 2009, Braybrook and Kuhlemeier 2010). The formation of the auxin patterns ensures a strict patterning of leaves around the stem known as phyllotaxy (Reinhardt, Pesce et al. 2003). In *er erl1 erl2* mutant leaf initiation is greatly reduced and the leaves that form do so in a disorganized manner rather than the consistent spiral patterning seen in wildtype (Chen, Wilson et al. 2013). The organ formation and patterning phenotypes of *er erl1 erl2* mutant are linked to the mislocalization of the polar auxin transport protein, PIN1 (Chen, Wilson et al. 2013).

ERf signal transduction pathway

The ligands of the ERf receptors are a family of small-secreted cysteine-rich peptides known as the EPIDERMAL PATTERNING FACTOR - LIKE (EPF/EPFL) family which function as agonists or antagonists (Richardson and Torii 2013). EPF1, the founding member of the family, was found in a screen designed to identify small signaling peptides that would affect stomata

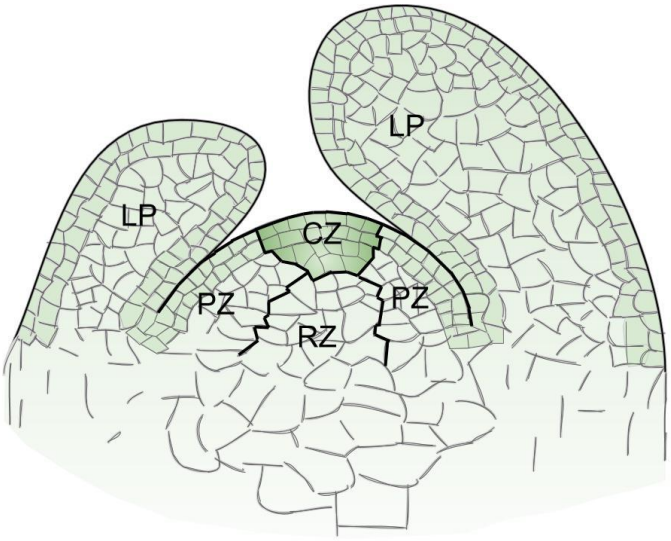

Figure 1.3 Cartoon depiction of the vegetative shoot apical meristem zones and emerging plant organs. LP, leaf primordia; CZ, central zone; PZ, peripheral zone; RZ, rib zone.

development (Hara, Kajita et al. 2007). When overexpressed using the 35S promoter *EPF1* decreased stomata density. Ten additional paralogues have been identified within the *Arabidopsis* genome (Hara, Kajita et al. 2007, Hara, Yokoo et al. 2009). *EPF2* was found to be another negative regulator of stomata and the phenotype is additive with *EPF1* (Hara, Yokoo et al. 2009, Hunt and Gray 2009). *EPF1* and *EPF2* have different roles in regulating stomata development; *EPF2* controls the initial asymmetric divisions whereas *EPF1* regulates the later asymmetric divisions. Both *EPF1* and *EPF2* were found to be expressed in stomata lineage cells but their temporal regulation is reflective of their function (Hara, Yokoo et al. 2009, Hunt and Gray 2009). EPFL9/STOMAGEN is an antagonist of the ERf receptors that positively regulates stomata density (Hunt, Bailey et al. 2010, Kondo, Kajita et al. 2010). Unlike *EPF1* and *EPF2*, *EPFL9/STOMAGEN* was found to be expressed in the mesophyll but functioned in the epidermis demonstrating how tissues such as the mesophyll could secrete peptides to regulate stomata development in the

epidermis (Sugano, Shimada et al. 2010). *EPFL4/CHALLAH-LIKE-2 (CLL2)* and *EPFL6/CHALLAH (CHAL)* have been shown to regulate the compact inflorescence phenotype of *erecta* by regulating vasculature development (Abrash, Davies et al. 2011, Uchida, Lee et al. 2012). Most recently, *EPFL2* was found to regulate a novel phenotype of *erecta*, which is the development leaf serrations (Tameshige, Okamoto et al. 2016, Tameshige, Okamoto et al. 2016). The leaf serration phenotype was found to be controlled by the plant hormone auxin which upregulated the *ERf* receptors and downregulated the expression of *EPFL2* (Tameshige, Okamoto et al. 2016). The EPF/EPFL ligands are expressed as propeptides that are cleaved to produce their bioactive form, the evidence for this was synthetic versions of EPFL9/STOMAGEN having different levels of activity depending on their form (Kondo, Kajita et al. 2010). The components of EPF/EPFL processing are largely unknown although one protease has been found to cleave the EPF2 propeptide (Engineer, Ghassemian et al. 2014). CO_2 RESPONSE SECRETED PROTEASE (CRSP) has been found to be upregulated by CO_2 and process EPF2 into its bioactive form thus allowing the plants to suppress stomata development in response to CO_2 (Engineer, Ghassemian et al. 2014).

ERf signal transduction is dependent upon the physical interactions of multiple proteins. *TOO MANY MOUTHS (TMM)* encodes a receptor like protein with an LRR extracellular domain and transmembrane domain but lacking the intercellular kinase domain therefore requiring a partner receptor to produce a signal (Yang and Sack 1995, Nadeau and Sack 2002). The *tmm* mutant forms stomata clusters and is insensitive to *EPF2,* which suggested it is part of the EPF2 sensing array (Hara, Yokoo et al. 2009). TMM was later shown to physically interact with the ERf proteins, and recent x-ray crystallography work has found that this interaction is a requirement for EPF1 and EPF2 to be able to bind with ERL1 (Lee, Kuroha et al. 2012, Lin, Zhang et al. 2017). From biochemical experiments we now know EPFL9/STOMAGEN competes against EPF1 and EPF2 for interactions with the TMM-ERf complex thus allowing plants

to fine tune stomata development (Lee, Hnilova et al. 2015, Lin, Zhang et al. 2017). NMR studies have revealed that different interactions comparing the positive and negative regulators of stomata are due to a variable loop region within the ligands, and swapping the loop of EPFL9/STOMAGEN onto a EPF2 scaffold swaps their function (Ohki, Takeuchi et al. 2011). EPFL4/CLL2 and EPFL6/CHAL also interact with ERf receptors but with no need for the TMM co-receptor (Lin, Zhang et al. 2017). Another family of receptors, known as the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family, act as co-receptors for ERf receptors, this interactions has been shown through co-immunoprecipitation (Meng, Chen et al. 2015). In vitro kinase assays demonstrated the ability of the receptors to transphosphorylate each other (Meng, Chen et al. 2015). Interestingly, *serk* family mutant produces the same compact inflorescence architecture as *erecta* and also produces stomata clusters which are insensitive to EPF1 and EPF2 (Meng, Chen et al. 2015). The association of ERf receptors with SERK receptors is of particular interest because SERK receptors are also known to act as co-receptors with a number of other signaling pathways such as brassinosteroid signaling, bacterial recognition, and phytosulfokine signaling (Li, Wen et al. 2002, Sun, Han et al. 2013, Sun, Li et al. 2013, Wang, Li et al. 2015). That association of so many different RLK receptors associating with each other might suggest they function as a multicomponent array of receptors allowing for the plant plasma membrane to function as a computer that modulates signals in a cell specific manner. Downstream of the ERf receptors and is a Mitogen-Activated-Kinase cascade made up of YODA, MAP KINASE KINASE 4/5 and MAP KINASE 3/6 (Bergmann, Lukowitz et al. 2004, Wang, Ngwenyama et al. 2007, Lampard, Lukowitz et al. 2009). For the EPF2-TMM-ER complex, the cascade leads to the destabilization of the transcription factor SPEECHLESS, however targets for other complexes or the initial cytoplasmic interactions remain unknown (MacAlister, Ohashi-Ito et al. 2007, Pillitteri, Sloan et al. 2007).

Introduction to chapters

The second chapter seeks to gain a better understanding of how the intracellular domains of ERECTA are regulated. Of all components involved in ERf signaling, the cytoplasmic kinase and its associated c-terminal tail and juxtamembrane domains are the least studied. We performed a structure function analysis to probe putative phosphorylation sites and regulatory regions in order to understand how they contribute to ERf signaling. Using site directed mutagenesis we produced altered versions of ERECTA and introduced them into the *erecta* single mutant and the *er erl1 erl2* triple mutant. By analyzing the phenotypes of transgenic plants we were able to dissect which regions of the cytoplasmic kinase domain have regulatory functions. The results in this chapter highlight the importance of ERECTA's ability to phosphorylate and the importance of the juxtamembrane domain for the receptor. Additionally, we found the C-terminal tail to be non-essential for signaling. We were able to analyze putative phosphorylation sites within the activation loop of the kinase and dissect their importance in regulating ERECTA signaling. Our data predicts threonine 807 to be an activation phosphorylation site and tyrosine 815 and 820 to be inhibitory phosphorylation sites. Lastly, we found nonfunctioning versions of ERECTA were still able to rescue some phenotypes of the *erf* mutant suggesting that there are multiple molecular mechanisms for ERf signaling to occur.

The third chapter further examines the role of ERf signaling in SAM development. Using heterologous promoters we were able to ectopically express *ERECTA* to confined regions of the vegetative SAM in the *er erl1 erl2* mutant to test which regions are affected by ERf signaling output. We found that CZ expression of ERECTA was best suited for rescuing the meristem width and leaf initiation phenotypes of the *er erl1 erl2* mutant. Using transcriptional reporters we examined expression patterns of the 11 ERf ligands to find the signaling components that control the *er erl1 erl2* SAM phenotype. Based on the expression patterns, we were able to generate mutant lines of the ERf ligands to

test their contributions to the phenotype. We found *EPFL1, EPFL2, EPFL4,* and *EPFL6* to be expressed in the meristem, and analysis of the quadruple mutant revealed that the four genes redundantly regulate the ERf receptors' role in the SAM. This lead to a model that EPFL ligands signal from the PZ to the ERf receptors in the CZ. This model was tested and supported with experiments using heterologous promoters to express *EPFL1* in different SAM regions.

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CHAPTER 2 IDENTIFICATION OF CRITICAL FUNCTIONAL RESIDUES OF RECEPTOR-LIKE KINASE ERECTA

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The dissertation writer performed the majority of the lab work and contributed to writing the manuscript.

Abstract

In plants, extracellular signals are primarily sensed by plasma membrane-localized receptor-like kinases (RLKs). ERECTA is a leucine-rich repeat RLK that together with its paralogs ERECTA-like 1 (ERL1) and ERL2 regulates multiple aspects of plant development. ERECTA forms complexes with a range of co-receptors and senses secreted cysteine-rich small proteins from the EPF/EPFL family. Currently the mechanism of the cytoplasmic domain activation and transmission of the signal by ERECTA is unclear. To gain a better understanding we performed a structure–function analysis by introducing altered ERECTA genes into erecta and erecta erl1 erl2 mutants. These experiments indicated that ERECTA's ability to phosphorylate is functionally significant, and that while the cytoplasmic juxtamembrane domain is important for ERECTA function, the C-terminal tail is not. An analysis of multiple putative phosphorylation sites identified four amino acids in the activation segment of the kinase domain as functionally important. Homology of those residues to functionally significant amino acids in multiple other plant RLKs emphasizes similarities in RLK function. Specifically, our data predicts Thr807 as a primary site of phosphor-activation and potential inhibitory phosphorylation of Tyr815 and Tyr820. In addition, our experiments suggest that there are differences in the molecular mechanism of ERECTA function during regulation of stomata development and in elongation of above-ground organs.

Introduction

Intercellular communications are essential for development of multicellular organisms where cell proliferation and differentiation must be cooperative and structured to attain a desired shape and function. Plants especially rely on intercellular communications as cell behavior is often position-dependent. To detect extracellular signals, plant cells have a large group of receptor-like kinases (RLKs). These receptors possess a structurally diverse extracellular ligand-sensing domain, a single-pass transmembrane domain, and a cytoplasmic serine/threonine/tyrosine kinase domain.

The ERECTA family (ERf) RLKs appeared early during land plant evolution and are involved in the regulation of multiple developmental processes (Villagarcia et al., 2012, Shpak, 2013). During embryogenesis they stimulate cotyledon elongation (Chen and Shpak, 2014). Post-embryonically, ERfs promote growth of all above-ground organs (Shpak et al., 2004). ERfs have been demonstrated to regulate stomata formation, the function of the shoot apical meristem (SAM), and the development of flowers (Shpak, 2013). In angiosperms, ERf consists of two or more genes, with Arabidopsis having three: ERECTA (ER), ERECTA-like 1 (ERL1) and ERECTA-like 2 (ERL2) (Shpak et al., 2004, Villagarcia et al., 2012). Although all three genes regulate above-ground organ elongation, they exhibit unequal redundancy. While erecta mutants have compact inflorescences due to shorter internodes and pedicels, single mutations in erl1 and erl2 confer no detectable phenotype (Torii et al., 1996; Shpak et al., 2004). Loss of all three genes leads to severe dwarfism (Shpak et al., 2004). The reduced growth of above-ground plant organs in ERf mutants is associated with a decrease in the cell proliferation rate (Shpak et al., 2003, 2004). An analysis of pedicel growth suggested that ERECTA accelerates the elongation of cells along the proximo-distal axis and shortens the duration of the cell cycle (Bundy et al., 2012). The asymmetric redundancy of ERf receptors is also evident during stomata development. In the initial stage of the stomata development process,

ERfs synergistically inhibit differentiation of protodermal cells into meristemoid mother cells. ERECTA plays a major role during this process, as an increased number of asymmetric cell divisions has been observed only in the erecta single mutant (Shpak et al., 2005). Once meristemoids are formed, ERL1 and ERL2 inhibit their differentiation into guard mother cells (Shpak et al., 2005). In the SAM, ERfs seem to be equally redundant; the receptors synergistically inhibit meristem enlargement, promote leaf initiation, and contribute to establishment of phyllotaxy (Chen et al., 2013; Uchida et al., 2013). Finally, ERfs play an important role in the regulation of ovule and early anther development (Pillitteri et al., 2007; Hord et al., 2008; Bemis et al., 2013). The er erl1 erl2 mutant is sterile with compromised male and female fertility (Shpak et al., 2004).

ERECTA, ERL1, and ERL2 receptors form homo- and heterodimers (Lee et al., 2012). They also make complexes with SOMATIC EMBRYOGENESIS RECEPTOR KINASEs (SERKs) and with the transmembrane receptor-like protein TOO MANY MOUTHS (TMM) (Lee et al., 2012; Meng et al., 2015). The activity of ERf receptors is regulated by a family of secreted cysteine-rich small proteins from the EPF/EPFL family, which can function as agonists or antagonists (Shimada et al., 2011). A MAP kinase cascade consisting of YODA, MKK4, MKK5, MPK3, and MPK6 functions downstream (Bergmann et al., 2004; Wang et al., 2007; Meng et al., 2012). Changes in the structure of receptor complexes upon ligand binding and the mechanism of signal transmission from ERfs to YODA are currently not clear.

ERf protein structure consists of an extracellular leucine-rich domain (LRR), a single-span transmembrane domain, and a cytoplasmic Ser/Thr kinase domain flanked by a juxtamembrane domain (JMD) and a C-terminal tail (Figure 2.1 A). Previously, it was shown that the cytoplasmic segment of ERECTA is functionally important as its deletion leads to a dominant negative phenotype (Shpak et al., 2003). To gain a better understanding of how ERECTA activates downstream signaling, we performed a structure–function analysis of this domain. These studies demonstrated that the cytoplasmic JMD is important for

ERECTA function, but the C-terminal tail is not. Our experiments further confirmed that ERECTA is a functional kinase and suggested that Thr807, Thr812, Tyr815, and Tyr820 in the activation segment of the kinase domain are functionally important. Based on our results, we hypothesize that phosphorylation of ERECTA at Thr812 might have a stimulatory effect on receptor activity, and at Tyr815 and Tyr820 an inhibitory effect. Our experiments also indicated that the molecular mechanism of ERECTA function is different during regulation of stomata development and in elongation of above-ground organs.

Materials and methods

Plant material and growth conditions

The Arabidopsis ecotype Columbia (Col) was used as the wild-type (WT). The er-105 and er-105 erl1-2 erl2-1 mutants have been described previously (Torii et al., 1996; Shpak et al., 2004). Plants were grown on a soil mixture of a 1:1 ratio of Promix PGX (Premier Horticulture Inc.) and Vermiculite (Pametto Vermiculite Co.) and were supplemented with Miracle-Gro (Scotts) and approximately 3.5mg cm–3 of Osmocoat 15-9-12 (Scotts). All plants were grown at 20 °C under long-day conditions (18 h light/6 h dark).

Generation of transgenic plants

In all plasmids except pPZK111 the substitutions/deletions were introduced into the genomic ERECTA-RLUC sequence by overlap extension PCR using pESH427 as a template (Karve et al., 2011). The amplified fragments were digested with PstI, inserted into pESH427, and sequenced. The constructs carry the endogenous ERECTA promoter and the 35S terminator. The pPZK111 was generated by overlap extension PCR using pKUT196 as a template (Godiard et al., 2003). The amplified fragment was digested with PstI, inserted into pKUT196, and sequenced. This construct carries the endogenous ERECTA promoter and terminator. The backbone of all plasmids is the vector pPZP222. The plasmids were introduced into Agrobacterium tumefaciens strain GV3101/ /pMP90 by

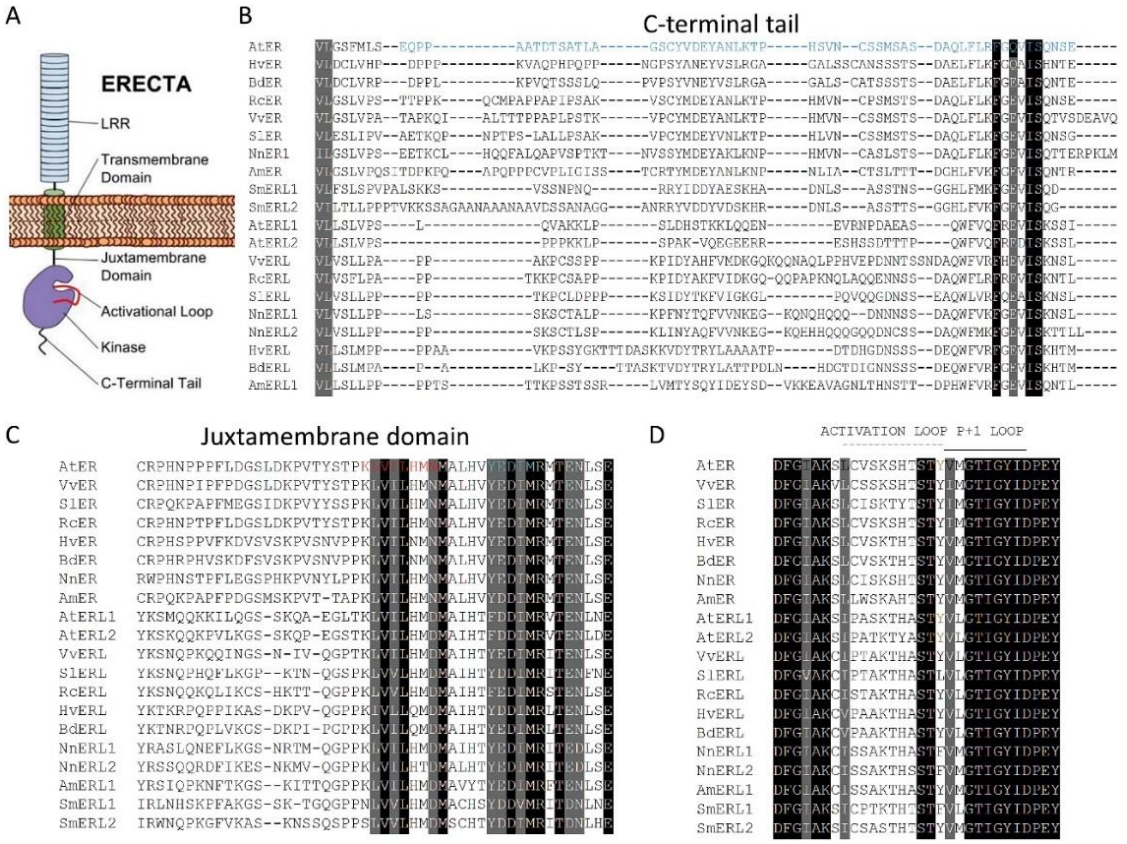

Figure 2.1 Comparison of amino acid sequences of ERECTA family proteins in different species. (A) Domain structure of the ERECTA receptor. (B–D) MAFFT alignment of the predicted amino acid sequences of *ERECTA* family genes from *Arabidopsis thaliana* (At), *Vitis vinifera* (Vv), *Solanum lycopersicum* (Sl), *Ricinus communis* (Rc), *Hordeum vulgare* (Hv), *Brachypodium distachyon* (Bd), *Nelumbo nucifera* (Nn), *Amborella trichopoda* (Am), and *Selaginella moellendorffii* (Sm). Residues that are identical among the sequences are shown with a black background, and those that are similar among the sequences are shown with a gray background. (B) The C-terminus. The blue residues have been deleted in pPZK110 and in pPZK111. (C) The juxtamembrane domain. The red residues have been deleted in pPZK104, the blue residues in pPZK105. Threonine in yellow has been substituted with Ala in pPZK102. (D) The activation loop. The predicted phosphorylation sites according to the Arabidopsis Protein Phosphorylation Site Database (PhosPht) are in yellow.

electroporation, and into Arabidopsis er-105 and er-105 erl1-2/+ erl2-1 plants by vacuum infiltration. The transgenic plants were selected based on gentamicin resistance and the number of rescued lines has been quantified based on general plant morphology (Tables 2.1 and 2.2, located in appendix). The er-105 erl1-2 erl2-1 mutants were selected based on kanamycin resistance and the homozygous status of the erl1-2 mutation was confirmed by PCR with the primers erl1g3659 (GAGCTTGGACATATAATC), erl1g4411.rc (CCGGAGAGATTGTTGAAGG), and JL202 (CATTTTATAATAACGCTGCGGACATCTAC). In addition, for transgenic lines transformed with pPZK102, pPZK110, and pPZK111 constructs, the homozygous status of the erl1-2 mutation was confirmed by analysis of kanamycin resistance in the progeny. The quantitative phenotypic analysis of er erl1 erl2 plants transformed with the described constructs has been done in T3 generation once their genetic status was established.

Measurement of Renilla luciferase activity

ERECTA-RLUC protein expression was measured by monitoring Renilla luciferase activity with a 20/20n single-tube luminometer in T1 inflorescences or in T2 8-d-old seedlings using the Renilla Luciferase Reporter Assay (Promega). The protein concentration in each sample was determined using the Bradford assay.

Analysis of mutant phenotypes

Measurements of stomata index and clustering were done on the abaxial side of cotyledons from 17-d-old seedlings using differential interference contrast (DIC) microscopy. For DIC, seedlings were incubated in a solution of 9:1 ethanol:acetic acid overnight, rehydrated with an ethanol series to 50% (v/v) ethanol, and then cleared in a mixture of 8:1:1 chloral hydrate:distilled water:glycerol.

Immunoblot analysis

The crude microsomal proteins were isolated from 11-d-old WT and T2 T807D seedlings (~0.4g per sample) using a method described by Zhang et al. (2011). The last step of this method, an enrichment for plasma membrane proteins, was omitted. Immunoblot analysis was performed as previously described with minor modifications (Shpak et al., 2003). Proteins were run on 8% or 10% SDS-PAGE. Primary anti-BAK1 polyclonal antibodies (Agrisera) were used at a dilution of 1:5000 followed by the secondary HRP Conjugated Goat Anti-Rabbit IgG antibody (Agrisera) at a dilution of 1:10 000. Primary anti-Rluc monoclonal antibodies (Millipore; clone 5B11.2) were used at a dilution of 1:5000 followed by the secondary HRP Conjugated Goat Anti-Mouse IgG antibody at a dilution of 1:7500. The detection of HRP was performed with a SuperSignal West Pico Rabbit IgG detection kit (Pierce).

Sequence alignment

Full-length amino acid sequences of ERECTA family proteins from different species were retrieved from the NCBI database and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results

The juxtamembrane domain (JMD) is important for ERECTA function, but the C-terminal tail is not

The activity of a RLK's kinase domain is often modulated by the flanking regions: the JMD and the C-terminal tail. In some receptors those regions inhibit kinase function, in others they are essential for the enzymatic activity (Wang et al., 2005b; Oh et al., 2009b, 2014). Phosphorylation of residues within these regions can often alter their function. For example, phosphorylation of Ser and Thr residues in the BRI1 C-terminal tail disables its inhibitory role (Wang et al., 2005b).

To examine whether regions flanking the ERECTA kinase domain have specific function, we created multiple constructs with modified genomic ERECTA sequences under the control of the native promoter (Figure 2.2). With the exception of one (pPZK111), all constructs contained Renilla Luciferase (RLUC) at the C-terminus of the receptor to monitor the level of protein expression. The luciferase assay is a fast, reliable, and relatively cheap method to measure protein levels. Most significantly, it reflects the protein concentration in Arabidopsis extracts (Ramos et al., 2001; Subramanian et al., 2006). Protein titration assays and immunoblot analysis confirmed that RLUC activity reflects the level of ERECTA-RLUC accumulation in transgenic seedlings (Figure. 2.12, located in chapter 2 appendix).

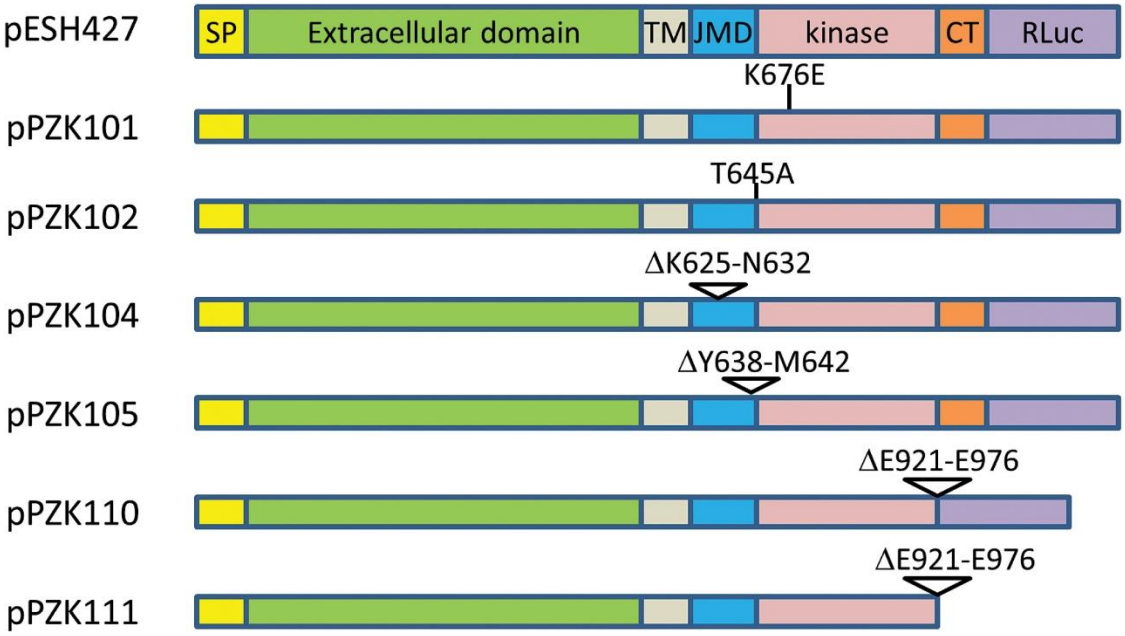

Figure 2.2 Schematic diagram of modifications introduced into the ERECTA protein. Triangles indicate deletions and lines indicate point mutations. SP, signal peptide; TM, transmembrane domain; JMD, juxtamembrane domain; CT, C-terminal tail; RLuc, *Renilla* Luciferase. In the constructs the genomic sequence of ERECTA is under the control of its native promoter and the 35S terminator. On the left are the names of the plasmids.

The unmodified ERECTA fused to RLUC (construct pESH 427) was used as a positive control. The constructs were transformed into *er-105* and into *er erl1/+ erl2* mutants and multiple independent transgenic lines were analyzed. Interestingly, we observed a decreased frequency of complementation in the T1 generation for constructs containing RLUC (Tables 2.1 and 2.2 appendix). In our earlier experiments, the genomic ERECTA (pKUT196) rescued 100% of transgenic *er-105* plants in the T1 generation (Karve *et al.*, 2011), while this time only 27% of T1 plants were rescued by ERECTA-RLUC (pESH427). Similarly, ΔE921-E976 ERECTA (pPZK111) rescued 58% of T1 *er-105* plants while ΔE921-E976 ERECTA-RLUC (pPZK110) rescued only 16% (Table 2.1 appendix). As a result, the frequency of complementation in the T1 generation has not been used as a measure of a construct functionality. Instead, for each construct we analyzed multiple T1 plants with the goal of finding three to four independent transgenic lines with relatively similar protein expression. While analysis of protein expression detected a variation in the amount of ERECTA produced in different transgenic lines, the general ability of a construct to rescue the ERECTA phenotype did not correlate with the level of protein expression in selected lines (Figure 2.3). For example, expression of ERECTA in non-complemented pPZK101, pPZK104, and pPZK105 transgenic lines is equal to or higher than that in complemented pPZK110 lines. Thus, we concluded that the inability of constructs to rescue *er-105*, *er erl2*, and *er erl1/+ erl2* mutants was due to modification of ERECTA structure and not to poor expression of the protein.

An analysis of ERf sequences from a broad variety of angiosperms suggests low conservation of the C-terminal tail except for a short stretch of amino acid residues at the very end (Figure 2.1B). Two constructs (pPZK110 and pPZK111) were created to examine the role of the C-terminal tail in ERECTA function. In both constructs the last 56 amino acids of ERECTA were deleted; in

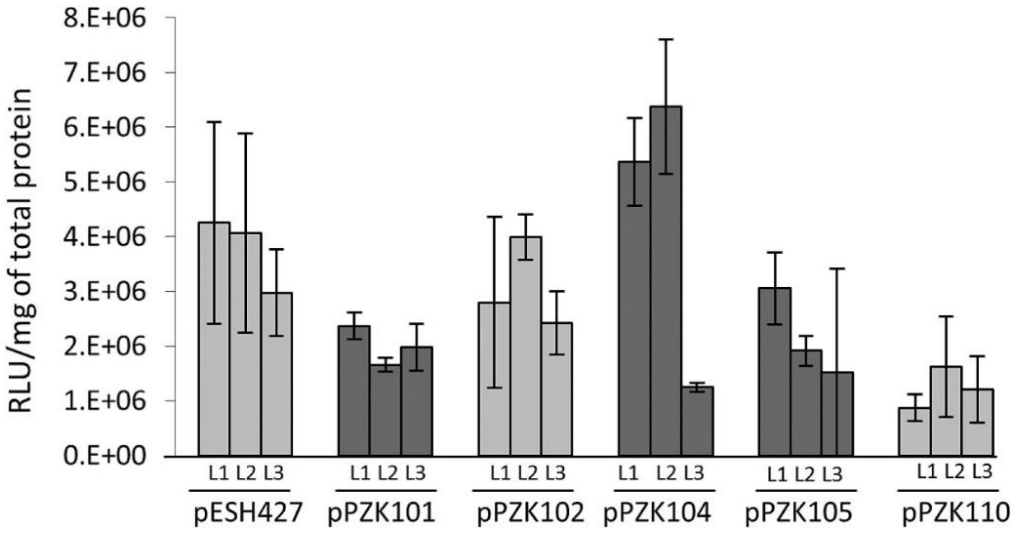

Figure 2.3 ERECTA-RLUC is expressed in the majority of transgenic lines. The level of ERECTA-RLUC expression was determined by measuring luciferase activity per milligram of total protein in inflorescences of T1 transgenic plants. RLU indicates relative light units. The mean of three biological replicates is plotted; error bars represent the SD. Three independent transgenic lines (L1–L3) were analyzed. The lines that rescue the *er-105* phenotype are in light grey and the lines that do not are in dark grey.

pPZK110, ERECTA was fused with RLUC and in pPZK111 it was not (Figure 2.2). We were concerned that RLUC at the C-terminus might interfere with receptor function and that its presence could conceal any possible increased activity of ERECTA without the C-terminus tail. However, both constructs rescued inflorescence structure and plant height of *er-105* and *er erl1/+ erl2*, similar to the positive control pESH427 (Figure 2.4; Figure 2.5A, B; Table 2.1). In addition, pPZK110 and pPZK111 fully rescued stomata development, plant height, and pedicel length phenotypes of the *er erl1 erl2* mutants (Figure. 2.5C–F). And while the stomatal index in the pPZK110 and pPZK111 *er erl1 erl2* lines was reduced below wild-type levels, it was not statistically significantly different from that in the pESH427 line and therefore this decrease cannot be due to the absence of the C-terminus tail (Figure. 2.5D). Thus, the ERECTA C-terminus seems to be dispensable for regulation of plant architecture and stomata formation.

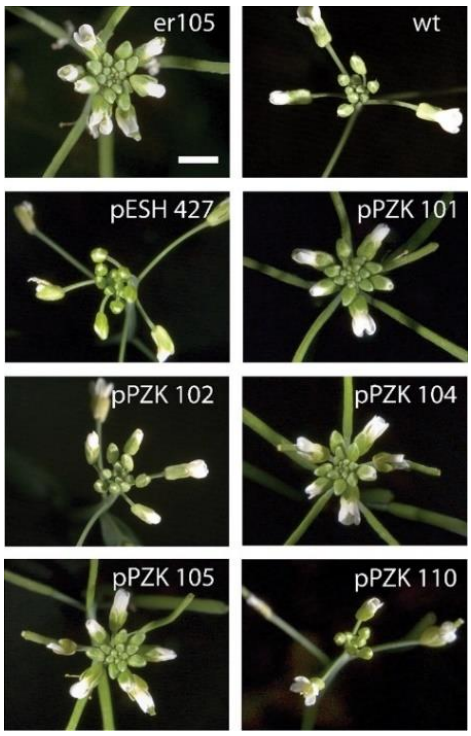

Figure 2.4 Inflorescence architecture reflects functionality of modified ERECTA receptors. Representative images of inflorescence apices of the wild-type (wt), er-105, and selected transgenic lines. All constructs were transformed into *er-105*. Scale bar =3 mm.

The JMD of the ERf receptors is 46–49 amino acids long. Comparison of this domain in different species revealed low conservation of the N-terminal half and high conservation of the C-terminal half (Figure. 2.1C). Several secondary structure prediction programs suggested the presence of a β-sheet and an α-helix in the conserved region of the JMD (Figure. 2.13, appendix). Two constructs were created: one with eight residues deleted in the region of a potential β-sheet (pPZK104), and another with five residues deleted in the region of a potential α-helix (pPZK105) (Figure. 2.2). While ERECTA containing these modifications was expressed, it did not rescue the elongation phenotype of above-ground organs in the *er-105* mutant (Figure. 2.3, Figure. 2.4). The deletions in the JMD also abolished the ability of ERECTA to inhibit stomata formation and to regulate stomata spacing (Figure. 2.6A, B).

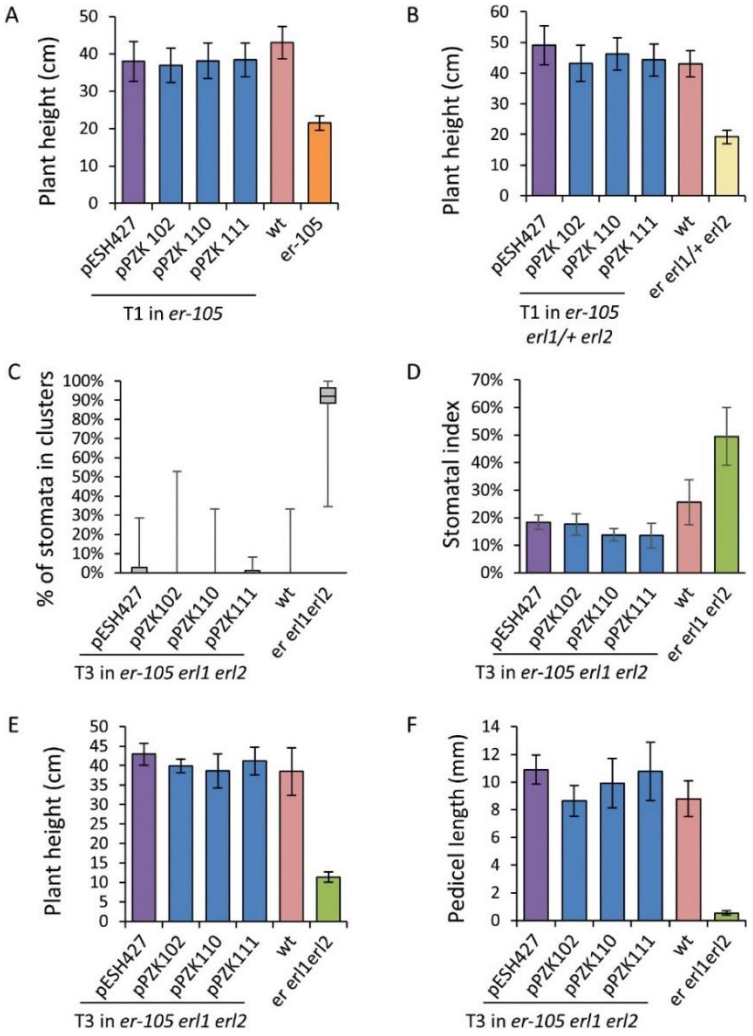

Figure 2.5 Deletion of the C-terminus domain (pPZK110 and pPZK111) or a point mutation in the JMD (T645A; pPZK 102) does not alter ERECTA's ability to regulate stomata development or above-ground organ elongation. (A, B) Height of mature plants (A, *n*=11–34; B, *n*=8–29). (C–F) Constructs were transformed into *er erl1/+erl2* mutants and transgenic *er erl1 erl2* plants were analyzed in the T3 generation. In (C) the median is indicated as a thick horizontal line, upper and lower quartiles are represented by the top and the bottom of the boxes, and the vertical lines designate the maximum and the minimum. Epidermal phenotypes were analyzed on the abaxial side of 17-d-old cotyledons (*n*=8–13). (E.) Height of mature plants (*n*=9–18). (F) Length of mature pedicels on the main stem (*n*=80; eight measurements per stem). In (A, B, D–F) values are means ± SD.

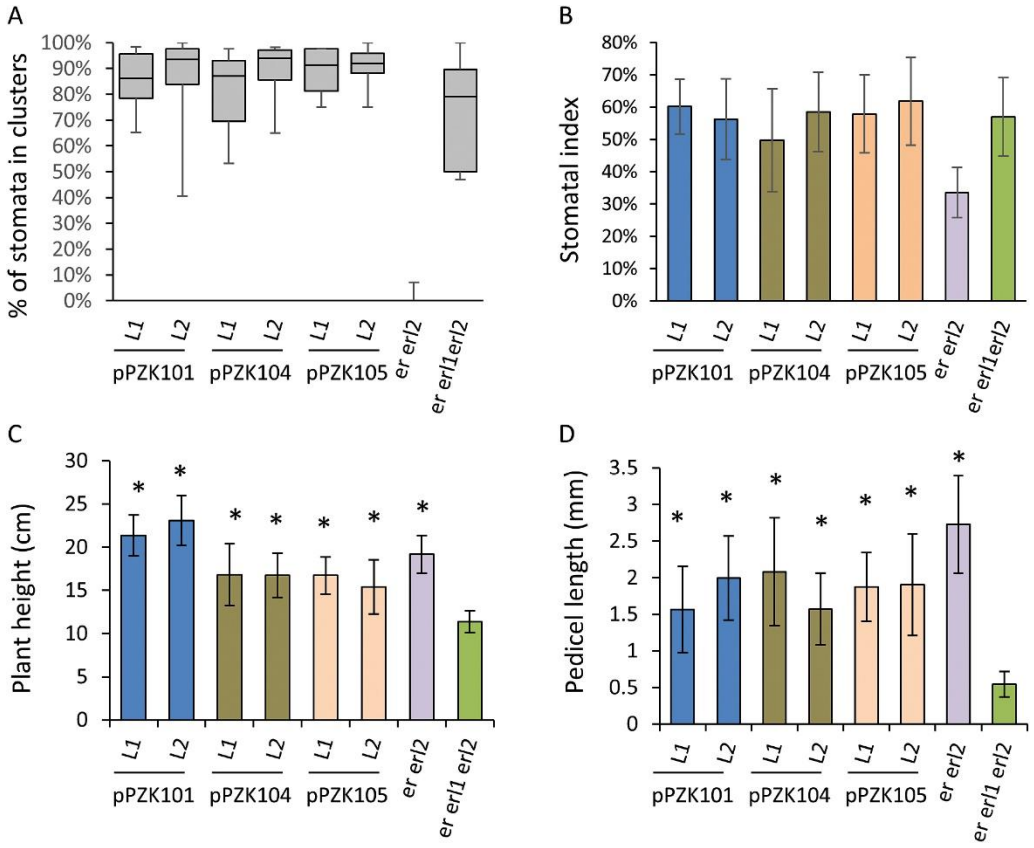

Figure 2.6 While substitution of the conserved lysine residue (K676E) in the ATP binding site of the ERECTA kinase domain (pPZK101) or deletion of short JMD segments (pPZK104 and pPZK105) disrupt ability of ERECTA to rescue stomatal phenotypes of *er erl1 erl2*, those constructs partially rescue elongation of above-ground organs. Constructs were transformed into *er erl1/+ erl2* mutants and two independent transgenic lines in the *er erl1 erl2* background were analyzed in the T3 generation. In (A) the median is indicated as a thick horizontal line, upper and lower quartiles are represented by the top and the bottom of the boxes, and the vertical lines designate the maximum and the minimum. (A, B) Epidermal phenotypes were analyzed on the abaxial side of 17-d-old cotyledons (n=8–16). (B–D) Values are means ± SD. (C) Height of mature plants (n=12–21). (D). Lengths of mature pedicels on the main stem (n=80; eight measurements per stem). (C, D) Values significantly different from *er erl1 erl2* (P<0.00001) are indicated by asterisks.

The phosphorylation and de-phosphorylation of JMD residues often regulates activity of RLKs, impinging on their enzymatic function or their ability to interact with downstream targets (Aifa et al., 2006; Heiss et al., 2006; Thiel and Carpenter, 2007; Chen et al., 2010). In Erf, the JMD contains only one conserved Thr (Thr645) and no conserved Ser or Tyr residues (Figure. 1C). This Thr is conserved not only in ERf but also in many other PELLE/RLK kinases (Figure. 2.14. appendix). In Pto and XA21, this Thr plays an important biological function and is essential for their autophosphorylation (Sessa et al., 2000a; Chen et al. 2010). The Arabidopsis thaliana phosphorylation site database PhosPhAt predicts phosphorylation of Thr645 (Durek et al., 2010). To test whether Thr645 is important for ERECTA function, this residue was substituted with Ala in the construct pPZK102 (Figure. 2.2). This substitution did not alter ERECTA functionality and the construct rescued organ elongation defects in er-105 (Figure. 2.4, Figure. 2.5A, Table 2.1), er-105 erl1/+ erl2 (Figure. 2.5B), and er-105 erl1 erl2 (Figure. 2.5E, F, Table 2.1). In addition, the pPZK102 construct fully rescued stomata formation defects in the *er erl1 erl2* mutant (Figure. 2.5C, D). These data suggest that Thr645 is not essential for ERECTA function. While our data suggest that the JMD is essential for ERECTA functionality, we were unable to identify critical phosphorylation sites in this region.

Importance of the kinase domain for ERECTA function

In vitro ERECTA is a weak kinase (Lease et al., 2001, Meng et al., 2015). The phenotypes of several mutants with substitutions and deletions in the ERECTA kinase domain suggest that the ability to phosphorylate might be important for ERECTA function (Lease et al., 2001). Alternatively, these mutations might lead to receptor instability or change ERECTA's capacity to bind co-receptors or downstream targets. The mutations are in the different α-helixes of the kinase domain and the exact function of those amino acids is not known. To further test whether the ability to phosphorylate is important for ERECTA function, a conserved lysine in the ATP-binding domain was replaced with a

glutamate (K676E; pPZK101; Figure. 2.2). We identified several pPZK101 transgenic lines in the er-105 background with sufficient expression of ERECTA (Figure. 2.3). However, organ elongation defects were not rescued in those lines (Figure. 2.4). The pPZK101 construct was also unable to rescue epidermal phenotypes in the er-105 erl1 erl2 mutant (Figure. 2.5C, D). Thus, ERECTA is likely an active kinase in vivo and its ability to phosphorylate has functional significance.

The PhosPhAt database predicts multiple phosphorylation sites in the kinase domains of ERECTA, ERL1, and ERL2. Based on these predictions and evolutionary conservation, two residues were selected for alanine substitutions preventing phosphorylation: T823, a residue at the end of λEF helix, and T906, a residue in the αI helix. T823 of ERECTA is homologous to T872 of the receptor-like kinase HAESA, a residue phosphorylated in vitro and contributing to enzymatic activity of HAESA in vitro (Taylor et al., 2016). However, these substitutions did not disrupt functionality of ERECTA (Figure. 2.15 appendix).

Next, we analyzed multiple Ser/Thr/Tyr in the activation segment by substituting them to Ala or as a phosphomimic to Asp. Alanine and aspartate substitution of Ser801, Ser803, Ser806, and Tyr808 in the activation loop did not have any effect on ERECTA function in control of organ elongation (Figure. 2.7). Substitutions of Thr812 to Ala and to Asp slightly, but statistically significantly, reduced functionality of ERECTA (Figure. 2.8). ERECTA with these substitutions was not able to fully rescue elongation defects of pedicels and stems when transformed into the er-105 mutant. These substitutions did not alter the expression level of ERECTA (Figure. 2.16 appendix). Interestingly, these two substitutions had a very similar negative impact on ERECTA function, and the phenotype of plants expressing T812A and T812D did not differ statistically. Therefore, phosphorylation of Thr812 is unlikely to play a major role in the activation of ERECTA.

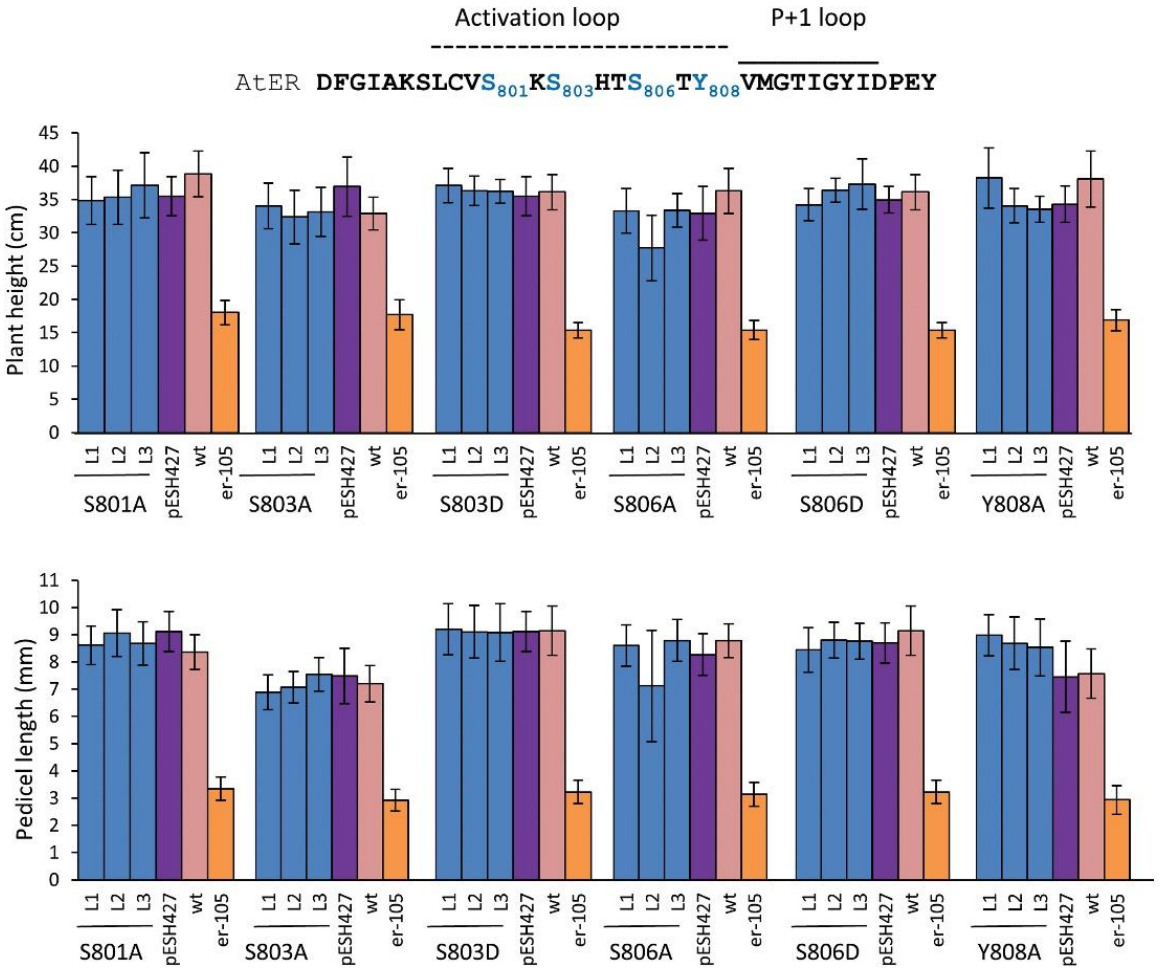

Figure 2.7 Site-directed mutagenesis of four potential phosphorylation sites in the activation loop of ERECTA suggests that these residues are not critical for ERECTA function. To determine ERECTA functionality, the constructs were transformed into *er-105*, and the height of mature plants (*n*=9–18) and the length of pedicels on the main stem (*n*=40; eight measurements per stem) were measured. Error bars represent ±SD. Three independent transgenic lines (L1–L3) were analyzed in the T2 generation. The mutated residues are in blue in the sequence at the top.

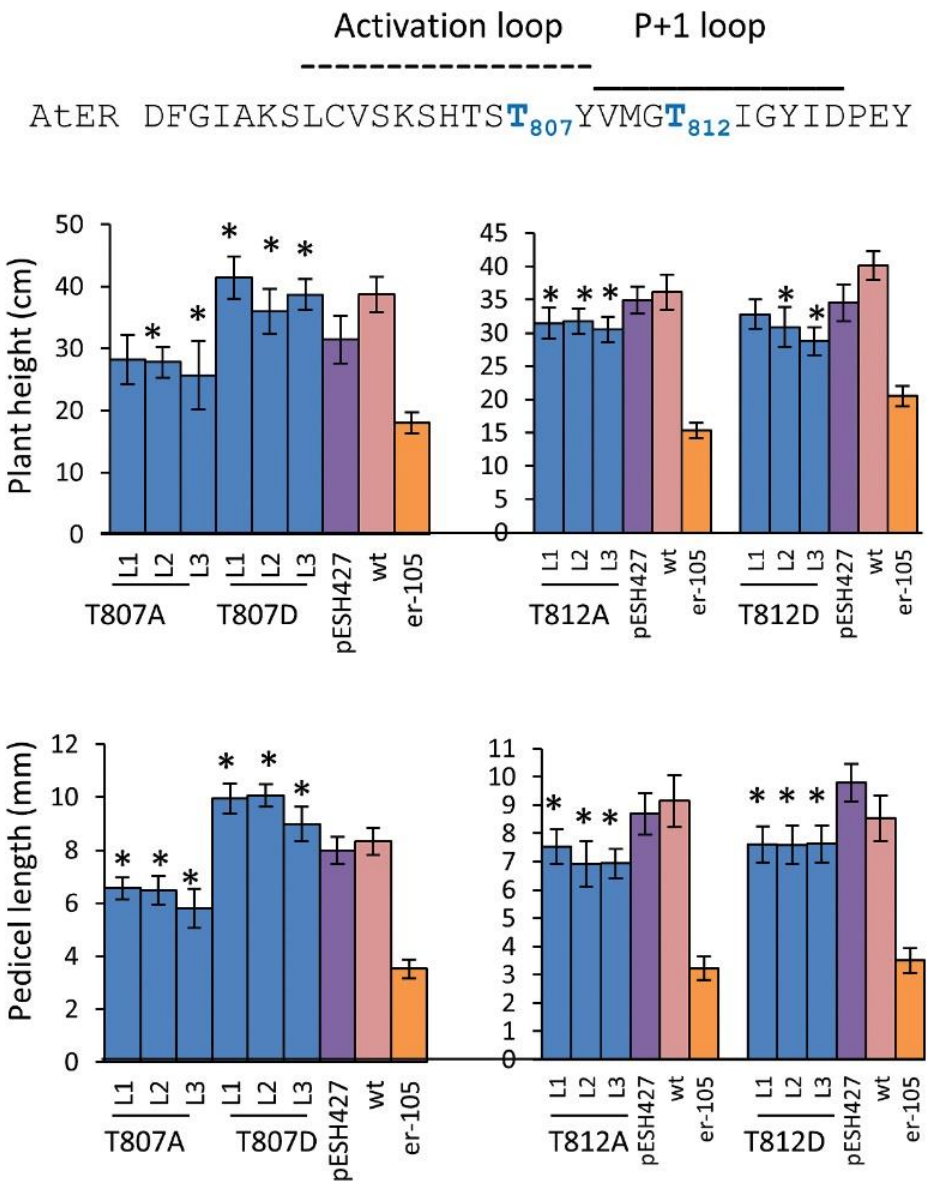

Figure 2.8 Site-directed mutagenesis of two conserved threonines in the activation segment impairs ERECTA function. To determine ERECTA functionality, the constructs were transformed into *er-105*, and the height of mature plants (*n*=9–18) and the length of pedicels on the main stem (*n*=40; eight measurements per stem) were measured. Error bars represent ±SD. Three independent transgenic lines (L1–L3) were analyzed in the T2 generation. The mutated residues are in blue in the sequence at the top. The transgenic line values significantly different from pESH427 (*P*<0.005) are indicated by asterisks.

Another residue that is important for ERECTA function is Thr807. The T807A substitution substantially reduced functionality of ERECTA, while organ elongation in plants expressing ERECTA with T807D substitution was similar to the wild-type or even greater (Figure. 2.8). Based on these data, we speculate that phosphorylation of Thr807 might have a positive impact on ERECTA function.

Finally, we observed that two Tyr substitutions had a very strong impact on plant growth. Both Y815A and Y820A strongly reduced ERECTA functionality, but were statistically different from er-105, suggesting that with those substitutions ERECTA retained a very low level of functionality (Figure. 2.9). Interestingly, substitutions of these Tyr to Asp resulted in a dominant negative phenotype (Figure. 2.9). Plants expressing ERECTA with Y815D or with Y820D were statistically shorter compared to er-105. This result suggests that these two Tyr are critical for ERECTA functionality and their phosphorylation might have a negative impact on ERECTA function.

Distinct signaling mechanisms of ERECTA in multiple developmental pathways

While deletions in the JMD (pPZK 104 and pPZK 105) or disruption of the kinase activity by the K676E substitution (pPZK 101) destroyed ERECTA's ability to regulate stomata development and fertility in er erl1 erl2 or to rescue er, er erl2, and er erl1/+ erl2 morphogenetic defects, these constructs were able to partially rescue stem and pedicel elongation in the er erl1 erl2 mutant (Figure. 2.6C, D; Figure. 2.10). In Figure. 2.6C, D the plant height and pedicel length are compared in fully mature plants. Because er erl1 erl2 grows slower and for a longer period of time, the plants are of different ages. If we compare plants of similar age as in Figure. 2.10, the ability of pPZK101, pPZK 104, and pPZK105 to partially rescue the er erl1 erl2 mutant becomes even more obvious. We speculate that in the er and er erl2 backgrounds the ability of pPZK101, pPZK 104, and pPZK 105 to alter organ elongation is not evident due to much stronger impact of ERL1 and ERL2 on plant growth. Taken together, these results suggest

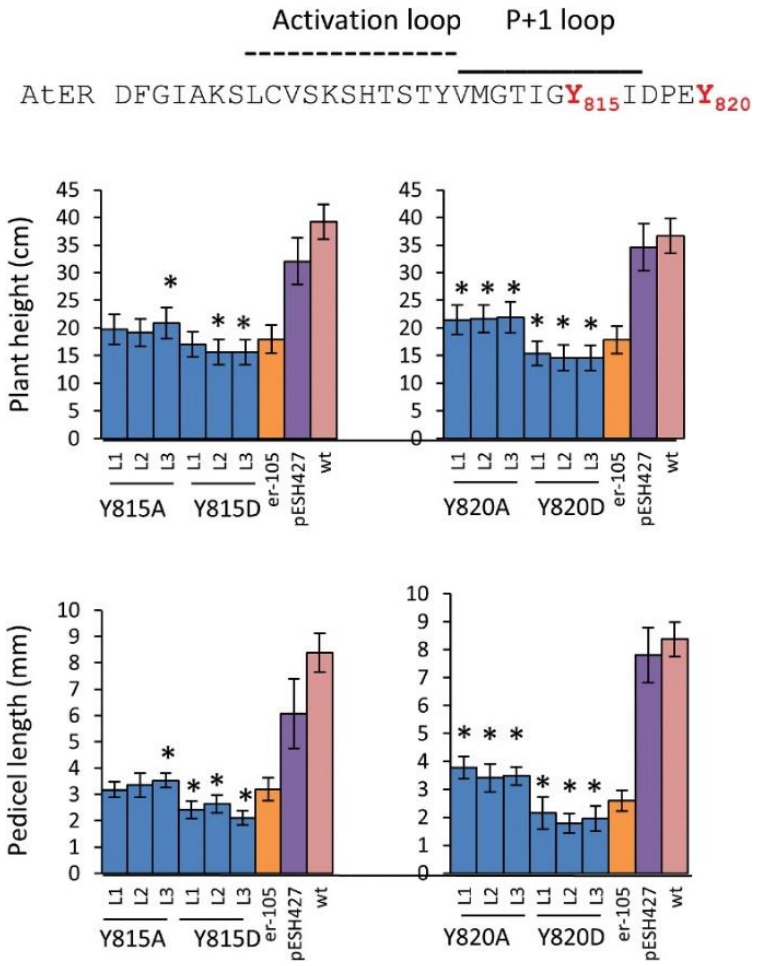

Figure 2.9 Site-directed mutagenesis of two conserved tyrosines in the activation segment of ERECTA suggests a negative role of their potential phosphorylation. To determine ERECTA functionality, the constructs were transformed into *er-105*, and the height of mature plants (*n*=9–18) and the length of pedicels on the main stem (*n*=40; eight measurements per stem) were measured. Error bars represent ±SD. Three independent transgenic lines (L1–L3) were analyzed in the T2 generation. The mutated residues are in red in the sequence at the top. The transgenic line values significantly different from *er-105*(*P*<0.005) are indicated by asterisks.

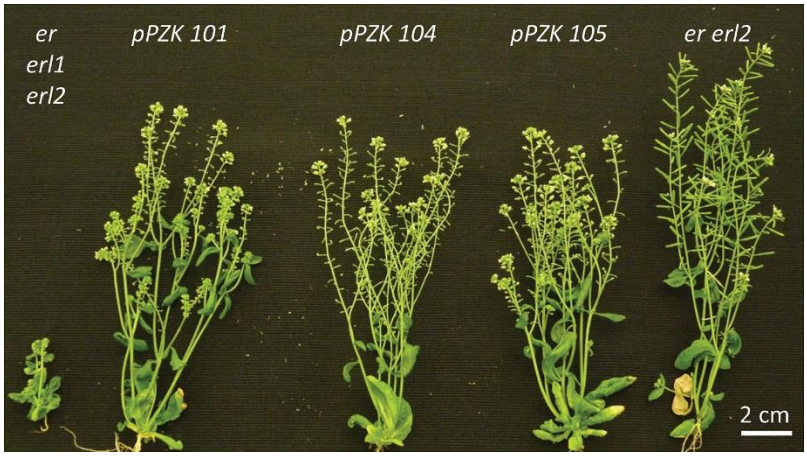

Figure 2.10 pPZK101, pPZK104, and pPZK105 constructs can partially rescue elongation of above-ground organs in the *er erl1 erl2* mutant. Representative 6-week-old plants from left to right: *er erl1 erl2*; T3 *pPZK101*, *pPZK104*, and *pPZK105* in *er erl1erl2* background; *er erl2*.

that the signal transduction by ERECTA is different in organ elongation versus control of stomata formation and development of flower organs.

Discussion

Considerable progress has been made recently in understanding the composition of receptor–ligand complexes formed by ERf in the plasma membrane (Lee et al. 2012, 2015). However, the mechanism of the cytoplasmic domain activation and transmission of the signal by ERf is still unclear. To explore the significance of ERECTA's kinase domain and the mechanism of its activation, we used a structure–function approach.

ERECTA is a RD kinase, which means that it has a conserved arginine (R) immediately preceding an aspartate (D) in the catalytic loop. Previous research has established that ERECTA is a weak kinase in vitro (Lease et al., 2001; Meng et al., 2015). Accordingly, we observed that ERECTA with substitution of the conserved lysine in the ATP-binding domain is unable to rescue the majority of developmental defects in mutants, and therefore the ability to phosphorylate is essential for ERECTA function. While inactive kinases adopt

a variety of distinct conformations, their activation often depends on a change in the structure of the activation segment, which in the RD kinases is the primary site of regulatory phosphorylation (Johnson et al., 1996). The activation segment is variable in length and sequence but it is restricted by highly conserved DGF and APE motifs, and in Ser/Thr kinases it almost always contains a characteristic GlyThr or GlySer dipeptide motif (Figure. 2.11). In Arabidopsis, more than 99% of RD LRR RLKs have a Thr or Ser in this motif (Wang et al., 2005a). In the active state, the hydroxyl group of the threonine or serine from the GlyThr/Ser motif forms hydrogen bonds with the catalytic aspartate of the HRD motif and the lysine one nucleotide behind this motif (Nolen et al., 2004). In IRAK4 and many other mammalian Ser/Thr kinases this Thr is important for the kinase activity, but is not a major phosphorylation site (Wang et al., 2009; Bayliss et al., 2012). Alanine or phosphomimetic substitutions of homologous Thr/Ser in plant kinases such as SERK1 (T468A, T468E), BRI1 (T1049A), SYMRK (T760A), BAK1 (T455A, T455D, T455E), BIK1 (T242A), ACR4 (T681A, T681D), HAESA (S861A), and PSKR1 (T899A) lead to loss of kinase function (Shah et al., 2001; Wang et al., 2005a, 2008; Yoshida and Parniske, 2005; Laluk et al., 2011; Meyer et al., 2011; Taylor et al., 2013; Hartmann et al., 2015). In BRI1, FLS2 (T1040A), BAK1, BIK1, and HAESA these substitutions were shown to decrease functionality of receptors in planta (Wang et al., 2005a, 2008; Robatzek et al., 2006; Laluk et al., 2011; Taylor et al., 2016). While our work demonstrates that both alanine or phosphomimetic substitutions of Thr812 alter functionality of ERECTA, the effect is surprisingly small. In this respect ERECTA resembles the RD receptor-like kinases PSKR1 and FERONIA where substitution of homologous S701 and T899, respectively, does not disrupt receptor function (Hartmann et al., 2015; Kessler et al., 2015).

Thr/Ser residues preceding the GlyThr motif are the primary phosphorylation sites that are essential for the activation of mammalian Ser/Thr kinases (Nolen et al., 2004; Bayliss et al., 2012). For example, the kinase activity of IRAK4 is regulated by the autophosphorylation of three sites, Thr342, Thr345,

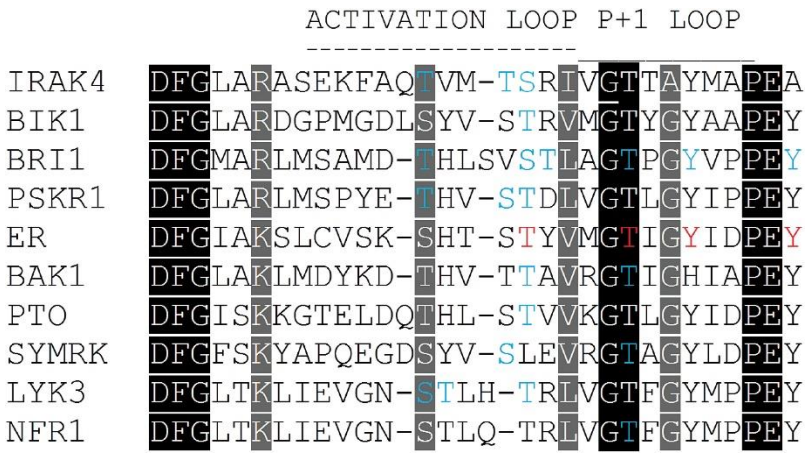

Figure 2.11 Alignment of the activation segments from plant receptor-like kinases ER, BAK1, LYK3, NFR1, BRI1, and PSKR1, plant kinases BIK1 and PTO, and human kinase IRAK4. Functionally significant amino acids are in red. Amino acids that are phosphorylated (either *in vitro* or *in vivo*) and functionally significant *in planta* or essential for the enzymatic activity are in blue. Residues that are identical among the sequences are given a black background, and those that are similar among the sequences are given a gray background.

and Ser346, located in front of the GlyThr motif (Cheng et al., 2007). Based on structural studies, phosphorylation of Thr345 is responsible for the activation of IRAK4 kinase while phosphorylation of Thr342 and Ser346 might stabilize the activation loop in the active state (Kuglstatter et al., 2007). Current literature suggests that residues homologous to Thr345 or Ser346 in IRAK4 are likely to be the primary phosphorylation sites in plant Ser/Thr kinases. Thus, Thr237 of BIK1 is necessary for full kinase activity and is a major phosphorylation site in response to flg22 (Lu et al., 2010; Laluk et al., 2011). In LYK3, T475A substitution leads to decreased kinase activity (Klaus-Heisen et al., 2011). The equivalent Thr233 in Pti1 is the major site of autophosphorylation and phosphorylation by Pto kinase (Sessa et al., 2000b). In BAK1, Thr450 is phosphorylated and T450A substitution reduces functionality of the receptor (Wang et al., 2008). Crystal structure confirmed the significance of T450 phosphorylation for enzymatic activity of BAK1 (Yan et al., 2012). Ser1044 is phosphorylated in BRI1 and, out of all Ser/Thr to Ala substitutions in the

activation segment, the S1044A substitution has the most severe negative impact on BRI1 functionality (Wang et al., 2005a, 2016). S856 in the activation segment of HAESA is a phosphorylation site that positively regulates kinase activity and contributes to the functionality of the receptor (Taylor et al., 2016). Thr807 of ERECTA is homologous to Ser346 of IRAK4 (Figure. 2.11). Therefore, it is not surprising that out of all Ser/Thr substitutions in the activation segment of ERECTA only T807A substitution significantly reduces receptor functionality, and ERECTA with a T807D substitution is fully functional. Based upon this data, we speculate that Thr807 is the primary phosphorylation site in the activation segment of ERECTA. Future structural and biochemical studies will be essential to confirm this hypothesis. In general, our findings resemble those obtained for HAESA where substitutions of only two Ser/The residues in the activation segment disrupt receptor function (Taylor et al., 2016). Those residues are homologous to Thr807 and Thr812 of ERECTA, although in ERECTA the substitution of T812 has a weaker effect on the receptor function.

Plant receptor-like kinases have dual specificity, phosphorylating both Ser/Thr and Tyr residues. Two tyrosine residues in the BRI1 P+1 loop, Tyr1052 and Tyr1057, have been shown in vivo to play an important role in BR signaling and their phosphorylation is predicted to have a negative impact on the kinase activity (Oh et al., 2009a, 2009b). Residues homologous to Tyr1057 of BRI1 have been shown to be critical for the function of other receptor-like kinases. Thus, Tyr463 of BAK1 is essential to its catalytic activity (Oh et al., 2010). Tyr250 in the activation segment of BIK1 can be autophosphorylated or transphosphorylated by BAK1 and is important for BIK1 function in plant defenses (Lin et al., 2014). Consistent with this, we observed that substitutions of homologous Tyr residues in the P+1 loop of ERECTA, Tyr815 and Tyr820, drastically reduced functionality of the receptor. As substitutions to Ala were less severe than substitutions to Asp, we hypothesize that phosphorylation of those residues could lead to inhibition of ERECTA function.

Phosphorylation events in the JMD and the C-terminal regions often alter activity of receptor-like kinases (Wang et al., 2005b; Oh et al. 2009b, 2014). However, this might not be the case for ERECTA: no changes in ERECTA functionality were observed after the deletion of the C-terminus. Thr645 is the only conserved Thr/Ser/Tyr in the JMD, yet its substitution had no effect on ERECTA function. The receptor-like kinase BAK1 associates with multiple receptor-like kinases including ERECTA family receptors (Meng et al., 2015). When BAK1 associates with BRI1 it increases its activity by phosphorylating the JMD and the C-terminus (Wang et al., 2008). The role of BAK1 during interaction with ERECTA is likely to be different as phosphorylation of ERECTA kinase's flanking regions is not likely to be significant for its function.

A majority of the RLK/Pelle kinases have an N-terminal extension in front of the N-terminal lobe of the kinase domain (Lei et al., 2005; Wang et al., 2006). The N-terminal extension is often an integral part of the overall fold of kinase and is essential for its activity. For example, the N-terminal extension is required for BRI1 enzymatic activity (Oh et al., 2012). While there is no sequence similarity between the N-terminal extensions of various kinases, there is some similarity of structure. The crystal structure of IRAK4 revealed a short β strand and an α-helix in the N-terminal extension region while those of BRI1 and BAK1 suggested the existence of an α-helix (Wang et al., 2006; Yan et al., 2012; Bojar et al., 2014). Homology modeling of LYK3 predicted an α helix in the N-terminal extension region (Klaus-Heisen et al., 2011). Four different programs (JPRED 4, NETSURFP, PSIPRED, and I-TASSER) predicted the existence of a short β strand and an α-helix in the N-terminal extension region of ERECTA. While we were unable to identify functionally significant putative phosphorylation sites in the JMD, our work determined that this domain is significant for ERECTA function. Two deletions in that region led to a functionally inactive receptor. This may be due to disruption of the N-terminal extension structure and, as a result, inactivation of ERECTA's enzymatic function.

Our structure–function analysis indicates that ERECTA function differs in the specific developmental processes in which it participates. The kinase function is absolutely essential for ERECTA's ability to regulate stomata formation and flower structure. Simultaneously, the kinase-dead ERECTA is able to partially rescue stem and pedicel elongation defects in the *er erl1 erl2* background. These results suggest that there are distinct signaling requirements for ERECTA in different developmental processes and imply that ERECTA might transmit the signal to downstream targets in different ways. The receptor-like kinases BAK1 and SCRAMBLED have also been shown to control multiple pathways using distinct signaling mechanisms with different requirements for their kinase domain function (Oh et al., 2010; Kwak et al., 2014). In addition, we observed that kinase-dead ERECTA and ERECTA without the cytoplasmic domain (Δkinase) function very differently. The Δkinase ERECTA confers dominant negative effects, probably titrating positive regulators of the signaling pathway through the extracellular domain (Shpak et al., 2003). The kinase-dead ERECTA is partially functional in regulation of organ elongation, which hypothetically could occur through titration of negative regulators by the kinase domain.

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Appendix

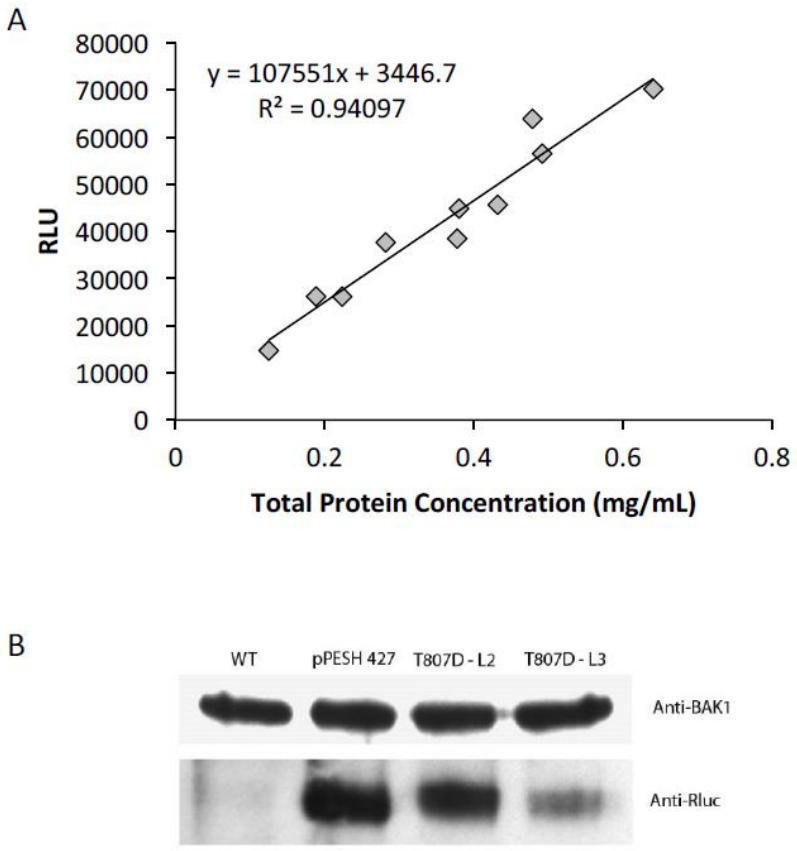

Figure 2.12 Both the luciferase titration curve and the immunoblot analysis confirm that the Renilla luciferase assay is a reliable method to test accumulation of RLUC-fused proteins in extracts from Arabidopsis seedlings. A. The luminescence signal increases linearly with increasing concentration of total protein in extracts from Arabidopsis seedlings expressing ERECTA-RLUC (pESH427). B. Immunoblot analysis of protein extracts from wild type seedlings and seedlings expressing ERECTA-RLUC (pESH427) and two independent lines expressing ERECTA-RLUC with T807D substitution. Accumulation of ERECTA-RLUC is consistent with the results obtained by the RLUC assay (Figure 2.S5). The immunoblot probed with anti-BAK1 antibody reflects uniform loading of microsomal proteins.

```
CRPHNPPPFLDGSLDKPVTYSTPKLVILHMNMALHVYEDIMRMTENLSE    JMD sequence

------------------------EEEEE----HHHHHHHHHHHH----    JPRED
----------------------HHHHHHHHHHHHHHHHHHHHHHHH---    SCRATCH
------------------------EEEEEE--HHHHHHHHHHHHHH---    NETSURFP
-----------------------EEEEEE---HHHHHHHHHHHHH----    PSIPRED
-----------------------EEEEEE---HHHHHHHHHHHHHH---    I-TASSER
```

Figure 2.13 The secondary structure of the 49 a.a. long ERECTA JMD as predicted by five different programs: JPRED 4, SCRATCH, NETSURFP, PSIPRED and I-TASSER. H represents α-Helix, E represents extended strand and a hyphen indicates no prediction.

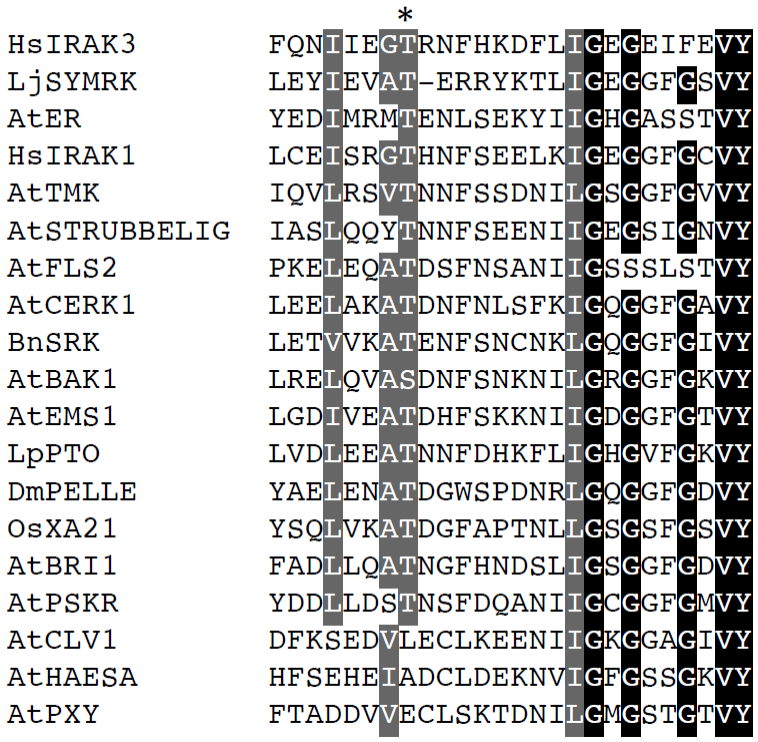

Figure 2.14 Comparison of amino acid sequences at the N-terminus of the kinase domain in the proteins belonging to the PELLE/RLK family of kinases. Residues that are identical among the sequences are given a black background, and those that are similar among the sequences are given a gray background. The Thr residues analogous to Thr645 of ERECTA are labeled with an asterisk.

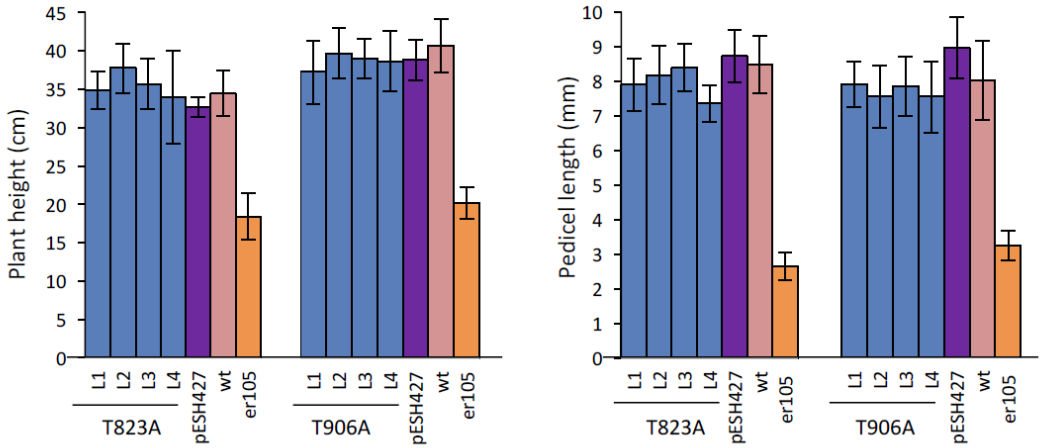

Figure 2.15 Site directed mutagenesis of two potential phosphorylation sites in the kinase domain of ERECTA suggests that these residues are not critical for ERECTA function. Two amino acids are predicted to be phosphorylated according to the Arabidopsis Protein Phosphorylation Site Database (PhosPht) and are conserved in ERECTA, ERL1, and ERL2. To determine ERECTA functionality the constructs were transformed into *er-105,* and the height of mature plants (n=9-18) and the length of pedicels on the main stem (n=40; eight measurements per stem) were measured. Error bars represent one SD. Four independent transgenic lines (L1-L4) were analyzed in the T2 generation.

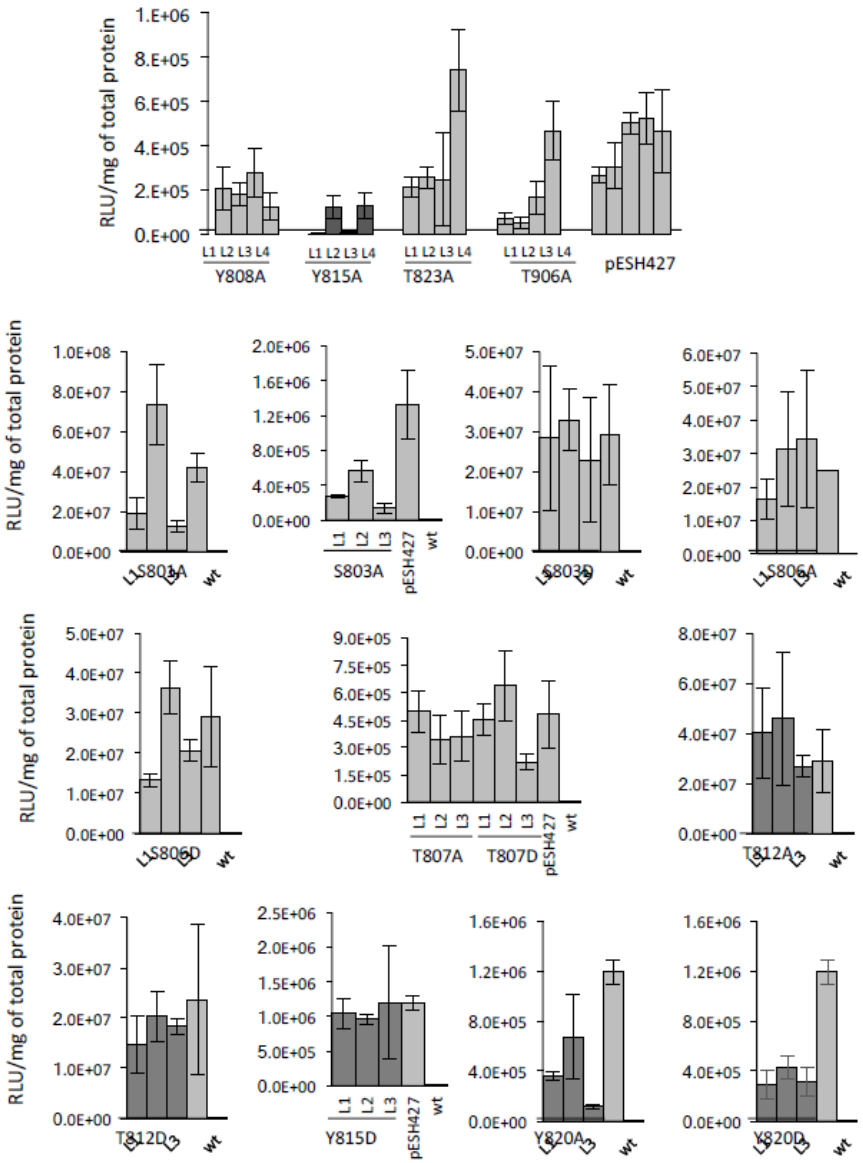

Figure 2.16 ERECTA-RLUC is expressed in the majority of transgenic lines. The level of ERECTA-RLUC expression was determined by measuring luciferase activity per milligram of total protein in 8 day old T2 seedlings. RLU indicates relative light units. The mean of three biological replicates is plotted; error bars represent one SD. Three to four independent transgenic lines (L1-L4) were analyzed. In light grey are lines that rescue the er-105 phenotype and in dark grey are lines that do not. WT indicates background RLU/mg of total protein.

Table 2.1 Ability of constructs to rescue er and er erl1 erl2 mutant phenotypes. Complementation of er and er erl1/+ erl2 is based on visual observation of plant height and pedicel length.

Name of the construct	Mutation	# of fully or partially complemented *er* plants/ total T1 plants analyzed	# of fully or partially complemented *er erl1/+ erl2* plants/ total T1 plants analyzed
pESH 427	none	13/48	30/44
pPZK 101	K676E	0/38	0/74
pPZK 102	T645A	13/50	25/45
pPZK 104	ΔK625-N632	0/27	0/22
pPZK 105	ΔY638-M642	0/27	0/24
pPZK 110	ΔE921-E976	7/43	24/33
pPZK 111	ΔE921-E976	36/62	20/24

Table 2.2 Ability of constructs to rescue *er* mutant phenotypes. Complementation of *er* is based on visual observation of plant height and pedicel length

Name of the construct	Mutation	# of complemented plants/ total T1 plants
pESH601	S801A	11/36
pESH602	S803A	5/36
pESH603	S803D	10/18
pESH604	S806A	6/24
pESH605	S806D	6/27
pPZK121	T807A	4/22
pPZK606	T807D	7/23
pESH607	T812A	10/54
pESH608	T812D	13/85
pPZP122	Y808A	8/17
pPZK123	Y815A	0/20
pESH609	Y815D	0/27
pESH610	Y820A	0/35
pESH611	Y820D	0/27
pPZK124	T823A	6/22
pPZK125	T906A	11/21

CHAPTER 3 CELL-CELL COMMUNICATIONS BETWEEN THE BOUNDARY REGION AND THE CENTRAL ZONE OF THE SHOOT APICAL MERISTEM ENABLED BY EPFL LIGANDS AND ERECTA FAMILY RECEPTORS REGULATE MERISTEM STRUCTURE AND LEAF INITIATION.

Chapter 3 represents unpublished work by Pawel Z. Kosentka, Alexander Overholt, Richard Maradiaga, Omar Mitoubsi, and Elena D. Shpak

The dissertation writer performed the majority of the lab work and contributed to writing the manuscript.

Abstract

The shoot apical meristem (SAM) enables the formation of new organs throughout the life of a plant. ERECTA family (ERf) receptors restrict SAM size and promote initiation of leaves while simultaneously supporting establishment of correct phyllotaxy. In the epidermis and during organ elongation ERf activity is regulated by a family of Epidermal Patterning Factor-Like (EPFL) secreted cysteine-rich small proteins. Here we show that ERfs play a critical role in communication between the SAM leaf boundary and the central zone. Ectopic expression of *ERECTA* in the central zone using *CLAVATA3* promoter is sufficient to restrict meristem size and promote leaf initiation. Genetic analysis demonstrated that four putative ligands: *EPFL1, EPFL2, EPFL4,* and *EPFL6* function redundantly in the SAM. These genes are expressed at the SAM-leaf boundary and in the peripheral zone. Previously *EPFL4* and *EPFL6* have been linked with elongation of aboveground organs. Here we demonstrate that *EPFL1* and *EPFL2* promote organ elongation as well. In addition, we show that expression of *ERECTA* in the central zone of the SAM has a strong impact on elongation of internodes and pedicels and growth of leaves. These results suggest that ERfs can stimulate organ growth cell non-autonomously.

Introduction

Cell-to-cell communications coordinate numerous processes during plant development. As message carriers plant cells use both small organic molecules and peptides. Plasma membrane localized receptor-like kinases sense the majority of peptides and some organic molecules and then activate appropriate developmental programs. The ability of a receptor to sense multiple signals and the variety of responses a signal may trigger enable the complexity and plasticity of developmental programs.

The ERECTA family (ERf) signaling pathway was initially linked to aboveground organ elongation (Torii et al. 1996). Since then it has become clear that ERf receptors also regulate numerous other developmental processes such as stomata formation, leaf initiation, shoot apical meristem (SAM) structure, and flower differentiation (Shpak, 2013). In *Arabidopsi*s the family consist of three genes: *ERECTA, ERECTA-LIKE 1 (ERL1),* and *ERL2* (Shpak et al. 2004). The contribution of an individual receptor to the regulation of a particular developmental response varies. For example, ERECTA is the primary receptor regulating organ elongation while ERL1 plays a leading role in the regulation of stomata spacing. In the SAM these receptors function redundantly with single and double mutants exhibiting extremely weak or no phenotypes (Chen et al. 2013). The activity of ERf receptors is regulated by a family of eleven secreted cysteine-rich small proteins from the EPF/EPFL family (Shimada et al. 2011). Three proteins, EPF1, EPF2, and STOMAGEN (EPFL9) regulate stomata development (Hara et al. 2007; Hara et al. 2009; Hunt et al. 2009; Hunt et al. 2010; Sugano et al. 2010). Based on the phenotypes of mutants and on the fact that EPF2 is able to induce phosphorylation of downstream signaling components, EPF1 and EPF2 are thought to activate the receptors (Hara et al. 2007; Hara et al. 2009; Hunt et al. 2009; Lee et al. 2015). STOMAGEN competes with EPF1 and EPF2 for binding to ERfs but is unable to activate the downstream cascade, and thus functions as an antagonist (Ohki et al. 2011; Lee et al. 2015).

Two ligands, EPFL4 and EPFL6, stimulate aboveground organ elongation (Abrash et al. 2011; Uchida et al. 2012). Another ligand, EPFL2, has been shown to regulate the shape of leaf margins (Tameshige et al. 2016). The function of the remaining five potential ligands has not been established. Selection of which ligands can bind to ERf receptors on a surface of an individual cell depends on the presence of the co-receptor TOO MANY MOUTHS (TMM) which promotes binding of EPF1, EPF2, and STOMAGEN and inhibits binding of EPFL4 and EPFL6 (Lin et al. 2017). The binding of ligands to ERfs or to ERf/TMM complexes does not cause significant conformational changes or induce homodimerization of ERfs (Lin et al. 2017). Recent work suggests that ERfs function in a complex with receptor-like kinases of the SERK family which could potentially assist ERfs in activation of downstream targets (Meng et al. 2015). A MAP kinase cascade consisting of YODA, MKK4/5/7/9, and MPK3/6 transmits the signal downstream of ERfs (Bergmann et al. 2004; Wang et al. 2007; Meng et al. 2012; Lampard et al. 2009; Lampard et al. 2014). How the signal is transmitted from the receptors to the cascade is not known.

Here we focus on ERf signaling in the SAM, a small but complex structure that must tightly control the proliferation and differentiation of its constituent cells. The SAM contains three different regions: the central zone with a pool of undifferentiated, slowly dividing cells; the peripheral zone where leaf and flower primordia are initiated; and the underlying rib zone that provides cells for internodes. As cells are continually transitioned from the central zone into the other two, cell-to-cell communications are essential to maintain a relatively constant number of stem cells. These communications are achieved through a negative feedback loop consisting of the receptor/ligand pair CLAVATA1 (CLV1)/CLAVATA3 (CLV3) and the transcription factor WUSCHEL (WUS) (Clark, 2001). Presumably the rate of cell proliferation and differentiation in the peripheral zone and the rib zone is also tightly controlled to ensure a consistent rate of organ initiation and uniformity of size; however, how this is achieved is not known. In addition, leaves and flowers develop in a specific geometric pattern. In

Arabidopsis the SAM forms leaves and flowers at 137.50 angles to each other, producing a spiral pattern of these organs around the stem. The formation of auxin maxima determines the position of organ primordia (Sluis et al. 2015). ERfs play a critical role in these processes - the vegetative SAM of er erl1 erl2 is dramatically wider and has a much broader central zone exhibiting increased expression of WUS (Chen et al. 2013; Uchida et al. 2013). Leaf primordia are initiated at a significantly reduced rate with almost random divergence angles (Chen et al. 2013). The changes in leaf initiation in *er erl1 erl2* correlate with abnormal auxin distribution as determined by DR5rev:GFP marker and decreased PIN1 expression in the vasculature (Chen et al. 2013).

To gain insight into the function of ERfs in the SAM we explored their pattern of expression and searched for ligands that are perceived by ERfs. While *ERfs* are expressed throughout the SAM, their expression in the central region by the *CLV3* promoter is the most efficient in rescuing the meristematic defects of *er erl1 erl2* compared to expression in the peripheral zone by the *KAN* promoter. Interestingly, *ERECTA* expression under the *CLV3* promotor is also able to rescue leaf size and stem elongation phenotypes, suggesting that those parameters might be controlled by ERfs indirectly from distant tissues. Based on the phenotype of the quadruple mutant, ERfs sense four ligands in the SAM, *EPFL1, EPFL2, EPFL4,* and *EPFL6.* Two of those ligands (*EPFL1* and *EPFL2*) are expressed in the boundary region in the embryo and in the vegetative SAM. Their expression on the periphery of the meristem is critical as the *epfl1 epfl2 epfl4 epfl6* mutant can be rescued by *EPFL1* expressed under the *KAN* promoter but not *CLV3*. Our data suggest that ERfs coordinate development of the central zone and the peripheral regions of the SAM.

Materials and methods

Generation of transgenic plants

Four different promoters were independently cloned into pPZP222 vectors that carried the genomic *ERECTA* sequence and the endogenous 7.4 kb

ERECTA terminator*. pSTM:ER* (pPZK 311) was generated by amplifying a 4.62 kb region upstream of the *STM* start site. A similar 4.5 kb STM promoter region has been used and analyzed previously (Verkest *et al.* 2005). *pWUS:ER* (pPZK 310) was created by amplifying a 4.5 kb region upstream of the *WUS* start site. This promoter region has been used previously by (Yadav *et al.* 2009). *pANT:ER* (pPZK 315) was created by amplifying a 4.3 kb region upstream of the *ANT* start site as in (Grandjean *et al.* 2004). *pKAN:ER* (pPZK 312) was generated by amplifying a 3.6 kb region upstream of the *KANADI* start site as in (Wu *et al.* 2008). The fifth construct *pCLV3:ER* (pPZK317) was generated slightly differently due to the presence of an enhancer in the terminator of *CLV3* (Brand *et al.* 2002). The genomic *ERECTA* sequence was inserted into pPZP222 between the 1.5 kb sequence upstream of the *CLV3* start site and the 1.2 kb sequence downstream of the *CLV3* stop codon. All created constructs were examined by the restriction analysis and sequencing of amplified regions.

The described plasmids were transformed into an *Agrobacterium tumefaciens* strain GV3101/pMP90 by electroporation and introduced into *er erl1/+ erl2* plants by the floral dip method. The *er-105 erl1-2 erl2-1* mutant has been described elsewhere (Shpak *et al.* 2004). The T1 transgenic plants were selected based on gentamicin resistance. Kanamycin resistance was used to identify *erl1-/+* or *erl1-/-* lines in the T2 generation. In the T3 or T4 generation we selected lines that are homozygous for the transgene based on gentamycin resistance.

To generate *pEPFL1:EGFP-GUS,* a 1.5 kb fragment upstream of the *EPFL1* start site was PCR amplified and inserted into p-ENTR/topo (Invitrogen) and recombined using LR recombinase (Invitrogen) into pKGWFS7 (Karimi *et al.* 2005). To clone the promoters of *EPF1* (2.7kb), *EPF2* (2.7kb), EPFL2 (3kb), *EPFL3* (2.9kb), *EPFL7* (1.5kb), *EPFL8* (2.4kb) and *EPFL9* (2kb) in front of *EGFP-GUS* a modified version of the Rapid one-step recombinational cloning method was used (Fu *et al.* 2008). The promoter regions were amplified by PCR using gene-specific primers that also contained shortened AttL1 or AttL2

sequences. Each fragment was extended using attL1-T2.1 and attL2-T2.1 primers to produce complete AttL sequences on both sides of each fragment. The generated fragments were recombined into pKGWFS7 using LR recombinase (Invitrogen). Primer sequences can be found in Table 3.1. (Located in chapter 3 appendix). The generated *pEPFL:EGFP-GUS* plasmids were introduced into wild type plants as described above. The transgenic plants were selected based on kanamycin resistance. *pEPFL4:GUS, pEPFL5:GUS* and *pEPFL6:GUS* transgenic plants were described previously (Abrash *et al.* 2010), (Abrash *et al.* 2011).

To generate *pEPFL1:EPFL1* a 3.3kb fragment encompassing a 2kb region upstream of the *EPFL1* start site and 0.8kb downstream of the stop codon was amplified and cloned into pPZP222. *pEPFL2:EPFL2* was generated by amplifying a 4.2 kb fragment including 2.5kb upstream of the *EPFL2* start codon and 1kb downstream of the stop codon. In *pKAN:EPFL1* and *pCLV3:EPFL1* constructs we used the same promoter regions as in *pKAN:ER* and *pCLV3:ER* and the *EPFL1* sequence that included introns. The *pKAN:EPFL1* construct contains the endogenous 0.8kb *EPFL1* terminator while *pCLV3:EPFL1* contains the 1.2 kb sequence downstream of the *CLV3* stop codon.

In situ analysis

In situ hybridization was performed as described previously (Hejatko *et al.* 2006) using 3-day old (post germination) T3 and T4 transgenic or WT seedlings. One kb cDNA region of ERECTA between the SacI and XhoI restriction sites was cloned into pBluescript II and used as the template for *in vitro* transcription with T3 (Promega) and T7 (Invitrogen) RNA polymerases to make the sense and antisense probes, respectively. To generate *EPFL* probes their full-length coding DNA sequences were amplified using WT cDNA and primers that contained the T7 promoter sequence near either the start or the stop codons. All probes were hydrolyzed to produce fragments of average length of about 0.3 Kb. Representative images were taken using DIC microscopy.

Analysis of mutant phenotypes

For measurements of leaf number and SAM size by DIC microscopy seedlings were grown on plates containing modified Murashige and Skoog supplemented with 13 Gamborg B5 vitamins and 1% (w/v) Sucrose. Selected 3- and 5-day old (post germination) seedlings were incubated in a solution of 9:1 ethanol: acetic acid overnight, rehydrated using an ethanol series (90%, 80%, 70%, and 50%) and cleared in a chloral hydrate solution (chloral hydrate:water:glycerol 8:1:1). The *pSTM:ER* transgenic lines were analyzed in the T4 or T5 generations, as they were homozygous for the transgene and the *erl1* mutation. The p*WUS:ER, pKAN:ER, pANT:ER* and *pCLV3:ERECTA* transgenic lines were analyzed in the T3 generation. These were homozygous for the transgene but were segregated for *erl1*. The *er erl1 erl2* plants used for analyses were identified based on the presence of stomata clusters in cotyledons. Microscopic observations were done using a Nikon Eclipse 80i microscope with differential interference contrast (DIC) optics and NIS-Elements BR imaging software (Nikon) was used for measurements. For measurement of plant height and pedicel length and to observe leaf growth plants were grown as described previously (Kosentka *et al.* 2017).

Generation of the epfl double, triple, and quadruple mutants

The *epfl1-1* (CS104435) transposon-insertion mutant (Col background) was obtained from the Arabidopsis Biological Resource Center. *epfl2-1* (CSHL_ET5721) transposon-insertion mutant (Ler background) was received from Cold Spring Harbor Laboratory and outcrossed three times to *epfl1-1* to obtain *epfl1-1 epfl2-1* in Columbia background. The absence of the *er-1* mutation in *epfl1-1 epfl2-1* was confirmed by sequencing. The *epfl1 epfl2* double mutant was crossed with *epfl4 epfl6 /cll2-1 chal-2* (Abrash *et al.* 2011) to obtain new combinations of mutations. *epfl* double, triple and quadruple mutants were identified by genotyping with *epfl1-1* and *epfl2-1* primers from Table 3.2 (appendix) and with *epfl4/cll2-1* and *epfl6/chal-2* specific primers described previously (Abrash *et al.* 2011). We used a three-primer PCR for genotyping of

epfl1-1 and *epfl2-1*. During genotyping of *epfl1-1* the primers epfl1.436.rev and 3dspm were used to amplify a ~ 200 bp fragment and the primers epfl1.436.rev and epfl1.74 were used to amplify a 387 bp fragment. During genotyping of *epfl2-1* the primers epfl2.1 and gus.43.rc were used to amplify a ~700 bp fragment and the primers epfl2.1 and epfl2.540.rev were used to amplify a 575 bp fragment. Because the *epfl1 epfl2 epfl4 epfl6* mutant is infertile, for the morphological analysis we obtained it from the progeny of *epfl1*/+ *epfl2 epfl4 epfl6* plants.

The GUS reporter gene and assay and microscopy

GUS staining was performed as described previously (Sessions *et al.* 1999) using 5-days post germination T2 or T3 transgenic seedlings. Multiple independent transgenic lines were analyzed for each construct to find a consistent pattern of expression. Depending of the level of the signal the concentration of ferricyanide and ferrocyanide in the staining buffer varied between 0.25mM and 2 mM. After staining, the samples were dehydrated with a graded series to 50% ethanol, fixed in FAA solution for 30 min, dehydrated with a graded series of ethanol to 100% ethanol, infiltrated with polymethacryl resin Technovit 7100 resin and then embedded and polymerized in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Eight-micrometer sections were prepared using a Leica RM-2255 microtome (Wetzlar, Germany). Pictures were obtained using a Nikon Eclipse 80i microscope and a 12 megapixel cooled color DXM-1200c (Nikon) camera. A C-FL B-2A (Nikon) filter cube was used to observe the GFP signal.

Reverse Transcription-PCR

Total RNA was isolated from 5 DPG seedlings and from fully expanded leaves using a Spectrum Plant Total RNA Kit (Sigma). RNA was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using 150 ng of RNA with a ProtoScript II RT-PCR Kit (New England Biolabs) according to the manufacturer's instructions. Quantitative PCR was performed using the CFX96 Real Time System (BioRad) with Sso Evagreen Supermix reagent (BioRad).

Each experiment used three technical replicates and three biological replicates to calculate relative fold difference of *ERECTA* to *ACTIN-2* expression. Bio-Rad CFX Manager was used to calculate cycle threshold values and the fold difference in gene expression was calculated using the delta-delta-Ct algorithm ($2^{-\Delta\Delta Ct}$). Primers and annealing temperatures are listed in Table 3.3 (appendix).

Results

Expression of ERECTA in the central zone is most efficient in regulating the SAM size.

Based on an *in situ* analysis and a reporter gene assay *ERfs* are expressed broadly in the vegetative SAM and throughout forming leaf primordia (Yokoyama *et al.* 1998; Shpak *et al.* 2005; Uchida *et al.* 2013). A gene expression profile of the inflorescence SAM suggests similar expression of *ERfs* in the central zone, the peripheral zone, and in the organizing center with only *ERL1* being upregulated in the central zone (Yadav *et al.* 2009). In that experiment the zones were defined by *CLAVATA3 (CLV3), FILAMENTOUS FLOWER (FIL),* and *WUSCHEL (WUS)* expression, respectively. We were interested in how the meristematic expression of ERfs affects plant morphology and whether ERECTA expression in a specific zone is sufficient to rescue defects observed in the *er erl1 erl2* mutant. With this goal in mind five different promoters were chosen. The *SHOOTMERISTEMLESS (STM)* promoter was used to express the gene throughout the SAM (Long *et al.* 1996). The *CLV3* and *WUS* promoters were used to drive *ERECTA* expression in the central zone and in the organization center, respectively (Fletcher *et al.* 1999; Mayer *et al.* 1998). The *AINTEGUMENATA (ANT)* promoter was used to induce ERECTA expression in the peripheral zone and broadly in the forming leaf primordia (Elliott *et al.* 1996). We expected the *KANADI (KAN)* promoter to express *ERECTA* at the outer edges of the peripheral zone and on the abaxial side of leaf primordia (Kerstetter *et al.* 2001; Yadav *et al.* 2014).

To examine *ERECTA* expression in the generated transgenic lines we performed *in situ* hybridization on three-day old seedlings (Figure 3.1A). In the

wild type *ERECTA* was detected throughout the SAM and in leaf primordia, although the signal was very weak. In the *pCLV3:ER* and *pWUS:ER* transgenic plants *ERECTA* was expressed as expected in the central zone and the organizing center, respectively. Based on both *in situ* and qRT-PCR *ERECTA* expression was considerably lower in the *pWUS:ER* lines compared to all other transgenic lines (Figure. 3.1). Most importantly, neither in *pCLV3:ER* nor in p*WUS:ER* transgenic lines was *ERECTA* detected outside of the SAM. In *pSTM:ER* transgenic lines a signal was observed throughout the SAM and sometimes on the abaxial side of leaf primordia. The strength of the *in situ* signal and its appearance outside of the meristem varied greatly, consistent with variable expression of *ERECTA* in those lines as determined by qRT-PCR (Figure. 3.1B). In the *pANT:ER* transgenic plants *in situ* analysis detected *ERECTA* in the L1 layer of the SAM and throughout young organ primordia. A similar pattern including expression in the L1 layer of the SAM was observed previously when 6.5 kb ANT promoter was used to drive β-glucuronidase (GUS) expression (An *et al.* 2004). In the *pKAN:ER* transgenic plants the majority of the signal was detected in the peripheral zone with very low expression in leaf primordia. Thus, only the *CLV3* and *WUS* promoters drove expression of *ER* as expected, with *STM, ANT,* and *KAN* expressing *ERECTA* in slightly different patterns, suggesting that expression of genes under exogenous promoters should always be coupled with analysis of their expression.

To understand how zone-specific expression of *ERECTA* affects the SAM size we analyzed transgenic seedlings 3 and 5 days post germination (Figure 3.2). In all cases there was very little variation in the SAM size between these two time samples, suggesting that all lines can maintain a specific meristem size. The size of the meristem in lines expressing *ERECTA* throughout the meristem (under the *STM* promoter) or in the central zone (under the *CLV3* promoter) was rescued more efficiently compared to the other transgenic lines (Figure 3.2A). On average, the width of the SAM in the *pCLV3:ER* transgenic lines is 1.14±0.03

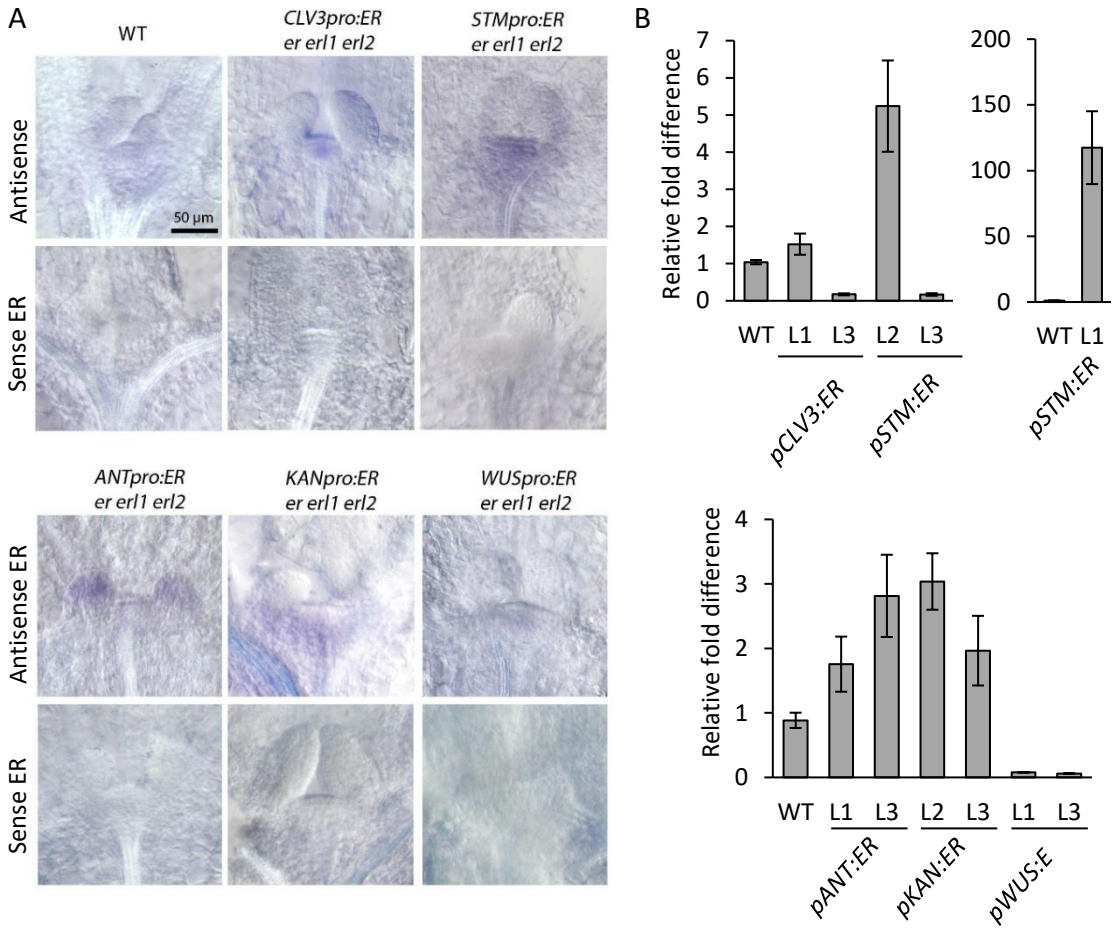

Figure 3.1 Ectopic expression of *ERECTA* in the SAM using heterologous promoters. A. Representative DIC images of in situ hybridization with a sense and an antisense probe for *ERECTA* using 3-day old T3 or T4 transgenic seedlings. B. Real time RT-PCR analysis of ER in 5-day old seedlings of wt and transgenic plants. The average of three biological replicates is presented. Error bars represent SE.

and in *pSTM:ER* is 1.13±0.03 times larger than the wild type while in the *er erl1 erl2* mutant it is 1.99±0.02 (± standard error). The expression of *ERECTA* in the organizing center under the *WUS* promoter was less efficient in rescuing the meristem size (Figure 3.2B) with an average width being 1.36±0.03 of the wild type. This is probably only partially due to the low expression of *ERECTA* in those lines as low expression of *ERECTA* in *pCLV3:ER* line #3 and *pSTM:ER* lines #3 is sufficient to rescue the SAM size (Figure 3.1B and Figure 3.2A). The *pANT:ER* construct was the least efficient in controlling meristem size with the average meristem width being 1.43±0.03 of the wild type and in this case it is not clear whether this poor rescue is related to low level of *ERECTA* expression in the L1 layer of the meristem or due to its expression in the leaf primordia (Figure 3.2B). The expression of *ERECTA* in the peripheral zone under the *KAN* promoter also led to a relatively inefficient rescue with the average meristem width being 1.34±0.04 of the wild type. This result cannot be attributed to the low expression of *ERECTA* in *pANT:ER* and *pKAN:ER* lines (Figure 3.1B). These data suggest that *ERECTA* can affect the meristem size when expressed in a variety of tissues but it is most efficient when expressed in the central zone.

Expression of ERECTA in the central zone of the SAM is most efficient in regulating leaf initiation

ERfs promote leaf initiation (Chen *et al.* 2013). At 3 and 5 days post germination the *er erl1 erl2* mutant forms on average 0.33± 0.03 and 0.42± 0.03 times as many leaves compared to the wild type. Out of the five promoters used, *CLV3* and *STM* were the most efficient in rescuing leaf initiation defects (Figure 3.3A). Plants expressing the *pCLV3:ER* and *pSTM:ER* constructs in the *er erl1 erl2* background formed on average 0.89±0.05 and 0.91±0.05 times as many leaves versus wild type at 3 days post germination and 0.92± 0.05 and 0.89± 0.05 times as many leaves at 5 days post germination, respectively. Expression of *ERECTA* in the peripheral zone using the *KAN* promoter was the least efficient in the enhancement of leaf initiation (Figure 3.3B) with those transgenic plants having on average 0.69±0.05 and 0.71± 0.04 times as many leaves than the wild

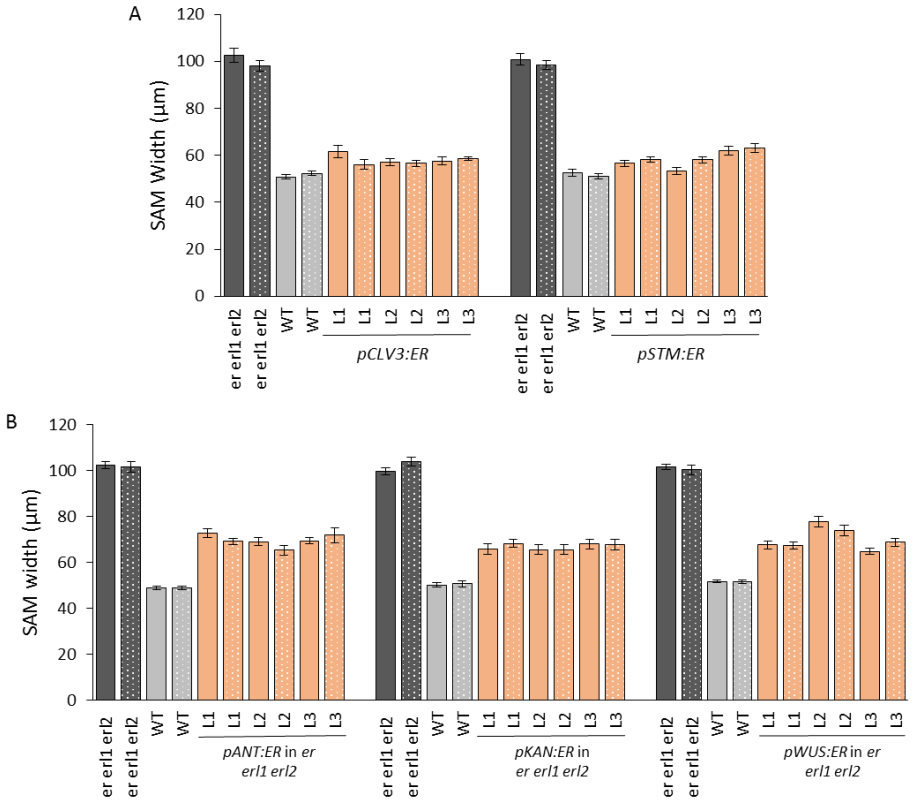

Figure 3.2 Expression of *ERECTA* in the central zone or broadly in the meristem rescues SAM size defects more efficiently (A) compared to when *ERECTA* is expressed in the leaf primordia, the peripheral zone, or in the organizing center (B). SAM size measurements were performed by DIC microscopy using 3 DPG (solid bars) and 5 DPG (dotted bars) seedlings. L1, L2, L3 are three genetically independent transgenic lines. N=7-11 Error bars represent SE.

type at 3 and 5 days post germination, respectively. The *pANT:ER* transgenic plants had on average 0.85±0.05 and 0.80±0.06 times as many at 3 days and 5 days, respectively. It is not clear whether expression in the leaf primordia or in the L1 layer of the meristem is responsible for this phenotype. The *pWUS:ER* transgenic plants had on average 0.79±0.07 and 0.72±0.05 times as many leaves than the wild type at 3 days and 5 days, respectively. It is interesting to note that expression of *ERECTA* in the organizing center has an effect at all on leaf initiation in the peripheral zone, suggesting that at least to some extent ERfs regulate leaf initiation indirectly. Based on the phenotypes of *pCLV3:ER*,

p*KAN:ER*, and *pWUS:ER* transgenic plants we conclude that ERfs can regulate leaf initiation indirectly and they do so the most efficiently when expressed in the central zone of the meristem.

ERECTA expression in the SAM can alter leaf expansion and stem elongation.

In addition to SAM size and leaf initiation, the expression of *ERECTA* under the utilized promoters altered other aspects of plant development. Two out of the five constructs, *pSTM:ER* and *pANT:ER*, were able to rescue infertility of *er erl1 erl2* (Figure. 3.10, located in chapter 3 appendix) consistent with their broad expression in developing flowers (Elliott *et al.* 1996), (Long *et al.* 1996). The expression of *CLV3* and *WUS* is much more restricted during flower development (Fletcher *et al.* 1999), (Mayer *et al.* 1998) and thus it is not surprising that *ERECTA* expressed under promoters of those genes cannot rescue fertility defects. While the *KAN* promoter is active on the abaxial side of initiating floral organs and in the tissue that gives rise to ovules (Kerstetter *et al.* 2001), that expression was not sufficient to rescue infertility of *er erl1 erl2* (Figure. 3.10 appendix).

The *ERf* genes are not only important for leaf initiation but also for leaf expansion (Shpak *et al.* 2004). In the three independent transgenic lines analyzed the *STM* promoter led to very different levels of *ERECTA* transcription from ~100-150 times more than in the wild type in L1 to ~5 times less in L3 (Figure 3.1B). The expression was observed in both young primordia (Figure 3.1A) and in mature leaves (Figure 3.4B). The different levels of *ERECTA* expression were reflected in the size and shape of leaves with fully rescued leaf expansion in L1 and a minor increase in leaf expansion in L3 (Figure 3.10 appendix). Two *pCLV3:ER* lines also varied in the levels of *ERECTA* expression. The *pCLV3:ER* L1 line expressed ~1.5 more *ERECTA* compared to the wild type and L3 about six times less (Figure 3.1B). This difference of expression again was reflected in different leaf sizes (Figure 3.10 appendix). Comparison of *L3*

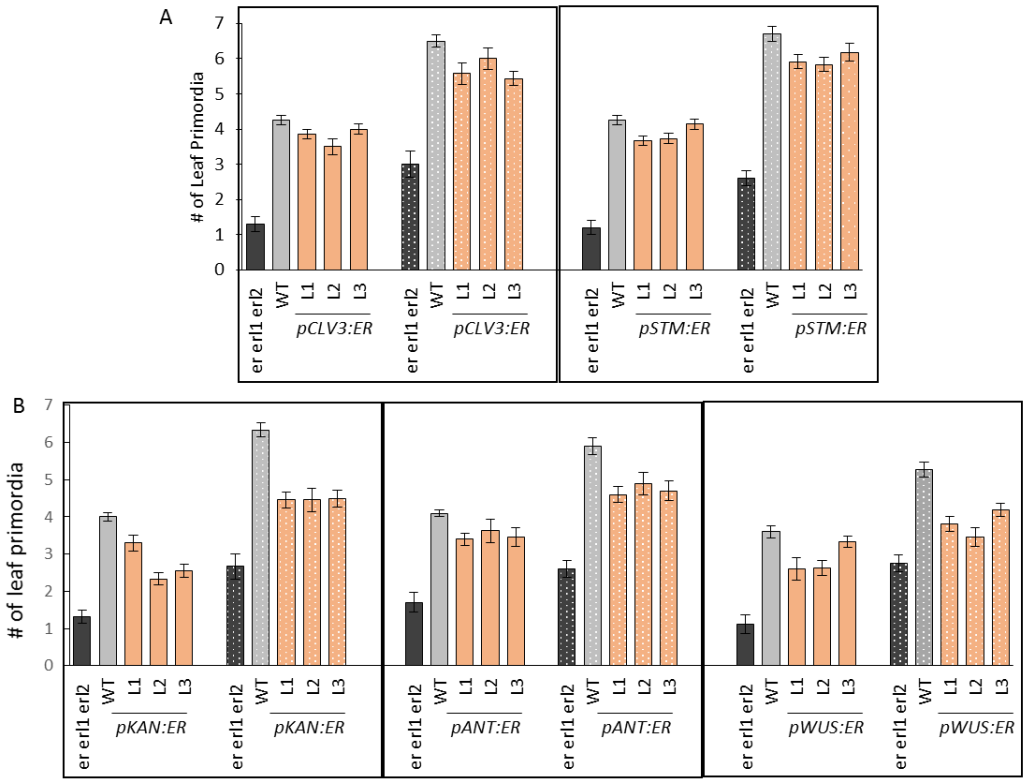

Figure 3.3 Expression of *ERECTA* in the central zone or broadly in the meristem rescues leaf initiation more efficiently (A) compared to when *ERECTA* is expressed in leaf primordia, in the peripheral zone, or in the organizing center (B). The number of leaf primordia formed was measured by DIC microscopy using 3 DPG (solid bars) and 5 DPG (dotted bars) seedlings. L1, L2, L3 are three genetically independent transgenic lines. N=7-11 Error bars represent SE.

pSTM:ER and *L3 pCLV3:ER*, which on the level of the whole seedling express similar amounts of *ERECTA*, suggests that expression under the *CLV3* promoter is more efficient in promoting leaf expansion (Figure 3.1B and Figure 3.10B). Interestingly, *ERECTA* expression directly in leaves using the *KAN* and *ANT* promoters only weakly altered leaf size (Figure 3.4). The leaf size in *pKAN:ER L2* that had twice as much *ERECTA* in mature leaves compared to the wild type was very similar to the size of leaves in *pWUS:ER L3* where *ERECTA* was barely detectable if even present. The most revealing line is *pANT:ER L1*, which expressed relatively high levels of *ERECTA* throughout both young primordia and

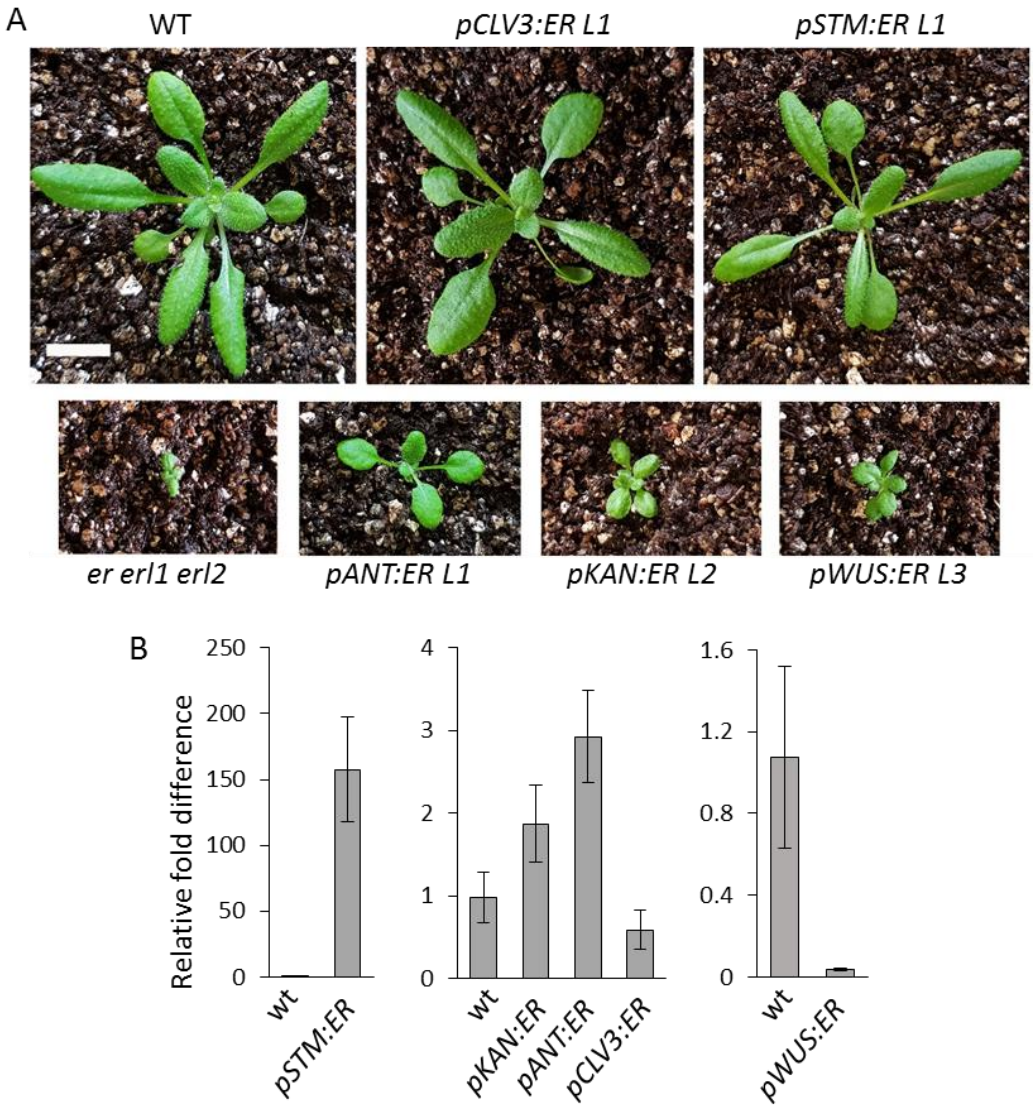

Figure 3.4 Expression of *ERECTA* using the *CLV3* or *STM* promoters most efficiently rescues leaf shape defects of the *er erl1 erl2* mutant. A. 20-day old plants, bar=1 cm. B. Real time RT-PCR analysis of *ER* in leaves of wt and T3-T6 transgenic plants. The average of three biological replicates is presented. Error bars represent SE.

older leaves but only partially rescued leaf size. Taken together, these data suggest that to induce leaf expansion either ERfs have to be expressed in a very specific pattern in a leaf which we inadvertently achieved with the *STM* and *CLV3* promoters or ERfs regulate leaf size indirectly from the SAM.

To further explore how ectopic expression of *ERECTA* affects plant growth we analyzed plant height and pedicel lengths. Previously it was shown that *ERECTA* expression in the phloem using the *SUC2* promoter was able to rescue height and pedicel length in the *erecta* mutant (Uchida *et al.* 2012). Here we show that expression in a variety of tissues rescues elongation defects of *er erl1 erl2* (Figure 3.5). *ERECTA* most efficiently stimulated stem growth when expressed under *CLV3* and *STM* promoters, which is most noticeable when one observes younger plants (Figure 3.10C appendix). Given enough time *ERECTA* under the control of four promoters, *CLV3, STM, ANT* and *KAN,* fully rescued final plant height (Figure 3.5A). *ERECTA* under the same four promoters also stimulated pedicel elongation in *er erl1 erl2* (Figure 3.5B), with *pCLV3:ER* and *pKAN:ER* being the least efficient. This may be at least partially related to their inability to rescue fertility, since we previously demonstrated that pedicels attached to unfertilized siliques are approximately 2 mm shorter compared to those attached to fertilized siliques (Bundy *et al.* 2012). Unexpectedly, even low expression of *ERECTA* in the organizing center of the SAM using the *WUS* promoter had a small but statistically significant effect on both stem and pedicel elongation (Figure 3.5 and Figure 3.S1C). These results are inconsistent with ERf function in the phloem and indicate that further analysis is necessary to understand the role of ERECTA in organ elongation.

Expression pattern of EPFL1, EPFL2, EPFL4, and EPFL6 near the SAM.

The activity of ERf receptors is regulated by a group of secreted small proteins from the EPF/EPFL family (Shimada *et al.* 2011). To narrow down the group of ligands that might be perceived by ERfs in the SAM we investigated *EPF/EPFL* expression patterns using the GUS and GFP transcriptional reporter assay.

Analysis of whole mount seedlings using GUS assay suggested that *EPF1, EPF2,* and *EPFL8* are expressed in epidermis and specifically in developing stomata but not in the shoot apical meristem (Figure 3.11). The expression of other genes near the meristematic region was further examined by sectioning (Figure 3.6A). Three genes, *EPFL3, EPFL5/CLL1*, and *EPFL7*, were expressed in different regions of leaf primordia: *EPFL3* on the adaxial side of leaves at some distance from the SAM; *EPFL5* at the bases of leaf primordia, especially on the abaxial size; and *EPFL7* in the internal tissues at the base of leaf primordia. Five genes were expressed near the meristematic region: *EPFL1*, *EPFL2, EPFL4/CLL2, EPFL6/CHAL,* and *EPFL9/STOMAGEN*. Both *EPFL4* and *EPFL6* were expressed throughout the meristem albeit at a very low level. *EPFL9* was expressed in the rib zone of the meristem. Because *EPFL9* is an antagonist of ERfs (Lee *et al.* 2015) and currently mutants in that gene are unavailable, we did not investigate it any further. We observed expression of *EPFL1* at the boundary of the SAM, and on the adaxial side of forming leaf primordia. There was also low level of expression in the rib zone. *EPFL2* was expressed at the boundary and in the peripheral zone of the meristem. Next we used epifluorescence microscopy to analyze *GFP* expression. Unfortunately, we could not detect the *EGFP* signal in the vegetative meristem presumably due to the low level of expression and high background autofluorescence. But we were able to analyze expression of all genes during embryogenesis. Out of 11 genes only *EPFL1* and *EPFL2* were expressed in the developing embryos. Both genes were expressed very highly in the peripheral regions of the embryonic SAM where two margins of cotyledons meet (Figure 3.6B). *EPFL1* was also expressed in the epidermis of hypocotyl and in the root apical meristem.

EPFL1, EPFL2, EPFL4, and EPFL6 partially redundantly regulate elongation of plant organs.

Due to their expression near the meristematic region we investigated the function of *EPFL1, EPFL2, EPFL4,* and *EPFL6* in plant development. The *epfl4/cll2-1* and *epfl6/chal-2* single mutants are null alleles carrying T-DNA

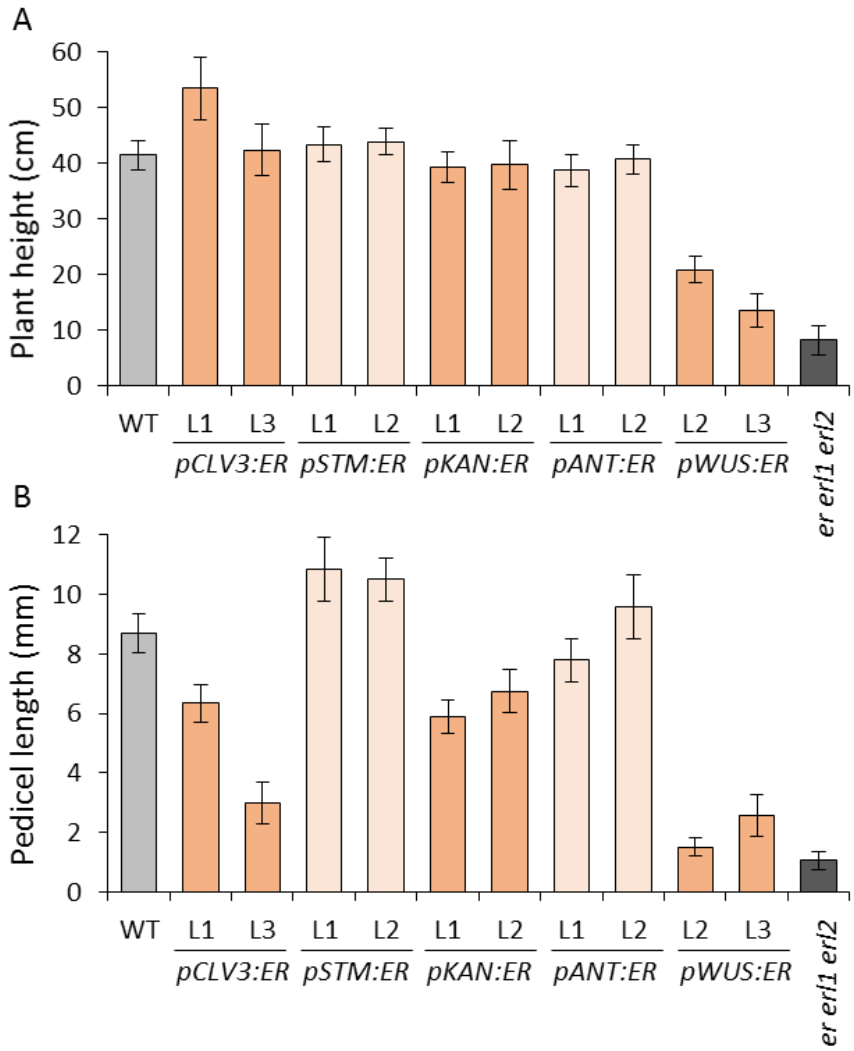

Figure 3.5 Expression of *ERECTA* under a variety of promoters can fully or partially rescue elongation of stem and pedicels in the *er erl1 erl2* mutant. Plant height (A) and pedicel length (B) were measured in mature 2 month old plants. Two independent transgenic lines were analyzed. N=10-30 for heights and n=64 for pedicel length. Error bars represent SD.

insertions with no visible phenotype (Abrash *et al.* 2010; Abrash *et al.* 2011; Uchida *et al.* 2012). The *epfl2-1* is a null allele carrying a transposon insertion which exhibits diminished leaf tooth growth (Tameshige *et al.* 2016). The *epfl1-1* is a null allele carrying a transposon insertion with no visible phenotype (Figure 3.12 and 3.13 appendix). To understand the function of these genes we created all possible combinations of double and triple mutants. The *epfl4 epfl6* plants are shorter in stature compared to the wild type but are slightly taller compared to *er-105* (Abrash *et al.* 2011; Uchida *et al.* 2012) and Figure 3.7A-B). None of the other double mutants displayed a significant reduction in elongation of stems or pedicels (Figure 3.7A-B). Addition of the *epfl1* mutation to *epfl4 epfl6* did not change stem and pedicel elongation while the presence of *epfl2* in the *epfl4 epfl6* background slightly reduced elongation of pedicels leading to formation of more compact inflorescence (Figure 3.7B and E). The *epfl1 epfl2 epfl4 epfl6* mutant reached a final height comparable to that of the *erecta* single mutant; however, it grew drastically slower and took an additional four weeks to achieve maturity compared to *erecta* (Figure 3.7A and D). In this respect *epfl1 epfl2 epfl4 epfl6* is similar to *er erl1 erl2* which is also characterized by an extended period of growth and a longer lifespan (Kosentka *et al.* 2017). We observed that the extended life span of *epfl1 epfl2 epfl4 epfl6* leads to increased number of siliques formed on the main stem (Figure 3.7C). Taken together these data suggest that while *EPFL4* and *EPFL6* play the primary role in stimulation of stem and pedicel elongation, *EPFL1* and *EPFL2* also contribute to this process.

In addition to changes in elongation of aboveground organs we also observed changes in silique growth, fertility, and apical dominance (Figure 3.13 appendix) Of all double mutants, *epfl1 epfl6* formed the shortest siliques suggesting that the primary role for these two genes is in fruit development (Figure 3.13B appendix). Fertility was reduced in the *epfl1 epfl2 epfl6* and *epfl1 epfl4 epfl6* mutants, and *epfl1 epfl2 epfl4 epfl6* plants are infertile (Figure 3.13A appendix). In addition, all four genes contribute partially redundantly to establishment of apical dominance (Figure 3.13C appendix). No obvious

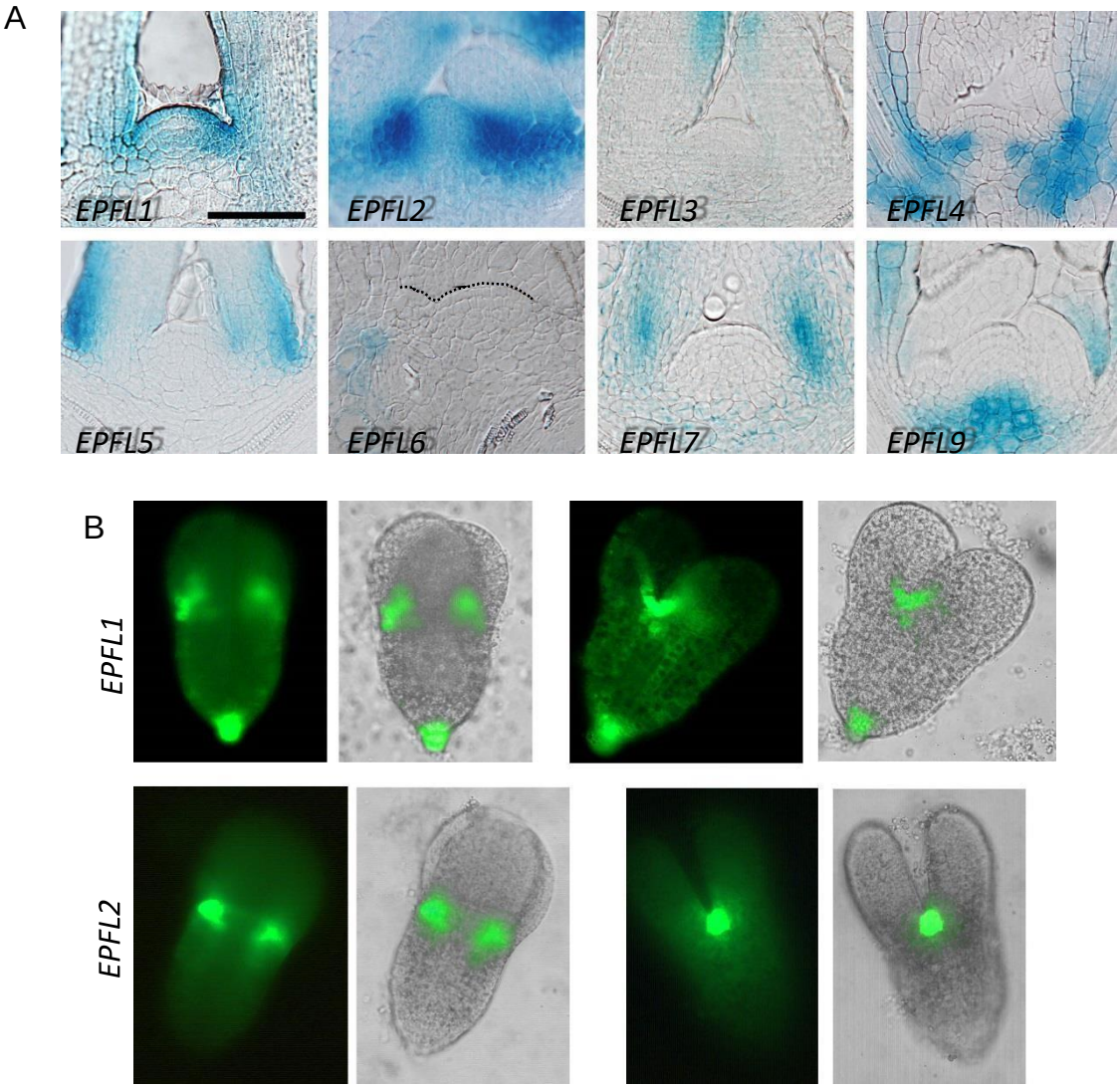

Figure 3.6 A reporter gene assay of the *EPF/EPFL* gene family in the SAM demonstrates distinct patterns of expression. A. Longitudinal sections of shoot apices of T2 7 or 10-day old wild type seedlings expressing indicated *pEPFL:EGFP-GUS* constructs. The dotted line in the *EPFL6* insert emphasizes the L1 layer of the SAM. B. Epi-fluorescence microscopy of plants expressing *pEPFL1:EGFP-GUS* and *pEPFL2:EGFP-GUS* in torpedo embryos. For each construct the same embryo is represented from two different perspectives.

changes in the formation of stomata were observed (Figure 3.14 appendix).

EPFL1, EPFL2, EPFL4, and EPFL6 redundantly regulate SAM size and leaf initiation.

Analysis of triple *epfl* mutants demonstrated a slight but statistically significant increase of meristem size in *epfl1 epfl2 epfl4*, *epfl 1 epfl2 epfl6,* and *epfl1 epfl4 epfl 6* (Figure 3.8A). There were no significant changes in the rate of leaf initiation (Figure 3.8B). Since the *epfl1 epfl2 epfl4 epfl6* mutant is infertile and the epidermal phenotype cannot be used to identify it in the progeny of *erfl1/+ epfl2 epfl4 epfl6* plants, 30 seedlings with slightly shorter petioles of cotyledons

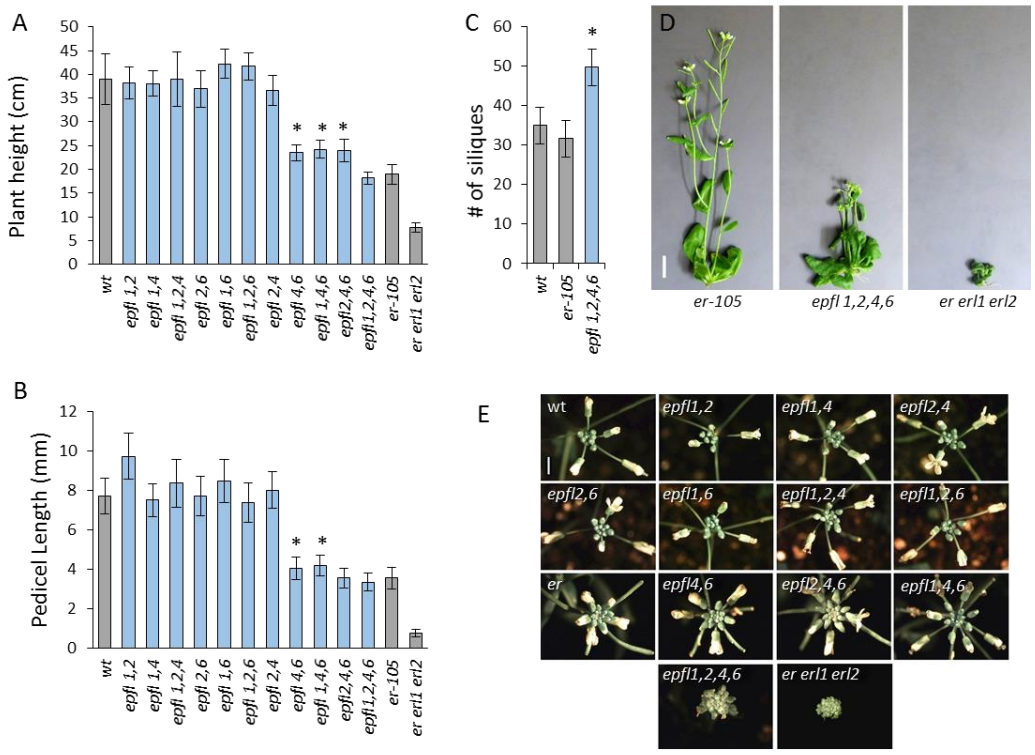

Figure 3.7 *EPFL1, EPFL2, EPFL4,* and *EPFL6* synergistically regulate stem and pedicel elongation with *EPFL4* and *EPFL6* playing the key role. (A) Height of fully grown plants (n=27-46 except er erl1erl2 n=12). (B) Lengths of mature pedicels on the main stem (n=100-120). (C) Number of siliques on the main stem (n=10). A-C. Bars represent the average; Error bars represent SD. Values significantly different from *er-105* when not obvious are indicated by asterisks ($P < 0.001$). (D) Six-week-old plants of *er-105*, *epfl1-1 epfl2-1 epfl4 epfl6* and *er-105 erl1-2 erl2-1*. Scale bar: 1 cm. (E) Inflorescence apices from the wild type, *er, er erl1 erl2* and various combinations of *epfl* mutants. Bar=25 mm.

were genotyped for *epfl1-1* prior to fixation for DIC microscopy. This allowed us to identify ten *epfl1 epfl2 epfl4 epfl6* mutants. The following analysis demonstrated that in terms of the meristem size and the leaf initiation rate *epfl1 epfl2 epfl4 epfl6* is indistinguishable from *er erl1 erl2* (Figure 3.8), suggesting that EPFL1, EPFL2, EPFL4 and EPFL6 are the ligands that are sensed by ERfs in the meristem. To confirm that the phenotype is due to mutations in the *EPFL* genes and not to some other overlooked mutations we independently expressed *EPFL1* and *EPFL2* under their endogenous promoters in *epfl1 epfl2 epfl4 epfl6.* Both constructs rescued meristematic defects in multiple independent transgenic lines (Figure 3.9). To test whether ligands have to be co-expressed with *ERfs* in the central zone or if they function from the peripheral zone *EPFL1* was expressed in *epfl1 epfl2 epfl4 epfl6* under *CLV3* and *KAN* promoters. The expression under *KAN* fully rescued both meristem size and leaf initiation while expression under *CLV3* had no effect on the meristem size and only partially rescued leaf initiation (Figure 3.9). Taken together these data suggest that four *EPFL* genes, *EPFL1, EPFL2, EPFL4,* and *EPFL6*, redundantly regulate maintenance of meristem size and promote leaf initiation with expression in the peripheral zone being sufficient for their function.

Discussion

The first indication that ERECTA signaling might contribute to regulation of SAM structure came from the analysis of higher order mutants. It was observed that the *er* mutation enhances meristematic defects of CLV pathway mutants and suppresses those of the uni-1D/+ mutant (Dievart et al. 2003; Durbak et al. 2011; Uchida et al. 2011). Later, analysis of the *er erl1 erl2* mutant demonstrated that ERfs synergistically inhibit expansion of the vegetative meristem and promote leaf initiation (Chen et al. 2013; Uchida et al. 2013). While the CLAVATA pathway regulates meristem height ERECTA signaling restricts the meristem width and functions independently of CLAVATA (Mandel et al. 2014; Mandel et al. 2016). Understanding a signaling pathway depends on knowing the identity of

cells involved in sending and receiving the signal. ERf receptors are expressed throughout the SAM and in forming leaf primordia (Yokoyama et al. 1998; Uchida et al. 2013) but that does not mean that their expression in all those areas is necessary for regulation of meristem expansion and/or leaf initiation. To uncover the regions where ERfs are critical for meristem maintenance and organ initiation we expressed *ERECTA* under a range of promoters in the *er erl1 erl2* mutant. Unexpectedly, expression of *ERECTA* under all 5 promoters, *STM, CLV3, KAN*, *ANT*, and *WUS,* in different and in some cases non-overlapping areas of the meristem reduced meristem size and promoted leaf initiation, suggesting that ERfs can have an impact on meristem function when expressed in variety of locations. Simultaneously, expression of *ERECTA* throughout the meristem under the *STM* promoter or in the central zone under the *CLV3* promoter had the strongest impact on the meristem width and organ initiation, implying that the function of ERfs in the central zone is paramount. It is interesting to note that expression of *ER* under the *WUS* promoter elements is insufficient to fully rescue meristematic defects of *er erl1 erl2* while expression of *CLV1* under the same promoter elements fully rescues the *clv1* mutant (Nimchuk et al. 2015) which reinforces the distinctiveness of these two signaling pathways.

The next question is: what signals are perceived by ERfs in the SAM? There are eleven *EPF/EPFLs* in *Arabidopsis*. Analysis of mutants suggests that four genes, *EPFL1, EPFL2, EPFL4,* and *EPFL6*, contribute to meristem size establishment and promotion of leaf initiation. These genes function redundantly with triple mutants exhibiting no or very weak meristematic phenotypes. *EPFL1, EPFL2* and *EPFL4, EPFL6* belong to two closely related clades with stomata-regulating *EPF1, EPF2,* and *EPFL9* genes being more distantly related (Takata et al. 2013). Both clades have one additional gene, *EPFL3* and *EPFL8,* that are neither expressed near the meristematic region nor seem to be essential for SAM regulation. EPFL4 and EPFL6 are verified ERf ligands as they bind directly to ERfs (Lee et al. 2012; Lin et al. 2017). EPFL2 has been shown to bind to ERf containing complexes which suggests that genes belonging to that clade are

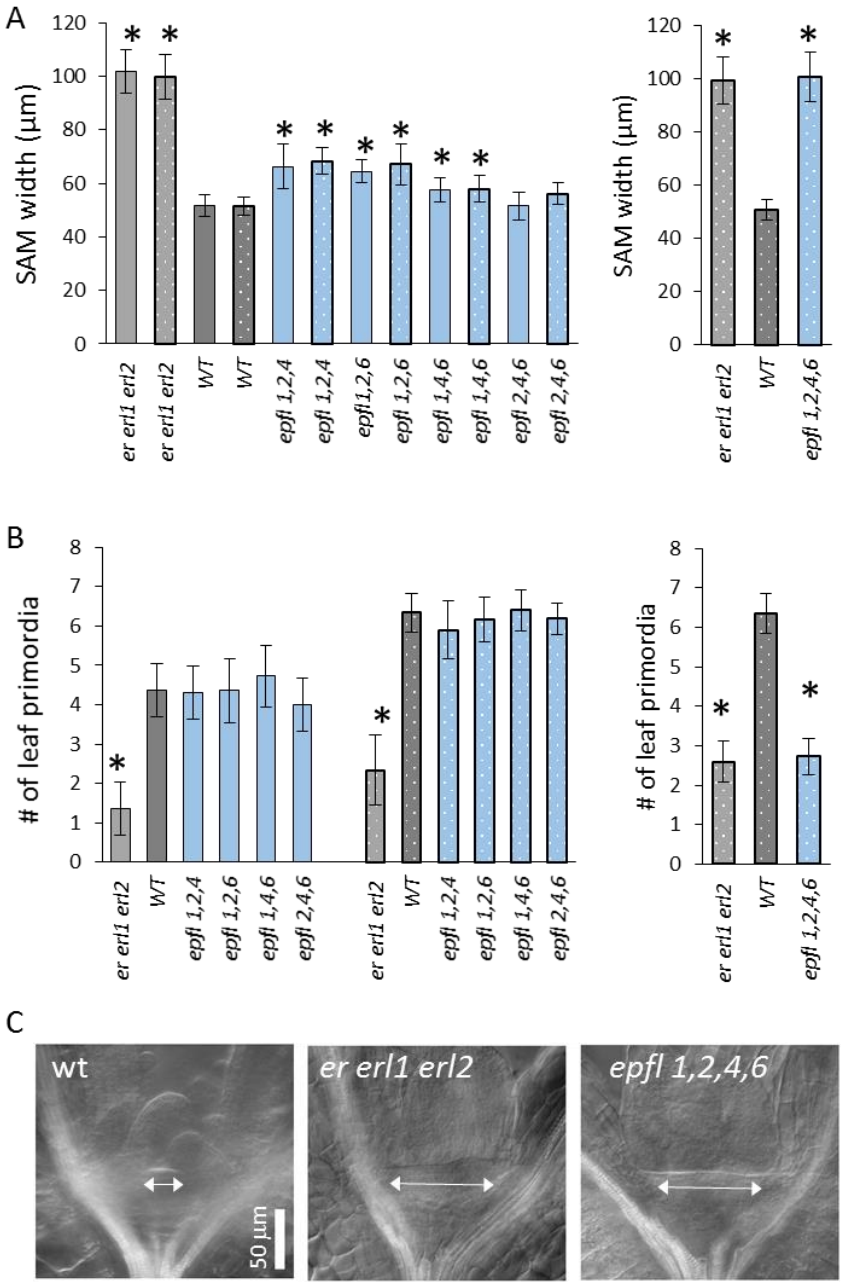

Figure 3.8 *EPFL1*, *EPFL2, EPFL4,* and *EPFL6* redundantly regulate the size of the shoot apical meristem and the rate of leaf initiation. Comparison of the SAM width (A) and the number of formed leaf primordia (B) in the wild type, *er erl1 erl2,* and *epfl* family mutants determined by DIC microscopy at 3 (solid bars) and 5 days post germination (dotted bars). Bars represent the average; Error bars represent SD. N=10-11. Values significantly different from the wild type are indicated by asterisks (P< 0.006). C. DIC images of meristematic regions in the wild type (wt), *er erl1 erl2,* and *epfl1,2,4,6* at 3 DPG. The meristem width is displayed with an arrow.

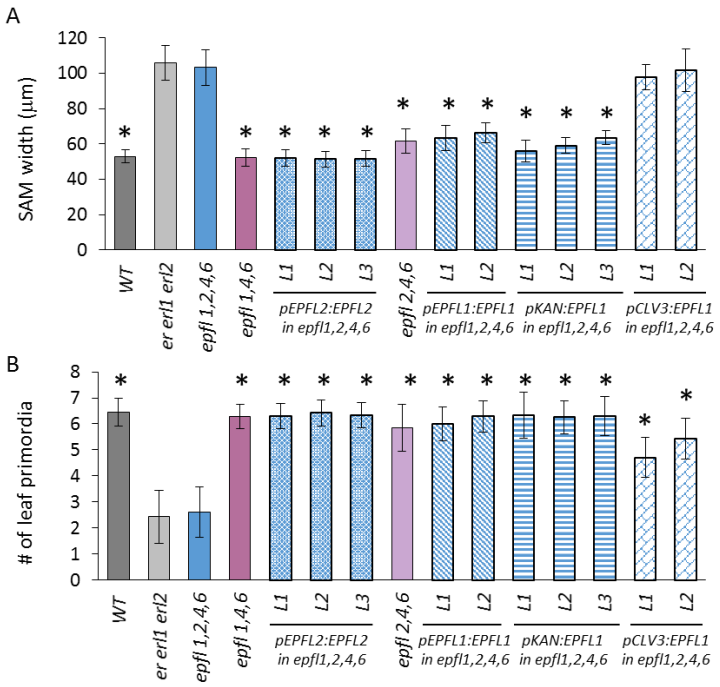

Figure 3.9 The meristematic phenotype of *epfl1,2,4,6* can be fully rescued by expression of *EPFL1* or *EPFL2* under endogenous promoters or by expression of *EPFL1* under *KANADI* promoter but not *CLV3*. Comparison of the SAM width (A) and the number of formed leaf primordia (B) in the wild type, selected mutants as indicated and in independent transgenic lines expressing indicated constructs in *epfl1,2,4,6* background as determined by DIC microscopy in 5-day post germination seedlings. Bars represent the average; Error bars represent SD. N=7-14 Values significantly different from *epfl1,2,4,6* are indicated by asterisks (P<0.05).

likely to encode ERf ligands (Tameshige et al. 2016). Since all four genes have the potential to suppress stomata development when expressed in the epidermal tissue layer they are likely to be agonists of ERf receptors (Abrash et al. 2011). The expression patterns of *EPFL1, EPFL2* and *EPFL4, EPFL6* differ. *EPFL1* and *EPFL2* are expressed during embryogenesis in the boundary region between two cotyledons at the periphery of the shoot apical meristem. After germination they are expressed in the analogous region at the border of the meristem and already formed leaf primordia. Expression of *EPFL1* in the border zone is consistent with the gene expression profiling of the inflorescence SAM which indicated

upregulated *EPFL1* expression at the periphery of the SAM (Yadav et al. 2009) and with the previously published pattern of its expression in the vegetative SAM (Kimura et al. 2018). *EPFL2* has been classified as a boundary enriched gene in a TRAP-seq experiment that was done using seven-day old seedlings (Tian et al. 2014). The boundary zone with a low rate of cell divisions, low auxin accumulation, and high expression of *CUC* genes is similar to another location where *EPFL2* is expressed – the sinus of leaf teeth (Wang et al. 2016; Tameshige et al. 2016). *EPFL4* and *EPFL6* are not expressed during embryogenesis. After germination *EPFL4* and *EPFL6* are expressed weakly throughout the entire SAM.

ERf expression in the central zone and the expression of *EPFLs* at the periphery of the meristem or at the bases of the leaf primordia suggests that the ERf signaling pathway enables communications between the border region and the central zone. This conclusion is also supported by the ability of *EPFL1* to rescue the quadruple mutant phenotype when expressed under the *KAN* but not the *CLV3* promoter. Taken together, our data suggest that EPFLs diffuse from the borders and activate ERfs at the outer boundary of the central zone of the meristem, restricting SAM width and promoting leaf initiation.

Recently it has been proposed that ERfs function in the L1 layer of the meristem where they sense signals coming from internal layers of the SAM (Kimura et al. 2018). Our data is not consistent with this conclusion. Kimura et al. utilized only two promoters to interrogate the function of ERfs and did not measure the meristematic parameters at multiple developmental points using numerous samples to obtain statistically significant data. Since the expression of *ERECTA* in many different regions of the meristem alters behavior of meristematic cells it is important to obtain quantitative measurements for precise comparisons. Moreover, it is necessary to take into account the differences in the expression levels of *ERECTA*. For example, while *ERECTA* expressed under the *ANT, KAN* and *WUS* promoters rescues meristem defects in a similar manner, the first two promoters drive *ERECTA* expression at much higher level

suggesting that the SAM is much more sensitive to ERECTA that is localized in the organizing center. In addition, our data suggests that the ligands are endogenously expressed at the boundary of the meristem and not in the internal layers. Their expression in the internal layers by *CLV3* cannot efficiently rescue the meristematic defects of *epfl1 epfl2 epfl4 epfl6* mutant. While Kimura and colleagues state that EPFLs are secreted by in the internal layers of the SAM data supporting that conclusion is not provided.

Expression of a gene under an exogenous promoter is a popular approach to interrogate gene function in a specific tissue. This approach has been effective in revealing the function of ERfs. Here, we would like to emphasize some issues associated with this approach. First, to prove that a gene controls a particular process from a specific tissue it is necessary to use a sizable range of exogenous promoters. Because expression of *ERECTA* in a variety of non-overlapping tissues has an effect on meristematic processes and elongation of organs, the use of a limited number of promoters we believe has been misleading (Kimura et al. 2018). Second, the expression pattern of a gene under an exogenous promoter can differ from what is expected and it is essential to evaluate the actual expression pattern. For example, while in situ data suggest that *ANT* is expressed in leaf and flower primordia (Elliott et al. 1996; Long et al. 2000), the commonly used 6.5k promoter of that gene drives expression in the L1 layer of the meristem as well (An et al. 2004). Finally some promoters can lead to variety of expression levels and expression patterns. An example is the *STM* promoter. In situ data indicates that *STM* is expressed throughout the SAM and is downregulated in the forming organ primordia (Long et al. 1996; Long et al. 2000). However, in transcriptional reporter assays the *STM* promoter induced diversity of expression patterns that differed from endogenous: the reporters were expressed underneath the shoot apical meristem in the cells of the hypocotyl, in the vascular cells of the leaf primordia, preferentially at the boundary of SAM, or in the peripheral region but not the central region of the SAM (Verkest et al. 2005; Landrein et al. 2015; Kim et al. 2003). Similarly in our

experiments we observed *STM* expression underneath the SAM and in leaf primordia. Moreover expression levels between created transgenic lines varied more than 500 times. Hypothetically these differences in expression could be due to inconsistent epigenetic regulation of the STM promoter in new locations (Katz et al. 2004).

By expressing *ERECTA* in different regions of the SAM we anticipated to rescue meristematic phenotypes. What we did not expect is to rescue the elongation of aboveground organs. *ERf* genes promote elongation of internodes, pedicels, petioles, siliques, leaves, and flower organs. Single *er* mutants have compact inflorescences as a result of shorter internodes and pedicels (Torii et al. 1996). The *erl1* and *erl2* mutations enhance organ elongation defect of *er* and the loss of all three genes results in severe dwarfism (Shpak et al. 2004). Previously it has been proposed that ERfs promote internode and pedicel growth by enabling cell-to-cell communication between the endodermis and phloem (Uchida et al. 2012). Our data suggests that ERECTA can promote organ elongation while expressed in a variety of locations including the central zone of the SAM. Most significantly our data suggest that expression in the phloem is not essential for ERfs to promote elongation of organs. Does ERfs regulate organ elongation from the SAM? We can envision several mechanisms that would allow this. Internodes are initially formed through activity of the peripheral zone that generates progenitor cells for epidermis and cortex and the rib zone that supplies cells for the central cylinder. As observed above the activity of ERECTA in the central zone promotes initiation of leaves in the peripheral zone. Thus it is not a big stretch to imagine that ERfs promote proliferation of cells surrounding forming leaf primordia. Alternatively ERfs might regulate growth of internodes indirectly, for example through controlling homeostasis of hormones such as auxin and gibberellin. This later possibility can account for the ERfs ability to regulate organ growth when expressed in a variety of tissues including from the phloem and the SAM. Our data indicates that understanding of the ERfs role in organ elongation is incomplete and requires further investigation.

The phenotype of *epfl1 epfl2 epfl4 epfl6* mutant suggests that in addition to regulation of meristem structure all four genes promote elongation of internodes and pedicels with *EPFL4* and *EPFL6* playing the major role in this process. The expression pattern of *EPFL1* and *EPFL2* in internodes and pedicels and their precise role in organ elongation is yet to be established. While quadruple mutant grows much slower compared to *epfl4 epfl6* its final size only slightly below *er* mutant and considerably bigger compared to *er erl1 erl2*. This result suggests that either other ligands contribute to regulation of organ elongation or perhaps ERf also can regulate organ elongation independently of ligands binding. Previously we demonstrated that the kinase dead ERECTA promotes organ elongation when expressed in *er erl1 erl2* (Kosentka et al. 2017). If the main outcome of EPF/EPFL binding is the activation of the ERf's kinase domain then it is consequent that the phenotype of *epfl1 epfl2 epfl4 epfl6* will resemble that of the kinase dead receptor.

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Appendix

Table 3.1 Primers used for cloning.

WUS.PRO.for	ccGGATCCGTATGATCTCTGTTGTACTCAC
WUS.PRO.rev.	GGCTCCATGGGTGTTTGATTCG
KAN.PRO.for	AAGGATCCAAGACCAACACAAACAAATTACC
KAN.PRO.rev.	GGCCATGGAATTAAAGAAACCTTTCTCTTG
STM.pro.for.v2	CCGGATCCTACAATTTCTCTAGCCTCCGTTTAATTT
STM.Pro.Rev.v3.	*ATATCTCTAAACAGAGC*CATCTTCTCTTTCTCTCACTAG
ER.pSTM.OH.for	CTAGTGAGAGAAAGAGAAGATG *GCTCTGTTTAGAGATAT*
ER.1100.xbal.rev.	ACATATGAAGTCTAGAAGCAGAATAACT
ANT.ER.OH.rev	ATATCTCTAAACAGAGCCATGGTTTCTTTTTTTGGTTTCT
ANT.PRO.for	CCGGATCCTATTATTGTGTTTCTCCTTTCTCT
ER.ANT.OH.for	AGAAACCAAAAAAAGAAACCATGGCTCTGTTTAGAGATAT
CLV3.Pro.for	CCGGATCC ATAAAATTAATCGAATTCCGG
CLV3.Pro.rev	*ATATCTCTAAACAGAGC*CATTTTTAGAGAGAAAG
ER.pCLV3.OH.for	CTTTCTCTCTAAAAATG *GCTCTGTTTAGAGATAT*
ER.tCLV3.OH.rev	AGCAACAAGAGATTAGG*CTACTCACTGTTCTGAGAA*
CLV3.ter.for	*TTCTCAGAACAGTGAGTAG* CCTAATCTCTTGTTGCT
CLV3.ter.rev	GG CTGCAGTCGAC ATTAAAAATAATACATTTATAATCAA
EPFL1-1	CACCTCTGTTCTCCCTGAGGAAA
EPFL1-2	TTTTGAGTTGGATTCAAGAATTACTACTATAA
attL1-T2.1	ccccTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTaagctt
attL2-T2.1	ggggTCTTATAATGCCAACTTTGTACAAGAAAGCTGGGTggatcc
Q.EPF1-1	AAAAAAGCAGGCTaagctt ACGACGATGTCCTCTTTTGTC
Q.EPF1-2	AAGAAAGCTGGGTggatcc GATATATTATCGCAAGTG
EPF2-2b	GTTTATAATCTTTTTTTTTAACAAGAAGAAAC
Q.EPF2-1	AAAAAAGCAGGCTaagctt TGGTCTAGAGAACAAGTGAAG
Q.EPF2-2	AAGAAAGCTGGGTggatcc GTTTATAATCTTTTTTTTT
Q.EPFL2-1	AAAAAAGCAGGCTaagctt GACATTTGTAGTACAACC
Q.EPFL2-2	AAGAAAGCTGGGTggatcc TTTCAGACACGAGATCGG
Q.EPFL3-1	AAAAAAGCAGGCTaagctt CGATTCATGGGTAGGTCCAT
Q.EPFL3-2	AAGAAAGCTGGGTggatcc TTTCTATGATTCTTTTTACT
Q.EPFL7-1	AAAAAAGCAGGCTaagctt TAAAATTGGATAATTGTGGGG
Q.EPFL7-2	AAGAAAGCTGGGTggatcc CTCTCTCTTTTCAAAGGCTT
Q.EPFL8-1	AAAAAAGCAGGCTaagctt TTTGGAGCTTCCCTTACAAGC
Q.EPFL8-2	AAGAAAGCTGGGTggatcc ATCATCACAATTTTCTCAAA
Q.EPFL9-1	AAAAAAGCAGGCTaagctt CTTGGAATTCAGTCGTCTAAC
Q.EPFL9-2	AAGAAAGCTGGGTggatcc TCTCTACTTCTTCTTCTTCT
Epfl1.us.bam	ACGGATCCTTAAGTCATGGTTATATAC
Epfl1.ds.pst1.rev	ACATAGAACTGCAGTTCAAAATTTAAG
Epfl2.us.bam	TTTGGATCCCTAAATCGCTCTAGAC
Epfl2.ds.Pst1.rev	CACACTCTGCAGTTTTCTTTATG
Epfl1.nco	ATCCAACTCAACCATGGTTGCTATATAC
Epfl1.clv3.ter	CCACTTTTATAATCCTTAACCTAATCTCTTGTTG
Clv3.ter.epfl1.rev	CAACAAGAGATTAGGTTAAGGATTATAAAAGTGG
Clv3.pro.epfl1	CTTTCTCTCTAAAAATGTTTGCTATATACAAATC
Epfl1.clv3.pro.rev	GATTTGTATATAGCAAACATTTTTAGAGAGAAAG

Table 3.2 Primers used for genotyping *epfl1-1* and *epfl2-1.*

Primer name	sequence	purpose
3' dSpm	TACGAATAAGAGCGTCCA TTTTAGAGTGA	Genotyping *epfl1*
epfl1.74	ATCCTTTCTTCAACCTATCCAACCTCCT	
epfl1.436.rev	TTAAGGATTATAAAAGTGGCCATTGCA	
epfl2.1	ATGGTGTGGAGCAGCAACATGTCAAGC	Genotyping *epfl2*
epfl2.540.rev	TCAAGGGTTGTAGATAGAGTTACCA	
GUS.43.rc	GTTTTTTGATTTCACGGG	

Table 3.3 Primers used for RT-PCR

Primer name	sequence	Annealing temperature
Act2-1	GCCATCCAAGCTGTTCTCTC	51.9°C
Act2-2	GCTCGTAGTCAACAGCAACAA	
qPCR ERF	TGAATGTGGCCAACAATGATCTGG	61.9 °C
qPCR ERR	TTTTGAAATGCTCGGGGTATAGTGC	
epfl1.1	ATGTTTGCTATATACAAATCAACCCTTCTTC	52 °C
epfl1.436.rev	TTAAGGATTATAAAAGTGGCCATTGCA	

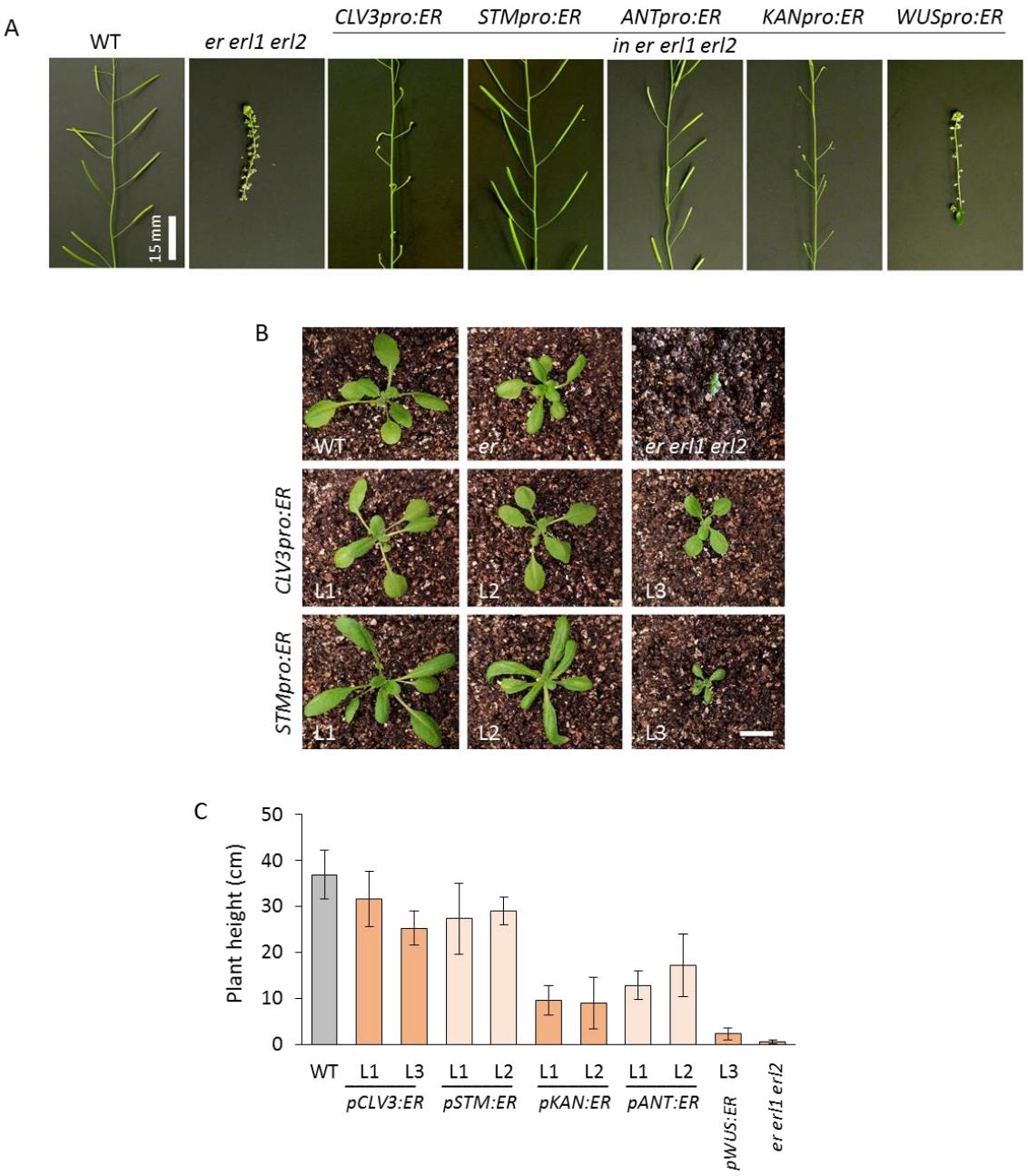

Figure 3.10. The effect of *ERECTA* expression under different promoters on plant morphology. A. Expression of *ERECTA* under *STM* and *ANT* promoters rescues infertility of *er erl1 erl2*. B. 20 days old plants, bar=1 cm. C. The plant height was measured in 5 week old plants. N=5-16 for heights.

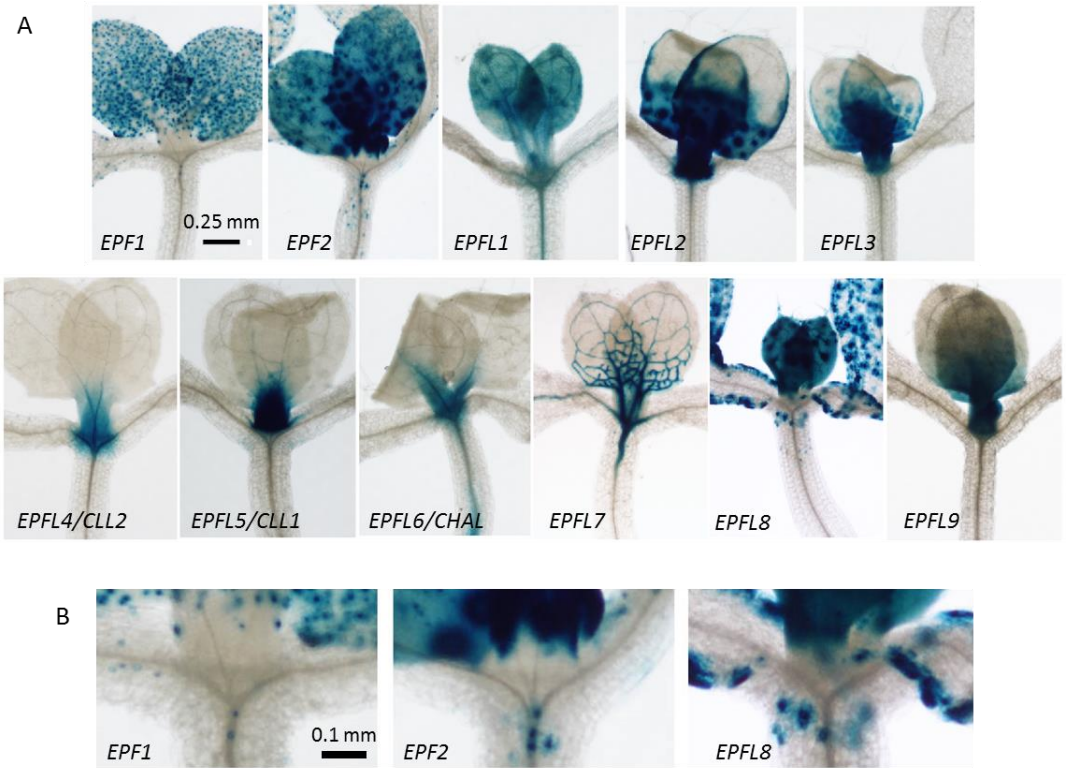

Figure 3.11 The GUS reporter gene assay of the *EPF/EPFL* gene family in the SAM demonstrates distinct patterns of expression in seedlings (A) and shows an absence of expression in the SAM for *EPF1, EPF2*, and *EPFL8* (B). Seedlings are 5 days post germination. In B are magnified images of the SAM region from seedlings depicted in A.

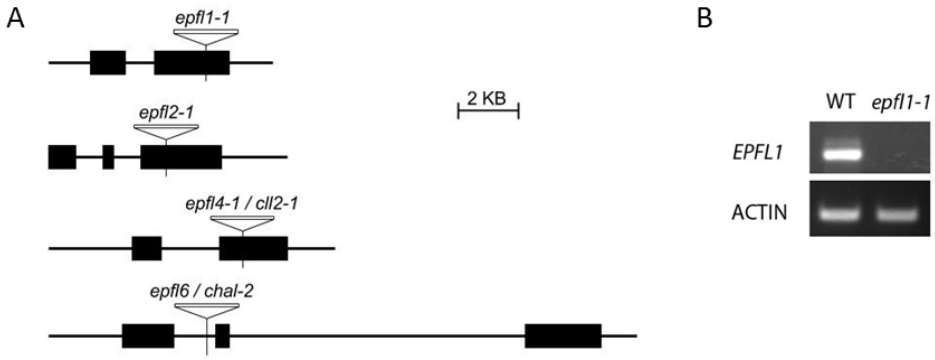

Figure 3.12 *epfl1-1* is a null mutant with a transposon insertion in the second exon. A. Schematic of the gene structure and insertion sites for mutants used in this study. Lines indicate introns or UTR regions, bars indicate exons, triangles indicate T-DNA insert position for *epfl4 and epfl6* or transposon site for *epfl1-1 and epfl2-1*. B. RT-PCR analysis of *epfl1-1*

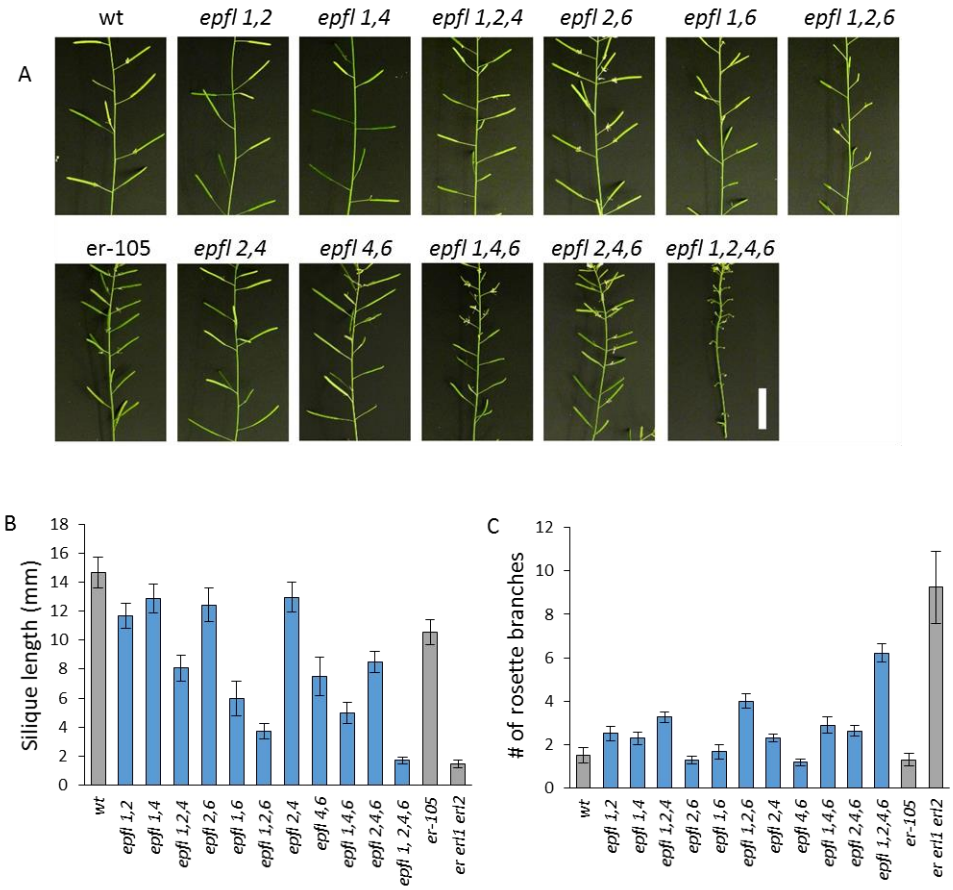

Figure 3.13. *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* partially redundantly regulate flower development and apical dominance. (A) The wild type, *er-105* and *epfl* family mutant inflorescence stems. Scale bar=15 mm (B) Length of mature siliques on the main inflorescence stem (n=78-158; 7-8 measurements per stem). (C) The number of rosette branches is increased in some of epfl family mutants and in the *er erl1 erl2* mutant (n=6-9). B and C. Bars represent the average; Error bars represent S.D.

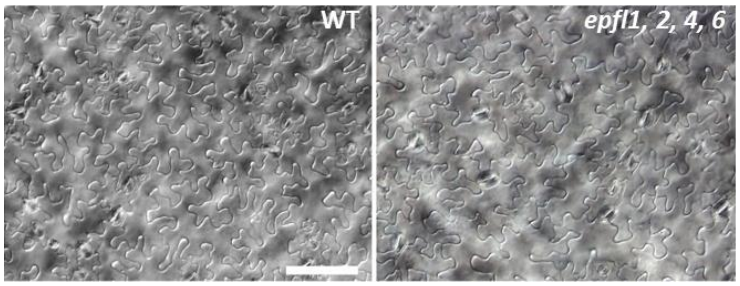

Figure 3.14. The *epfl1, 2, 4, 6* mutant does not exhibit obvious stomata patterning defects. The scale bar=100µm. The abaxial epidermis of cotyledons from 12 days post germination seedlings.

CHAPTER 4 CONCLUSION

Central to understanding the development of multicellular organisms is understanding how cells are able to communicate with one another. The ERECTA family signaling pathway is of particular importance in plant development due to the wide range of developmental processes it is involved in. The works presented in this dissertation have provided novel biological information about how the ERECTA receptor functions and how its use is integrated into regulating the SAM. Understanding how signaling pathways function opens the door for engineering improved crop plants that are better suited for harsh environments and increased yields.

In Chapter 2, a structure function analysis of ERECTA's cytoplasmic domain was presented. This is the first study that specifically examines the cytoplasmic kinase of ERECTA to understand how it is regulated. This work highlighted the importance of the juxtamembrane domain and kinase activity for ERECTA signaling. Analysis of putative phosphorylation sites in the activation loop of the kinase reveled that that threonine 807 phosphorylation could be a means to activate and increase kinase signaling. Additionally, tyrosine residues 815 and 820 showed a dominant negative response when mutated to aspartate which suggests their role as inhibitory phosphorylation sites of ERECTA signaling. Lastly, this chapter showed that nonfunctional versions of ERECTA were still able to partially rescue organ elongation in *er erl1 erl2* mutants suggesting that there are multiple molecular mechanisms that control different aspects of ERECTA family signaling.

Additional work related to chapter 2 should include an analysis of how the juxtamembrane domain and kinase activation loop interact with other cytoplasmic components of ERECTA signaling. There are several possibilities for how the juxtamembrane domain (JMD) contributes to signaling: as a docking site for phosphorylation of downstream components, a pivot arm that positions the two receptor kinase domains into an active conformation, or the JMD could serve as a regulatory region for the ERECTA kinase (Jura, Endres et al. 2009, Sengupta, Bosis et al. 2009, Oh, Clouse et al. 2012). Creating a chimeric version of

ERECTA where the JMD is swapped with one from another plant RLK would help in understanding if its role is specific to ERECTA signaling or a general feature that other receptors share. Unfortunately, no direct downstream targets of ERECTA are currently known, therefore JMD function as a docking site cannot be studied. The cytosolic kinase of the receptor-like kinase SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)3 has been shown to phosphorylate the kinase domain of ERECTA in vitro (Meng, Chen et al. 2015). A biochemical approach to examine which residues are phosphorylated by SERK3 and whether the JMD contributes to this would provide insight into how the two receptors function in terms of regulating each other. Lastly, the fact that inactive kinase versions of ERECTA were able to rescue some of the ERf related phenotypes should be re-examined to understand what portions of the receptor contribute to this role.

In Chapter 3, an analysis of ERECTA family signaling components in the SAM was presented. It was shown that ectopically expressing the ERECTA receptor in the CZ restores meristem phenotypes in the *er erl1 erl2* mutant. Expression analysis of the 11 member ligand family revealed that *EPFL1, EPFL2, EPFL4* and *EPFL6* were endogenously expressed near the SAM. Analysis of the *epfl1 epfl2 epfl4 eplf6* mutant revealed that it shares the same meristematic phenotype as *er erl1 erl2* mutant with regards to SAM width and leaf initiation. Lastly, expressing *EPFL1* in the peripheral zone restored SAM function which led to the model that ERECTA ligands in the peripheral zone signal to the receptor in the central zone, thus allowing for communication between the two zones in the SAM.

Additional work related to the ectopic expression of *ERECTA* part of chapter 3 should address the non-cell autonomous function of *ERECTA* expression in the central zone. The result of CZ expression of the receptor rescuing the rate of leaf initiation was exciting because organ generation is believed to be controlled in the PZ. One explanation for ERECTA's non-cell autonomous action is through the plant hormone auxin which marks the sites and

regulates organ initiation (Reinhardt, Pesce et al. 2003). The *er erl1 erl2* mutant has been shown to over accumulate auxin in the outermost layer of the SAM, presumably preventing auxin sequestration and not allowing leaves to form (Chen, Wilson et al. 2013). Examining auxin distribution using auxin reporters in lines where *ERECTA* is expressed in the CZ would address if this phenotype is rescued through auxin sequestration in the CZ. Auxin distribution could also be an explanation for the partial complementation of SAM phenotypes in other lines ectopically expressing *ERECTA*. Another possibility for how ERECTA regulates leaf initiation from the CZ is through regulating the differentiation of stem cells. As stem cells become displaced away from the CZ they undergo differentiation and eventually become the cells that make up organs. If the differentiation step is blocked then the PZ would be made up of stem cells that are unable to become leaves. A major challenge in examining the cell identity in the SAM is that the lack of a true stem cell marker. Historically, the stem cells in the SAM have been defined by *CLAVATA3* expression but recent examination of cell division rates has revealed that slowly dividing cells make up a much smaller portion of cells than previously thought (Fletcher, Brand et al. 1999, Burian, Barbier de Reuille et al. 2016). Investigating the chromatin states of cells in the SAM could lead to an improved stem cell marker and aid in understanding the phenotype of the *er erl1 erl2* mutant.

Additional work related to the EPFL ligands regulating SAM functions should be aimed to examine how the ligands are regulated and whether there are differences in receptor activation. One study examining the role of *EPFL2* on leaf serrations found the ligand to form a negative feedback loop between auxin response and *EPFL2* which would allow for a highly localized response (Tameshige, Okamoto et al. 2016). Understanding how *EPFL1, EPFL2, EPFL4* and *EPFL6* are regulated in terms of expression patterns would help in understanding their exact role in SAM regulation. Another interesting feature of the four SAM related ligands is their redundancy in function even though they greatly differ in peptide sequence. EPFL1 and EPFL2 contain roughly 20

additional amino acids in their loop region when compared to the other 9 EPF/EPFL ligands (Ohki, Takeuchi et al. 2011). The loop region has been shown to control specificity of the ligands; swapping the loop from a positive regulator of stomata (EPFL9) onto a scaffold of a negative regulator of stomata (EPF2) reverses their function (Ohki, Takeuchi et al. 2011). Further dissecting the differences between EPFL1/EPFL2 and EPFL4/EPFL6 would help in understanding the specificities of the ligand family.

The findings presented in this work have the potential to impact the engineering of agriculturally relevant plants. Reducing water loss from transpiration and optimizing crop yield are two major targets that ERf signaling could contribute to in engineering improved crop varieties. Using the results from chapter 2, the signaling output of ERf receptors could be increased which should decrease stomata density. This method of water loss has already been shown through *EPF2* overexpression but modifying the kinase could have the benefits of fine tuning the signaling output (Franks, T et al. 2015). Using the results from chapter 3, crop yield could be optimized by decreasing EPFL signaling in the SAM to increase its size. The components that regulate WUS/CLV3 signaling and impact SAM size have already been shown to have a dramatic impact on crop yield in both tomato and maize (Xu, Liberatore et al. 2015, Je, Gruel et al. 2016). Both methods of modifying ERf signaling to engineer improved crop varieties require a better understanding of the mechanisms in the less agriculturally relevant species, *Arabidopsis thaliana.*

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VITA

Pawel Zbigniew Kosentka was born 1989 in Opole Poland. He graduated from Farragut High School in 2007. He attended the University of Tennessee in Knoxville where he graduated in 2011 with a Bachelor of Arts and Sciences degree in Biology (biochemistry, cellular, and molecular). He stayed at the University of Tennessee to pursue a doctorate degree specializing in plant development and molecular biology