

***ERECTA* Family Genes Regulate the Shoot Apical Meristem
and Organ Formation**

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Dedication

This work is dedicated to my mother, Andrea, and my father, Ray. They are my first and greatest teachers.

Above all, this work is dedicated to my wife, Erin. She always believes in me, even when I sometimes do not believe in myself.

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Abstract

Plants are sessile and must adjust their organ growth to their environments. A reservoir of stem cells in the shoot apical meristem (SAM) supplies cells for differentiation into organs. The SAM must balance organ production with stem cell maintenance. The *ERECTA* family (ERfs) encodes the leucine-rich repeat receptor-like kinases *ERECTA* (*ER*), *ERECTA-LIKE 1* (*ERL1*), and *ERL2*. Erf signaling regulates organ initiation and stem cell maintenance. Results presented in this work include the following:

1) *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) make up a negative feedback loop to maintain SAM size. *WUS* and *CLV3* expression localization is critical for their function. Using genetic analysis, we show that ERfs and EPIDERMAL PATTERNING FACTOR ligands (EPFL) synergistically regulate SAM size with *CLV3*, and the *wus* mutation is epistatic to Erf signaling. The *erf* triple mutant has enlarged *CLV3* and *WUS* expression domains, and Erf activation by EPFL causes a decrease in *WUS* and *CLV3* transcript levels independently of protein translation. These findings show that Erf-EPFL signaling is required to restrict lateral stem cell overproliferation in the SAM.

2) Leaf initiation is regulated by the hormone auxin. Auxin peaks precede organ initiation. The auxin transport protein, PIN1, concentrates auxin in the L1 layer of the SAM during polar auxin transport. Mutants deficient in ERfs have decreased leaf formation and altered auxin signaling. The auxin responsive reporter, *DR5rev::GFP*, shows that *er erl1 erl2* has elevated auxin signaling in the L1 layer, but cannot initiate leaves. Exogenous auxin or auxin efflux inhibition elevates auxin accumulation in the L1 layer of the *er erl1 erl2* SAM and rescues leaf initiation, indicating the threshold for leaf initiation is elevated in the mutant. ERfs regulate organ formation synergistically with PID, a PIN polarity regulator. Analysis of *DR5rev::GFP* in *pid er erl1 erl2* indicates leaves do not form due to decreased auxin response. Taken together, we show that ERfs have a dual role in organ initiation by promoting auxin responses and inhibiting auxin accumulation.

This work shows *ERECTA* family signaling functions in the SAM to prevent lateral overproliferation of stem cells and promotes auxin response to drive leaf initiation.

Table of Contents

Chapter 1: Introduction.....	1
Role and organization of the SAM.....	2
Establishment and maintenance of the SAM.....	5
ERf-EPFL signaling pathway regulates plant development.....	9
ERf-EPFL signaling pathway regulates the SAM.....	13
The role of auxin in organ initiation.....	15
The role of auxin transport in organ initiation.....	17
Additional factors in organ initiation.....	21
ERfs regulate organ initiation.....	22
Chapter 2: ERECTA family signaling constrains <i>CLAVATA 3</i> and <i>WUSCHEL</i> to the center of the shoot apical meristem.....	29
Abstract.....	30
Introduction.....	31
Results.....	33
Discussion.....	40
Materials and methods.....	45
Chapter 3: ERfs regulate auxin response in the shoot apical meristem to promote leaf initiation.....	49
Abstract.....	50
Introduction.....	51
Results.....	54
Discussion.....	77
Materials and methods.....	84
Chapter 4: Conclusion.....	90
References.....	96
Vita.....	107

List of Figures

Figure 1.1. SAM organization and regulatory factors.

A. A cross sectional diagram of the SAM showing the localization of meristematic regulators. The L1 and L2 layers extend across the entire SAM, and the L3 layer makes up the internal tissues. The central zone (CZ) (red dashed line), the peripheral zone (PZ) (gray), and rib zone (RZ) (beneath SAM) are labeled. Leaf primordia (LP) are emerging from the PZ. Cytokinin is synthesized in the upper layers of the SAM (blue diagonal lines). *CLV3* is expressed in the CZ (green, including L1 layer of CZ). *WUS* is expressed in the organizing center (yellow diagonal lines). *HAM* is expressed in the organizing center and below (pink, including the organizing center). **B.** Cytokinin and *WUS* make up a positive feedback loop. *WUS* activates *CLV3*. *CLV3* inhibits *WUS* expression in a negative feedback loop. *HAM* prevents *WUS* activation of *CLV3* to exclude *CLV3* from the organizing center. This centers the organizing center on the vertical axis.....3

Figure 1.2. Leaf formation on the SAM through auxin peaks.

A. Auxin accumulates into peaks (green) on the L1 layer of the SAM through PIN1 (green arrows) to mark incipient primordia (IP). **B.** Primordia (P) emerge with a reversal in PIN1 polarity at the base of the to direct auxin toward the surrounding tissue away from the primordia. On the primordia, PIN1 is oriented toward the distal end of the forming organ. The internal tissues also express PIN1 and mark the forming vasculature. The primordium then becomes a source of auxin.....18

Figure 1.3. Key boundary markers of the SAM.

Lateral view of the SAM depicting KAN1 (blue) and REV (red), which straddle a ring of cells that are eligible for primordia initiation (Green).....23

Figure 1.4. ERfs regulate leaf initiation and have altered auxin distribution.

A. Number of leaves initiated are decreased in *er erl1 erl2* compared to the wild type (wt) as determined by DIC microscopy of fixed samples. DPG = days post germination. Values are means $\pm$ SD. n = 22-25 plants. Values significantly different from the control are indicated by asterisks ($P < 0.05$).

B. Auxin accumulation as shown by the auxin responsive *DR5rev::GFP* reporter in WT and *er erl1 erl2*. Auxin maxima are present in WT and ingress into the internal tissues. In *er erl1 erl2* auxin accumulation is elevated on the epidermal layer and is disjointed from the internal tissues. Adapted from Chen et al., 2013.....24

Figure 1.5. Auxin signaling pathway.

A. In the absence of auxin, Aux/IAA receptors in their unbound state repress auxin response factors (ARF). **B.** The E3 ubiquitin-ligase Skip/Cullin/F-Box containing complex (SCF) and TRANSPORT INHIBITOR RESPONSE 1 (TIR1), collectively SCF^{TIR1}, binds to the repressor Aux/IAA when exposed to auxin. **C.** Aux/IAA repressors are ubiquitinated and targeted to the proteasome for degradation. **D.** ARFs are free to promote the transcription of AREs, and an auxin response follows.....26

Figure 2.1. Expression of *CLV3* is expanded laterally in the *er erl1 erl2* mutants.

A. Confocal images of the SAM region of 1 DPG wild-type (wt) and mutant seedlings transformed with *CLV3p:H2B-GFP:CLV3t* (green). The cell walls were stained with SR2200 (blue). The white arrow indicates extended expression of the reporter in the L1 layer. **B.** Measurements of *CLV3p:H2B-GFP:CLV3t* expression in the SAM of WT, *er erl1 erl2* mutants and the segregating siblings of *er erl1 erl2* (*er erl2*). L.A and L.B are two independent WT lines, L.1 and L.2 are two independent mutant lines. The median is indicated as a horizontal line between two boxes, upper and lower quartiles are represented by the boxes, and the vertical lines designate the maximum and the minimum. Grey dots represent individual data points. n=6-8. For multiple comparisons (one-way ANOVA with Tukey post-hoc test), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum P-value of <0.01. **C.** Schematic of the seedling meristematic zone demonstrating how width #1 and width #2 of the SAM were measured in **B**.....35

Figure 2.2. Expression of *WUS* is expanded laterally in the *er erl1 erl2* mutants.

A. Confocal images of the SAM region of 1 DPG seedlings transformed with *WUSp:H2B-GFP* (green). The cell walls were stained with SR2200 (blue). **B.** Measurements of *WUSp:H2B-GFP* expression in the SAM of *er erl1 erl2* mutants, their segregating siblings (*er erl2*) and F1 generation seedlings from the cross of the L.1 line with the wild type (WT). L.1 and L.2 are two independent lines. The median is indicated as a horizontal line between two boxes, upper and lower quartiles are represented by the boxes, and the vertical lines designate the maximum and the minimum. Grey dots represent individual data points. n=5-9. For multiple comparisons (one-way ANOVA with Tukey post-hoc test), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum P-value of <0.01.....37

Figure 2.3. *WUS* and *CLV3* are targets of the ERF signaling pathway.

A. *CLV3* relative expression levels in above-ground parts of 5 DPG seedlings in genotypes as indicated. **B-D.** Relative expression levels of selected mRNAs in above-ground organs of 3 DPG *epfl1 epfl2 epfl4* (**B**), *er erl1 erl2* (**C**), or *clv3 epfl1 epfl2 epfl4 epfl6* (**D**) seedlings after treatment with 10 μ M EPFL4 or EPFL6 peptides as indicated compared with mock treatment. Seedlings were treated with peptides for 6 h (**B and C**), and from 1 to 6 h as indicated (**D**). +CHX indicates treatment with 10 μ M cycloheximide. Data are mean $\pm$ S.D. *ACTIN2* was used as an internal control. *P<0.05 (unpaired two-tailed Student's t-test).....39

Figure 2.4. Model for the role of ERF signaling in regulation of shoot meristem maintenance.

EPFL signals originating from the periphery of the SAM activate the ERF signaling cascade that restricts the expression of *CLV3* and *WUS* to the center of the SAM. LP, leaf primordium.....44

Figure 3.1. Leaf initiation in the *er erl1 erl2* mutant is limited by auxin availability.

A. Inhibition of auxin biosynthesis by kynurenine (Kyn) inhibits leaf initiation in both wild type (WT) and *er erl1 erl2*. **B.** Exogenous auxin (2,4D) promotes leaf initiation in *er erl1 erl2* but not in the WT. **A, B.** The rate of leaf primordia initiation was determined by DIC microscopy of fixed samples. Seedlings were grown in light or dark as indicated. DPG = days post germination. The mean is indicated as a thick horizontal bar. Blue is WT and red is *er erl1 erl2*. The vertical lines designate the maximum and the minimum. Each grey point represents number of leaves from one individual plant. Different lowercase letters above bars indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test. NS = not significant. n=9-20.....55

Figure 3.2. Decreased auxin transport partially rescues leaf initiation in *er erl1 erl2*.

A. The *pin1* mutation partially rescues leaf initiation defects of the *er erl1 erl2* mutant. Data are mean $\pm$ S.E. Two independent experiments were performed. Experiment #1 in light grey n=23-30; experiment #2 in dark grey n=10-11; Asterisks above bars indicate significant difference at $p < 0.005$ (*) and $p < 0.0005$ (**) as measured by unpaired two-tailed Student's t-test. **B.** While *pin2*, *pin3*, *pin4* and *pin7* mutations do not change leaf initiation on their own, *pin2*, *pin3*, and *pin7* partially rescue leaf initiation of *er erl1 erl2* n=10-20. An asterisk above a bar indicates significant difference at $p < 0.01$ compared to the wt as measured by unpaired two-tailed Student's t-test. NS – no statistical difference. **A and B.** Seedlings were grown on solid MS media. Data are mean $\pm$ S.E. **C and D.** Inhibition of auxin transport with NPA promotes leaf initiation in *er erl1 erl2* while it inhibits leaf initiation in the WT seedlings in light and in dark. NPA cannot promote leaf initiation in *er erl1 erl2* seedlings in the presence of 10 μ M kynurenine (+Kyn) (turquoise or magenta). The rate of leaf primordia initiation was determined by DIC microscopy of fixed samples. Seedlings were grown in liquid MS media in light or dark as indicated. DPG = days post germination. The mean is indicated as a thick horizontal bar. Blue is WT and red is *er erl1 erl2*. The vertical lines designate the maximum and the minimum. Each grey point represents number of leaves from one individual plant. Different lowercase letters above bars indicate significant difference at $p < 0.05$, as determined by one-way ANOVA with Tukey's post test. n=8-20.....57

Figure 3.3. Auxin signaling is altered at early stages of seedling development in *er erl1 erl2*.

A-C. Confocal micrographs depicting auxin localization in the SAM of WT and *er erl1 erl2* in 1 DPG seedlings using the *DR5rev::GFP* auxin responsive reporter. **A.** In WT, *DR5rev::GFP* is expressed in the distal tips of the leaf primordia. Incipient primordia show expression in punctate maxima and signal ingresses downward into the lower tissues. **B.** In *er erl1 erl2*, only one leaf has formed, and expression is elevated and restricted to the epidermal layers of the emerging primordia. **C.** Expression in *er erl1 erl2* is elevated,

sometimes patchy, and does not always coincide with primordia formation.

A-C. Green = *DR5rev::GFP* expression. Blue = DAPI. Scale bar = 25 μ m. A and B are maximum projections of multiple optical sections, and C is a single optical section.....61

Figure 3.4. Inhibiting auxin efflux increases auxin accumulation in the L1 layer of *er erl1 erl2*

A. Optical sections of 1 DPG mock and NPA treated *er erl1 erl2* seedlings expressing *DR5rev::GFP*. Auxin accumulation is elevated on the L1 layer of *er erl1 erl2* in the presence of the auxin efflux inhibitor NPA (right) compared to mock (left). **B.** Quantitative measurements of the L1 layer (left) and the L2 layer (right) of the *er erl1 erl2* SAM. The presence of NPA increases the *DR5rev::GFP* signal in the L1 layer of *er erl1 erl2*, but there is no significant difference on the L2 layer. Data points are mean signal intensity $\pm$ S.E over the distance indicated on the x-axis. White circle = mock. Black diamond = 5 μ M NPA. Asterisks above data points indicate significant difference at $p < 0.05$ as measured by unpaired two-tailed Student's t-test. NS = not significant. **C.** Diagram of quantitative measurement process shown in B. The L2 layer measurements are slightly shorter due to the tight radius of the L2 layer in the dome-shaped SAM. Red arrows = direction of measurement from leaf primordia (zero) to center of SAM. Blue = L1 layer measurement. Turquoise = L2 layer measurement. LP = leaf primordia. **D.** Representative confocal micrograph of 1 DPG *pin1 er erl1 erl2* expressing *DR5rev::GFP* with elevated auxin accumulation on the L1 layer. **A and D.** Blue = DAPI. Green = *DR5rev::GFP*. Scale bar = 25 μ m.....62

Figure 3.5. ERfs and *PID* synergistically promote initiation of cotyledons and leaves.

A. Representative images of 13 DPG seedlings demonstrate reduced number of cotyledons and leaves in *pid er erl1 erl2*. Scale bar = 2 mm. **B.** Comparison of cotyledon number in selected mutants versus WT. *Pid* and ERfs strongly, and synergistically interact in cotyledon initiation, but *PIN1* and ERfs weakly interact. **C.** Representative image of a one-month-old *pid er erl1 erl2* plant demonstrates the presence of a shoot apical meristem, and an absence of cotyledon and leaves. Scale bar = 0.5 mm. **D.** Comparison of formed leaf primordia in WT and mutants using 5 DPG seedlings. Data are mean $\pm$ S.E; $n=10-15$. Different lowercase letters above bars indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test.....65

Figure 3.6. ERfs and *PID* synergistically promote auxin signaling during cotyledon initiation.

Optical sections of WT, *er erl1 erl2*, *pid*, and *pid er erl1 erl2 er erl1 erl2* embryos expressing *DR5rev::GFP*. Each genotype has a DIC image overlaid with corresponding fluorescent image (left) and the fluorescent image alone (right). In WT, *DR5rev::GFP* expression is elevated at the tips of cotyledon primordia, and there is clear expression in the forming vasculature of the internal tissues. The *er erl1 erl2* mutant is similar to wild type, but expression is increased and there is variation in the forming vasculature expression. The

<i>pid</i> mutant forms broad peaks in the epidermis of the cotyledons, and the internal expression is absent. Cotyledons do not form in <i>pid erl1 erl2</i> , but auxin signaling is elevated on the flank surrounding the SAM and is absent in the internal tissues. Green = <i>DR5rev::GFP</i> . Scale bar = 20 μ m.....	68
Figure 3.7. ERfs and <i>PID</i> synergistically promote auxin signaling in the SAM.	
The <i>DR5rev::GFP</i> reporter indicates auxin signaling is present and elevated in periphery of the vegetative SAM of the 3 day post germination <i>pid erl1 erl2</i> seedling, but leaves cannot initiate. Green = <i>DR5rev::GFP</i> . Blue = DAPI. Scale bar = 25 μ m.....	70
Figure 3.8. Aboveground tissue differentiation in <i>pid erl1 erl2</i> is partially rescued by auxin.	
A. Primordia tissue initiates in 3 DPG <i>pid erl1 erl2</i> in a ring-like pattern when germinated on 2,4-D supplemented media. Scale bar = 50 μ m. B. Callus tissue with leaf-like qualities initiate in a ring-like pattern in <i>pid erl1 erl2</i> after transfer to standard media following 16 days of 2,4-D treatment. The meristematic region forms bulging green organs with trichomes. Plants shown are 33 DPG. Transfer took place at 13 DPG. Scale bar = 1 mm.....	72
Figure 3.9. Downregulation of ERf signaling in the <i>pin1</i> mutant partially rescues organ initiation and increases auxin signaling in the epidermis of the inflorescence meristem.	
A. The <i>erl2</i> mutations partially rescue flower organ initiation defects observed in <i>pin1</i> . The <i>pin1 erl1 erl2</i> mutant is an extreme dwarf which is able to form numerous randomly positioned carpeloid organs. B. Auxin signaling distribution in the inflorescence SAM of <i>pin1</i> and <i>pin1 erl2</i> mutants, as determined by the activity of the synthetic auxin response element <i>DR5rev::GFP</i> . Auxin signaling in the <i>pin1</i> mutant is elevated with the loss of <i>erf</i> signaling. Scale bar = 100 μ m.....	74
Figure 3.10. <i>PIN1</i> and ERfs synergistically promote inflorescence growth.	
A. Representative images of <i>erl1 erl2</i> and <i>pin1 erl1 erl2</i> . The <i>pin1 erl1 erl2</i> takes longer to bolt. Scale bar = 5 mm. B. Average length of the main stem at maturity. Data are mean $\pm$ SD, n=8-30. Different lowercase letters above bars indicate significant difference at P < 0.05, as determined by one-way ANOVA with Tukey's post test. C. Representative images of two-month-old plants. <i>Quad</i> = <i>pin1 erl1 erl2</i> . Two quad plants are depicted. Pictures are at the same magnification. Scale bar = 3 cm.....	76
Figure. 3.11. ERfs have a dual role in auxin regulation.	
A. Functional ERfs inhibit endogenous auxin signaling and promote L1 layer auxin response in the SAM to efficiently initiate leaf formation. B. Loss of all three <i>erfs</i> causes elevated auxin signaling, and decreased L1 layer auxin response, which prevents efficient leaf initiation. C. Inefficient leaf initiation in <i>erl1 erl2</i> due to inadequate L1 layer auxin responses can be partially rescued by elevating auxin accumulation in the L1 layer through exogenous auxin or decreased auxin efflux. D. The <i>erl1 erl2</i> mutant with inadequate auxin response in the L1 layer is further exacerbated by decreasing levels with the auxin biosynthesis inhibitor, Kynurenine (Kyn). Organ formation is decreased and leaves cannot form.....	79

List of abbreviations

ARE – Auxin Response Element
ARF – AUXIN RESPONSE FACTOR
Aux/IAA – Auxin/Indole-3-Acetic Acid
AUX/LAX – AUXIN/LIKE-AUXIN
CLV1/CLV3 – CLAVATA 1/3
DPG – Days post germination
DR5 – DR5 auxin response promoter
EPF/EPFL – EPIDERMAL PATTERNING FACTOR/EPF-LIKE
ER/ERL1/ERL2/ERf – ERECTA/ER-LIKE 1/ERL2/ER family
HAM – HAIRY MERISTEM
KAN - KANADI
Kyn – Kynurenine
LRR-RLK – Leucine rich repeat receptor-like kinase
MAPK – MITOGEN ACTIVATED PROTEIN KINASE
ML1 – MERISTEM LAYER 1
MP – MONOPTEROS
NPA - N-1-Naphthylphthalamic Acid
PAT – Polar auxin transport
PID - PINOID
PILS – PIN-LIKES
PIN – PIN-FORMED
REV - REVOLUTA
SAM – Shoot apical meristem
SERK - SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE
STM – SHOOT MERISTEMLESS
TAA/TAR - TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS/TRYPTOPHAN
AMINOTRANSFERASE RELATED
TIR1/AFB - TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX family
TPL – TOPLESS
WUS – WUSCHEL
YUC – YUCCA
2,4-D – 2,4-Dichlorophenoxyacetic acid

Chapter 1: Introduction

Role and organization of the SAM

Size and shape of plants are essential for their survival. Organs such as leaves, flowers, and stems must be produced at rates that they can nourish and support the plant, without expending unnecessary resources. Plants cannot move and must precisely initiate organs to have the correct architecture to maximize organ function in the context of the environment. For example, leaves must be generated in specific patterns to maximize photosynthesis for the plant. Furthermore, plant organs are generated throughout their entire life, and a plant's embryonic body plan is dramatically different from their final body plan.

Plants control their architecture and are able to constantly form organs using self-renewing populations of stem cells known as meristems. The shoot apical meristem (SAM) is a collection of undifferentiated stem cells reserved at the apex of all land plants that gives rise to aboveground organs (Doerner, 2003; Truskina and Vernoux, 2018; Shi and Vernoux, 2019; Uchida and Torii, 2019). Because the SAM produces new organs throughout the entire life of the plant, the maintenance of the stem cell pool must be perfectly balanced with tight regulation of organ differentiation. If the SAM does not produce enough stem cells to keep up with organ formation, the SAM will terminate, no more leaves will form, and the plant will be unable to keep up with its photosynthetic demands.

The structure of the SAM is divided into zones of cells with different differentiation statuses and division rates (Figure 1.1, A). Pluripotent cells in the meristem are located in the central zone and are undifferentiated. These cells divide slowly and are gradually

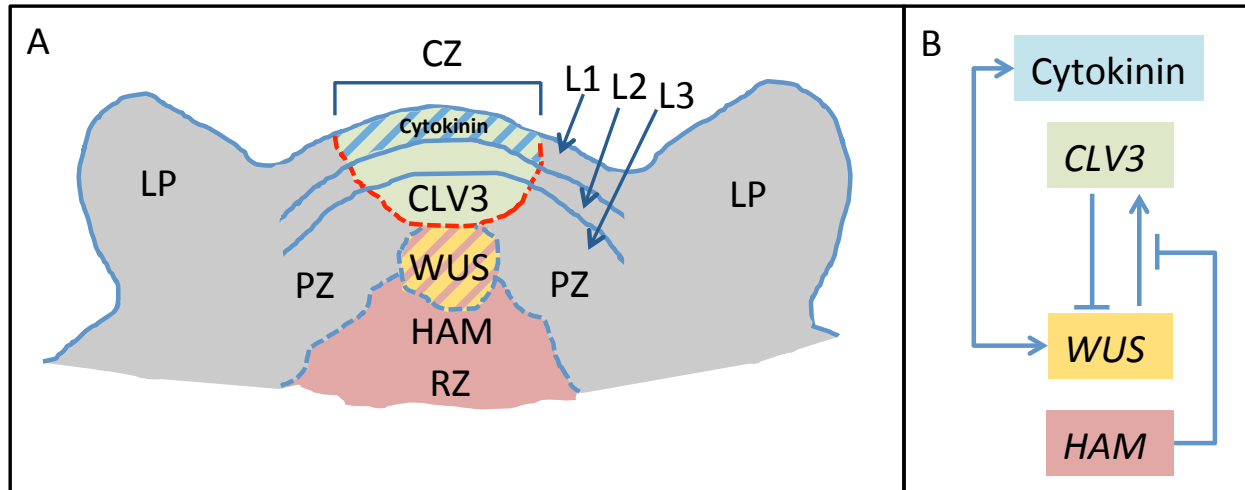


Figure 1.1. SAM organization and regulatory factors.

A. A cross sectional diagram of the SAM showing the localization of meristematic regulators. The L1 and L2 layers extend across the entire SAM, and the L3 layer makes up the internal tissues. The central zone (CZ) (red dashed line), the peripheral zone (PZ) (gray), and rib zone (RZ) (beneath SAM) are labeled. Leaf primordia (LP) are emerging from the PZ. Cytokinin is synthesized in the upper layers of the SAM (blue diagonal lines). *CLV3* is expressed in the CZ (green, including L1 layer of CZ). *WUS* is expressed in the organizing center (yellow diagonal lines). *HAM* is expressed in the organizing center and below (pink, including the organizing center). **B.** Cytokinin and *WUS* make up a positive feedback loop. *WUS* activates *CLV3*. *CLV3* inhibits *WUS* expression in a negative feedback loop. *HAM* prevents *WUS* activation of *CLV3* to exclude *CLV3* from the organizing center. This centers the organizing center on the vertical axis.

displaced from the central zone. The displaced cells will produce leaf and flower primordia and internodes. As the central zone cells divide and are displaced downward into the rib zone, they will form the vasculature of the internodes (Miyashima et al., 2013). If the central zone cells are displaced laterally into the peripheral zone, the cells will contribute to leaf primordia and the outer layers of internodes (Furner I and Pumfrey, 1992). The farther away cells move from the central zone, the faster they divide, and differentiate (Reddy et al., 2004). The cells in the two outermost layers of the SAM, the L1 and L2 layers, are restricted in their division patterns (Satina et al., 1940). Chimeric plants with genetic markers indicate that these layers remain distinct from each other, and only divide anticlinally, or perpendicular to the surface of the tissue (Satina et al., 1940; Marcotrigiano and Bernatzky, 1995). L2 layer cells displaced from the central zone can occasionally undergo periclinal divisions, or divisions parallel to the surface of the tissue (Zhang et al., 2021). The innermost cells of the SAM, known as the L3 layer, divide in all directions to give rise to the interior tissues of the plant (Satina et al., 1940).

The molecular signals that determine the rate and orientation of SAM cell divisions are studied extensively, because these parameters influence the availability of cells to contribute to differentiating tissue without depleting the pool of cells in stem cell niche. The stem cells in the SAM do not have a predetermined lineage fate as they often have in other multicellular organisms. Their fate is instead governed by their location with respect to the molecular signals exchanged from cell to cell (Vochting, 1898; Poethig, 1989). Signals that maintain the apical stem cell niche and signals that promote aboveground organ formation will be discussed in this work.

Establishment and maintenance of the SAM

The late torpedo embryonic stage is the earliest stage of development where stem cells are distinguishable by their large nuclei and small vacuoles (Barton and Poethig, 1993; Laux et al., 1996). The genetic markers of the meristem are present, even before this stage. *SHOOT MERISTEMLESS (STM)* is one of the first genetic meristem regulators that appears during the globular stage of embryogenesis (Long et al., 1996). The maize homolog to *STM*, *KNOTTED 1 (KN1)*, was first identified as a stem cell regulator through a gain of function mutation that caused overproliferation of stem cells (Smith et al., 1992). In *Arabidopsis*, *STM* encodes a class I knotted-like homeodomain transcription factor (Barton and Poethig, 1993), and the *stm* loss of function mutant lacks a SAM entirely (Barton and Poethig, 1993). The mechanism with which *STM* prevents cell differentiation is not fully understood, but the plant hormone cytokinin likely plays a role. *STM* expression causes an increase in cytokinin biosynthesis through upregulation of ISOPENTENYL TRANSFERASE 7 (*ipt7*) expression, and an application of cytokinin to *stm* mutants can ectopically induce meristem formation (Yanai et al., 2005), suggesting cytokinin can promote stem cell formation independently of *STM*. However, *STM* may inhibit cell differentiation independently of cytokinin. Loss of function *stm* mutants grown in cytokinin cannot fully rescue meristem function, and the meristem will eventually terminate (Scofield et al., 2014).

STM is expressed throughout the SAM, excluding emerging organ primordia (Long et al., 1996; Lenhard et al., 2002; Lau et al., 2012). The cells in the meristem differentiate as they divide and are displaced from the central zone, indicating there are signals that locally

regulate their behavior besides the broadly expressed *STM*. One of these meristematic regulators is the homeodomain transcription factor WUSCHEL (*WUS*) (Mayer et al., 1998). *WUS* is one of the earliest meristematic markers. It is visible in the 16-cell stage of embryogenesis (Mayer et al., 1998). From the late heart stage onward, *WUS* is expressed in a narrow set of cells that are three to four cell layers below the epidermis of the meristem in the L3 layer of the central zone (Mayer et al., 1998). *WUS* expression marks the organizing center – a group of pluripotent stem cells that inhibits differentiation of the surrounding cells. Loss of function *wus* mutants cannot maintain the stem cell niche, and the organ formation quickly depletes the stem cell pool (Laux et al., 1996). The *WUS* protein is mobile, and can travel through plasmodesmata (Yadav et al., 2011; Daum et al., 2014), or small membranous channels connecting adjacent cells (Sager and Lee, 2018). This allows *WUS* to move upward to maintain a pluripotent stem cell identity of the L1 and L2 layers, and the upper cells of the L3 layer central zone.

Molecular signaling is required to keep the *WUS/CLV3* feedback loop at the correct depth from the surface of the SAM (Figure 1.1, A and B). *WUS* activity is strongly influenced by cytokinin. Application of exogenous cytokinin increases *WUS* expression (Lindsay et al., 2006; Gordon et al., 2009). *WUS* then targets and inhibits the transcription of type-A *ARABIDOPSIS RESPONSE REGULATORS (ARRs)* that negatively regulate cytokinin signaling (To et al., 2004; Leibfried et al., 2005). The absence of the negative regulator promotes cytokinin signaling, which reinforces *WUS* expression in a positive feedback loop. It was shown that the cytokinin biosynthesis enzyme, LONLEY GUY (*LOG*), promotes meristem activity in rice (Kurakawa et al., 2007). In *Arabidopsis*, *LOG4* is expressed in the upper

layers of the SAM, creating a gradient of cytokinin that activates *WUS* expression (Chickarmane et al., 2012).

Because *STM* promotes cytokinin biosynthesis, *STM* may indirectly target *WUS* through the cytokinin biosynthesis pathway (Jasinski et al., 2005; Gordon et al., 2009). However, *WUS* expression precedes *STM* during embryogenesis (Long et al., 1996; Mayer et al., 1998), indicating that other mechanisms may establish *WUS* expression, and *STM* may only act to reinforce *WUS* in later stages. Interestingly, inducible ectopic expression of *WUS* and *STM* expression is sufficient to induce stem cell proliferation, showing the strong influence of these two genes on meristem activity (Gallois et al., 2002).

While it is important for *STM* and *WUS* to maintain the pluripotency of stem cells in the stem cell niche, experiments such as the ectopic expression of *WUS* and *STM* highlight the importance of restricting stem cell proliferation to the SAM. Overproliferation of the SAM results in the uncontrolled formation of organ primordia (Gallois et al., 2002). *STM* and cytokinin promote *WUS*, but to prevent overproliferation of stem cells, *WUS* must be held in check and restricted to the organizing center. *WUS* expression remains restricted to several cell layers below the epidermis in the SAM for the entire life of the plant because of the inhibitory effect of the small peptide CLAVATA 3 (CLV3) (Mayer et al., 1998; Schoof et al., 2000). The loss of function *clv3* mutant has an enlarged meristem due to the overproliferation of stem cells (Leyser and Furrer, 1992). The CLV3 peptide is secreted to neighboring cells where it is proposed to bind to a large number of receptor-like kinases (RLKs) including CLV1, CLV2, CORYNE (CRN), BARELY ANY MERISTEM 1, 2, and 3 (BAM), RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), and CLAVATA3 INSENSITIVE RECEPTOR

KINASES (CIKs) (Clark et al., 1993; Clark et al., 1997; Kayes and Clark, 1998; Jeong et al., 1999; DeYoung et al., 2006; Müller et al., 2008; Kinoshita et al., 2010; Hu et al., 2018). Once CLV3 is secreted and bound by the cell surface receptor, it activates a signaling cascade to inhibit *WUS* expression and prevent stem cell overproliferation. *WUS* travels to neighboring cells to directly bind to the *CLV3* promoter and activate its expression in the L1, L2, and upper cells of the L3 layer (Fletcher et al., 1999; Daum et al., 2014). However, *WUS* expression is excluded from the upper cell layers despite cytokinin biosynthesis genes active in the L1 and L2 layers. Therefore, in the current model of *CLV3/WUS* signaling, *CLV3* prevents *WUS* from being expressed in the upper cell layers of the SAM (Chickarmane et al., 2012).

The interaction between cytokinin, *WUS*, and *CLV3* accounts for the correct distance of the organizing center from the apex of the SAM. Another signal from the basal cell layers confines the position of the organizing center. The HAIRY MERISTEM (*HAM*) family of transcription factors were first identified in petunia and named for the terminating *ham* mutant meristems that give rise to trichomes on the L1 layer (Stuurman et al., 2002). In *Arabidopsis*, the *ham1 ham2 ham3* mutant inflorescence meristem also lacks indeterminate organ formation, and in some cases the inflorescence meristem arrests entirely (Engstrom et al., 2011). *HAM* is expressed below the organizing center in the rib zone of the SAM and prevents *CLV3* expression in the deeper layers (Zhou et al., 2018). In the *ham1 ham2 ham3* vegetative meristem, *CLV3* expression shifts downward, indicating that *HAM* transcription factors are necessary to restrict *CLV3* expression to the uppermost layers (Zhou et al., 2018). The shift in *CLV3* expression in *ham1 ham2 ham3* also corresponds to an expansion of the vegetative meristem (Engstrom et al., 2011). *HAM1* and *HAM2* are physical

interacting partners with WUS, and HAM prevents WUS from activating *CLV3* expression (Zhou et al., 2015). More work must be done to understand what the protein-protein interaction between HAM and WUS does to regulate their activities, but the discovery of HAM signaling in meristem regulation shows the WUS/*CLV3* feedback loop requires signaling from adjacent cells to center the organizing center.

ERf-EPFL signaling pathway regulates plant development

Recently, the *ERECTA* family genes (ERfs) have been shown to regulate meristem function. The *ERECTA* mutation was first named after the Landsberg *erecta* (*Ler*) accession that has a compact, upright stature (Rédei, 1992). It is now known to be the result of a mutation in a leucine-rich repeat receptor-like kinase (LRR-RLK) (Torii et al., 1996). Later, two other LRR-RLKs in the *ERECTA* family were found: *ERECTA-LIKE 1* and *ERECTA-LIKE 2*. The three receptors synergistically regulate many developmental processes (Shpak, 2013). Due to redundancy, the only single mutant with a phenotype is *er* (Shpak et al., 2004). It is shorter in stature, with elongation of siliques and the stalks that connect organs to the stem, or pedicels (Torii et al., 1996; Shpak et al., 2004). The length of internodes, pedicels and siliques is further reduced in *er erl1* and *er erl2* double mutants (Shpak et al., 2004). The triple mutant *er erl1 erl2* phenotype is the most dramatic. It is a severe dwarf, and its flower organs are defective and sterile (Shpak et al., 2004). ERfs also regulate stomata density and distribution. The single mutant *er*, and the double mutants *er erl1* and *er erl2* have increased stomata density, and stomata clusters occur in *er erl1 erl2* (Shpak et al., 2005). Because the *er erl1 erl2* mutant phenotype is so much more severe than single and

double *erf* mutants, it is evident that *ER*, *ERL1*, and *ERL2* synergistically regulate many aspects of plant development (Shpak et al., 2004; Shpak et al., 2005).

Consistent with their function, *ER*, *ERL1*, and *ERL2* have overlapping, but unique expression patterns in young proliferating tissues (Shpak et al., 2004). In leaves and cotyledons, ERfs are strongly expressed in the younger organs, but expression decreases as the organs age and become more established (Shpak et al., 2004; Chen and Shpak, 2014). ERfs are expressed in all young flower organs, but as the flower matures, expression is restricted to developing ovules (Shpak et al., 2004).

ERfs consist of an extracellular LRR receptor domain, a transmembrane domain, and an intracellular kinase domain (Torii et al., 1996; Lease et al., 2001). Erf receptors function as heterodimers with other LRR receptor-like kinases from SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (Meng et al., 2015; Fan et al., 2016). When the ligands of Erf are bound, ERfs and SERKs transphosphorylate one another (Meng et al., 2015). Higher order *serk* mutants mimic *erf* mutant phenotypes indicating a high level of redundancy between them (Meng et al., 2015). During stomata formation, the LRR receptor like protein TOO MANY MOUTHS (TMM) also binds ERfs to modulate their affinity to the ligands (Lee et al., 2012; Lin et al., 2017). Upon ligand binding, the Erf cytoplasmic kinase domain activates a downstream MITOGEN ACTIVATED PROTEIN KINASE (MAPK) signal cascade shown through genetic experimentation to be made up of *YODA*, *MKK4*, *MKK5*, *MPK3*, and *MPK6* (Bergmann et al., 2004; Wang et al., 2007; Meng et al., 2012).

The extracellular domain of ERfs binds a family of small secretory peptides known as EPIDERMAL PATTERNING FACTOR/EPF-LIKE (EPF/EPFL) (Abrash et al., 2011; Lee et

al., 2012; Uchida et al., 2012a; Lee et al., 2015; Tameshige et al., 2016). Eleven EPF/EPFL ligands exist in *Arabidopsis* (Hara et al., 2009). While many EPF/EPFLs function redundantly, specific groups of ligands occupy unique functions.

Phylogeny analysis of EPF/EPFLs indicates that several structural clades of EPF/EPFLs exist: *EPF1/EPF2/EPFL7*, *EPFL9*, *EPFL1-3*, and *EPFL4-6/EPFL8* (Takata et al., 2013). The ligands within each clade often have overlapping functions. *EPF1* and *EPF2* are expressed in the epidermis in stomata and stomata lineage cells to regulate stomata density (Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009). *EPFL9* also regulates stomata development, but it is thought to be an antagonist rather than the agonists as *EPF1* and *EPF2* (Sugano et al., 2010; Shimada et al., 2011). The *EPFL1-3* and *EPFL4-6/EPFL8* clades regulate organ elongation. *EPFL2* regulates leaf shape, and loss of function *epfl2* cannot elongate leaf teeth (Tameshige et al., 2016). *EPFL4* and *EPFL6* regulate plant height and flower organ elongation. The loss of function *epfl4 epfl6* mutant is shorter and has a more compact inflorescence (Abrash et al., 2011). The additional loss of *epfl5* mutant conveys lower fertility, and more compact inflorescences in the triple mutant *epfl4 epfl5 epfl6* compared to *epfl4 epfl6* (Uchida et al., 2012a). Recently, the *epfl1 epfl2 epfl4 epfl6* quadruple mutant was found to have a similar phenotype to *er erl1 erl2*. Both mutants are short, compact, and produce sterile deformed flowers (Kosentka et al., 2019).

EPF/EPFLs have several structural and posttranslational modifications required to be biologically active. The nascent propeptides carry an N-terminal secretory signal sequence that targets the peptide for secretion to the extracellular space (Kondo et al., 2009). Once the N-terminal signal sequence is cleaved and the propeptide is secreted,

EPF/EPFLs are thought to be cleaved again by subtilisin-like proteases to make the final mature peptide. The subtilase SBT5.2/CO₂ RESPONSE SECRETED PROTEASE (CRSP) is able to cleave a synthetic EPF2 peptide (Engineer et al., 2014), and CRSP is required for EPF2 stomata signaling (Engineer et al., 2014). EPF/EPFLs have a number of conserved cysteine residues that are required for their activity as shown by *in vivo* site directed mutagenesis (Kondo et al., 2009; Rychel et al., 2010). The final processed peptides are small – under 50 amino acids in size – with a β -sheet scaffold domain, a loop region, and an N-terminal tail. The N-terminal tail is extended in EPFL1 and EPFL2 hinting at subfunctionalization in their activity (Rychel et al., 2010; Lin et al., 2017). Although more work must be done to show how EPF/EPFLs differentially activate their receptors, a breakthrough was achieved by engineering chimeric EPF/EPFL ligands. By switching the loop domains of EPF2, an agonist, with EPFL9 an antagonist, it was shown that the loop regions in EPF/EPFLs are major drivers in their functional specificity (Ohki et al., 2011).

The function of EPF/EPFLs has been studied thoroughly during stomata formation (Shpak, 2013; Zoulas et al., 2018; Endo and Torii, 2019; Chen et al., 2020). Upon binding of the agonists EPF1 and EPF2, ERfs activate the MAPK cascade to inhibit stomata proliferation through the negative regulation of the transcription factor SPEECHLESS (SPCH) (Hara et al., 2007; Lampard et al., 2008; Hara et al., 2009; Hunt and Gray, 2009). ERfs are inhibited by STOMAGEN/EPFL9, which prevents binding of EPF1 and EPF2 and as a result SPCH is not phosphorylated (Lee et al., 2015).

ERf-EPFL signaling pathway regulates the SAM

ERfs regulate aboveground organ formation and growth. The loss of function *er erl1 erl2* mutant has shortened petioles, reduced leaf expansion, and cannot elongate the stem which contributes to its dwarfed appearance (Shpak et al., 2004). Maintenance of procambium cells in the vasculature is disrupted in *er* and *er erl1* mutants, which may contribute to the decreased stem height (Uchida and Tasaka, 2013). ERfs have been proposed to regulate the proliferation of stem cells and differentiation of stem cells for organ formation in the SAM (Uchida et al., 2012c). It was suggested that this is due to the loss of an unknown factor required for cytokinin response buffering, but the relationship between ERfs and cytokinin is unclear (Uchida et al., 2012b).

Single and double *erf* mutants have a wild type SAM size, but when all three *erfs* are lost the SAM is dramatically enlarged (Chen et al., 2013). The *er erl1 erl2* SAM is significantly larger than wild type as early as one day post germination (DPG), and remains this way throughout the life of the plant (Chen et al., 2013). The transcript levels of two crucial meristematic regulators, *WUS* and *STM*, are increased in *er erl1 erl2*, suggesting the enlarged meristem may be the result of increased stem cell proliferation (Uchida et al., 2012b; Chen et al., 2013).

The MAPK cascade that is genetically downstream of ERfs in the stomata development pathway might also be downstream of ERfs in SAM maintenance. A constitutively active variant of the MAPKKK, YODA (CA-YODA), reduces *er erl1 erl2* mutant SAM width to near wild type size (Chen et al., 2013). However, YODA has many interacting

partners, and more data is required to identify the final target of ERfs that regulates SAM size.

Four ligands, EPFL1, EPFL2, EPFL4, and EPFL6, redundantly regulate ERF function in the SAM. The *epfl1 epfl2 epfl4 epfl6* quadruple mutant has increased meristem width that is identical to *er erl1 erl2* (Kosentka et al., 2019). These *EPFL* genes are expressed and function in the border of the SAM. Ectopic expression of *EPFL1* or *EPFL2* under the peripheral zone promoter *KANADI*, can rescue *epfl1 epfl2 epfl4 epfl6* SAM width, but using the central zone promoter *CLV3* cannot (Kosentka et al., 2019). Additionally, the *er erl1 erl2* enlarged meristem is rescued by expressing *ERECTA* under the *CLV3* promoter, but not under the *KAN* promoter (Kosentka et al., 2019). This indicates that EPFLs maintain meristematic function by signaling from the periphery to the more centralized meristematic cells.

ERfs and EPFLs regulate SAM size, but it was previously unclear how this occurs molecularly. The cells that are displaced from the central zone by cell division are no longer subject to WUS/CLV3 regulation and are free to differentiate. The interactions between WUS, CLV3, HAM, and cytokinin regulate the apical basal patterning of the organizing center, but lateral organization of the SAM must also be regulated. Without a lateral signal to maintain the organizing center, cells might prematurely differentiate in the peripheral zone, or lateral overproliferation of stem cells could occur. We have recently acquired evidence that the ERF signaling cascade targets the *WUS/CLV3* feedback loop to maintain SAM size. These results will be detailed in Chapter 2.

The role of auxin in organ initiation

Up to this point, we have discussed stem cell maintenance in the SAM. The role of the SAM is to provide a constant supply of stem cells to emerging organs. The cells that exit the stem cell niche can acquire two different fates: to differentiate into either internode tissue or organ primordia. Organs are not initiated in the central zone in order to preserve the stem cell pool. Instead, organ primordia initiation occurs in the surrounding cells in the peripheral zone. As leaves initiate, it is important that they are not so close that they prevent other leaves from absorbing sunlight. To ensure organs do not form too close, leaves initiate at regular distances from one another.

The regular spacing between consecutive organs is known as phyllotaxy. The cotyledons and the first two true leaves are the first organs formed by the SAM in *Arabidopsis*. These first organs are formed opposite one another in an intersecting pattern (Irish and Sussex, 1992; Medford et al., 1992). The subsequent leaf and flower organs initiate 137.5° from a previously formed organ (Clarke et al., 1999). Phyllotaxy is established by a plant hormone signal known as auxin (Hofmeister, 1868; Snow and Snow, 1962; Douady and Couder, 1996). Auxin was first shown to influence organ formation in tomato by excising shoots and intermittently applying a lanolin paste containing auxin to the apex during regrowth (Laibach and Mai, 1936). Abnormalities such as fusions of leaves occurred in newly emerging organs. Auxin was later shown to influence phyllotaxy through direct topical application of a small point of the hormone paste to the shoot apex of *Lupinus albus* to cause leaf fusions and changes in divergence angles (Snow and Snow, 1937).

Major advances have been made toward understanding organ formation in the SAM. Genetic reporters that take advantage of the auxin signaling pathway show areas where auxin is accumulating in live plants. The synthetic *DR5* promoter was created by fusing together multiple auxin response elements (ARE), or genetic sequences known to bind the AUXIN RESPONSE FACTOR (ARF) family of transcription factors (Ulmasov et al., 1997). *DR5rev::GFP* expression in the SAM is seen in the peripheral zone where incipient primordia initiate (Benková et al., 2003; Heisler et al., 2005). This contributes to the hypothesis that areas of high auxin accumulation in the SAM, or auxin peaks, are the first molecular signal that induces cells to differentiate into organ primordia.

The *YUCCA* (*YUC*) family and the *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS/ TRYPTOPHAN AMINOTRANSFERASE RELATED* (*TAA/TAR*) family genes are the primary enzymes responsible for auxin biosynthesis in the SAM (Kasahara, 2016). Auxin is required for organ initiation, and higher order *yuc* mutants have severe organ initiation defects (Cheng et al., 2007a). Auxin biosynthesis genes are expressed throughout the SAM and do not correlate with auxin peaks or predict the formation of incipient organ primordia (Yadav et al., 2020). Thus, it is unlikely that auxin biosynthesis drives the precise auxin peak formation required for positioning organ primordia. Auxin must be transported into peaks. Auxin was first found to move from shoots to roots by applying radiolabeled auxin to the apex of corn coleoptiles and measuring the amount moved to the base (Goldsmith, 1967; Wilkins and Martin, 1967). This provided evidence that auxin is a mobile signal. Auxin movement is now known as polar auxin transport (PAT), or the movement of auxin from cell to cell to achieve long-range transport of the signaling hormone. Later, PAT was implicated in organ formation in the SAM.

The role of auxin transport in organ initiation

One significant breakthrough in understanding auxin transport in the SAM was the discovery of the *pin-formed* (*pin*) mutant, which lacks flowers creating pin-like stems (Okada et al., 1991). The lack of flowers is the result of defective auxin regulation. Placing lanolin paste containing auxin on *pin* apices, rescues flower formation (Reinhardt et al., 2000). In *Arabidopsis*, the *pin* mutant showed a dramatic decrease in basipetal auxin transport compared to wild type (Okada et al., 1991). Later, it was determined that the *pin* phenotype is due to a mutation in a plasma membrane localized carrier protein, and the gene was named *PIN1* for the phenotype it caused (Gälweiler et al., 1998). In *Arabidopsis*, there are eight PIN family auxin transporters that are divided into two groups: long PINs and short PINs (Křeček et al., 2009; Sauer and Kleine-Vehn, 2019). The long PINs – PIN1, PIN2, PIN3, PIN4, and PIN7 – have an intracellular loop region and are localized to the plasma membrane. The short PINs – PIN5, PIN6, and PIN8 – lack the intracellular loop and are embedded in the endoplasmic reticulum membrane. The short PINs regulate auxin homeostasis by modulating auxin flux from the endoplasmic reticulum to the cytosol (Mravec et al., 2009; Ding et al., 2012; Simon et al., 2016). The long PINs are auxin efflux transporters that accomplish PAT through their polar localization in the plasma membrane (Sauer and Kleine-Vehn, 2019).

The current understanding of organ initiation in the SAM is that PIN1 creates auxin peaks in the peripheral zone on the L1 layer (Figure 1.2, A) (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005). The depletion of auxin from the space adjacent to the incipient primordia prevents leaves from forming too close to one another (Traas

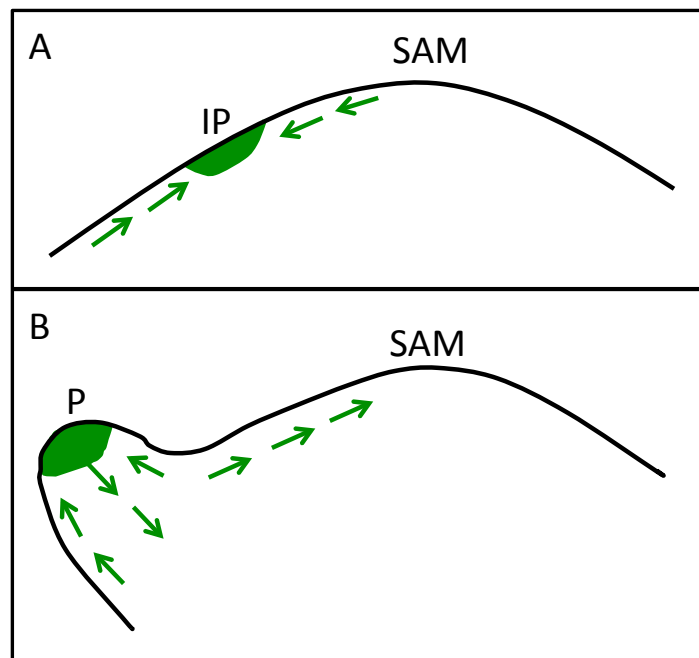


Figure 1.2. Leaf formation on the SAM through auxin peaks.

A. Auxin accumulates into peaks (green) on the L1 layer of the SAM through PIN1 (green arrows) to mark incipient primordia (IP). **B.** Primordia (P) emerge with a reversal in PIN1 polarity at the base to direct auxin toward the surrounding tissue away from the primordia. On the primordia, PIN1 is oriented toward the distal end of forming organs. The internal tissues also express PIN1 and mark the forming vasculature. The primordia then become a source of auxin.

and Monéger, 2010; Qi et al., 2014). After peak formation, organ primordia will emerge concomitantly with a reversal of PIN1 polarity at the base of the emerging primordia to direct PAT toward the surrounding epidermal tissue away from the primordia (Figure 1.2, B) (Heisler et al., 2005). Once a primordium bump emerges, PIN1 can be seen on the epidermis oriented towards the distal end of the forming organ. As primordia emerge, the internal tissues also express PIN1 and mark the eventual formation of the vasculature. The newly formed organ then becomes a source of auxin (Cheng et al., 2007a).

PIN1 is the lone member of the PIN family transporters expressed in both the vegetative SAM and forming leaves (Guenot et al., 2012). PIN2, PIN3, PIN4, and PIN7 are expressed in leaves, but are absent from the vegetative SAM (Guenot et al., 2012). Only *pin1* single mutants have decreased leaf formation and altered phyllotaxy (Guenot et al., 2012). In *pin* double mutants, only *pin1 pin4* double mutants have fewer leaves and more disrupted phyllotaxy than *pin1* indicating that *pin4* weakly contributes to organ formation. PIN1 is expressed in the inflorescence SAM, but the other PIN family transporters are absent. Additionally, driving PIN1 expression with the *MERISTEM LAYER 1 (ML1)* promoter in the L1 layer of the SAM is sufficient to rescue inflorescence formation and phyllotaxy in *pin1* mutants (Kierzkowski et al., 2013). This data collectively shows that PIN1 is the primary auxin PIN family transporter regulating flower formation in the inflorescence SAM, and leaf formation in the vegetative SAM.

Other families of auxin transport proteins facilitate the movement of auxin. There is growing evidence that a family of seven auxin transport facilitators known as PIN-LIKES (PILS) can alter cytosolic auxin levels (Barbez et al., 2012; Sauer and Kleine-Vehn, 2019).

Similar to the short PINs that reside primarily on the endoplasmic reticulum, PILS are thought to regulate auxin homeostasis by compartmentalizing auxin to the endoplasmic reticulum (Barbez et al., 2012). Root morphology and apical hook development have been connected to PILS activity (Barbez et al., 2012; Béziat et al., 2017), but PILS mediated auxin transport has not been connected to shoot organ primordia formation.

The AUXIN 1/LIKE-AUX1 (AUX/LAX) family is localized to the plasma membrane and facilitates auxin influx (Péret et al., 2012). Higher order *aux/lax* mutants do have organ formation defects. Phyllotaxy is most severely altered *aux1 lax1 lax2* (Bainbridge et al., 2008). *DR5rev::GFP* shows weaker auxin peaks in the inflorescence SAM of *aux1 lax1 lax2* compared to wild type (Bainbridge et al., 2008). However, these plants can still effectively initiate organ primordia. Therefore, current evidence indicates that AUX/LAX is needed more to reinforce auxin peaks on the SAM, but they do not directly drive peak formation. The final known auxin transport protein family is the ATP-BINDING CASSETTE B (ABCB) which uses ATP hydrolysis to facilitate movement of auxin across the plasma membrane (Cho and Cho, 2013; Wilkens, 2015). ABCB have been suggested to contribute to auxin efflux along with PIN in root tissue (Mravec et al., 2008), but there is no evidence of ABCB contributing to auxin peak formation on the SAM. While other transporters contribute to auxin patterning on the SAM, PIN family efflux transporters, especially PIN1, are the most important for organ formation.

Additional factors in organ initiation

Auxin signaling is not the only factor in organ formation. Some others are mechanical forces and transcription factors from two families: KANADI, and Class III HD-ZIP. It is possible to stimulate leaf initiation in *Nicotiana tabacum* using a lanolin paste on the L1 layer containing activators that cause inducible expression of expansins, or enzymes that loosen the cell wall (Pien et al., 2001). This indicates that expansins contribute to organ initiation and cell wall loosening can induce organ formation.

Genetic polarity cues are important for leaf initiation. The *KANADI* family genes (*KAN1* to *KAN4*) encode the GARP transcription factors and are expressed in a circular arrangement around the meristem marking the abaxial side of incipient primordia, or facing away from the central axis of the plant (Kerstetter et al., 2001). Loss of function *kan1 kan2 kan4* mutants can ectopically form leaves outside the peripheral zone on hypocotyl tissue (Izhaki and Bowman, 2007). The expression of KAN in the periphery of the SAM and *kan1 kan2 kan4* forming leaves outside the boundary region indicate *KAN* family may mark the peripheral zone boundary in the SAM.

Working in concert with *KAN* family genes are the Class III HD-ZIP genes, which encode the transcription factors *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), and *CORONA* (*CNA*). Class III HD-ZIP genes are expressed across the top of the SAM and on the adaxial side of primordia, or facing toward the central axis of the plant (McConnell et al., 2001; Otsuga et al., 2001; Caggiano et al., 2017). *KAN1* and *REV* expression straddles the ring of cells around the SAM on the L1 layer where organ primordia are initiated, further

supporting the role of KAN and REV in marking the peripheral zone (Figure 1.3) (Caggiano et al., 2017).

ERfs regulate organ initiation

Another regulator of leaf initiation is the *ERECTA* family. The *ER* locus was first suggested to have reduced leaf number in the *Ler* accession (El-Lithy et al., 2004). Due to the redundancy of ERf signaling, the effect was subtle. It was not until the triple *er erl1 erl2* mutant was analyzed that dramatically decreased leaf formation was seen (Chen et al., 2013) (Figure 1.4, A). After further analysis, *er erl1* and *er erl2* double mutants were also found to have decreased leaf formation compared to wild type (Chen et al., 2013). *Er erl1 erl2* has fewer leaves as early as 1 day post germination (DPG), and this persists throughout the life of the plant (Chen et al., 2013). Organ phyllotaxy is random in *er erl1 erl2* and does not resemble the wild type divergence angle of 137° (Chen et al., 2013). SAM size alone can influence phyllotaxy in ecotypes that have larger meristem size than Columbia (Landrein et al., 2015). However, larger SAM ecotypes initiate more primordia compared to Columbia wild type, indicating that the decreased leaf initiation in *er erl1 erl2* is likely the result of factors unrelated to SAM size.

A major breakthrough in understanding how ERfs promote leaf formation occurred by using the *DR5rev::GFP* reporter to observe auxin response in the *er erl1 erl2* mutant (Chen et al., 2013) (Figure 1.4, B). In seedlings that were 9 days old, expression is visible in peaks on the L1 layer in wild type, but *er erl1 erl2* has elevated expression that covers the entire L1 layer. Wild type expression of *DR5rev::GFP* should be visible in the vasculature of

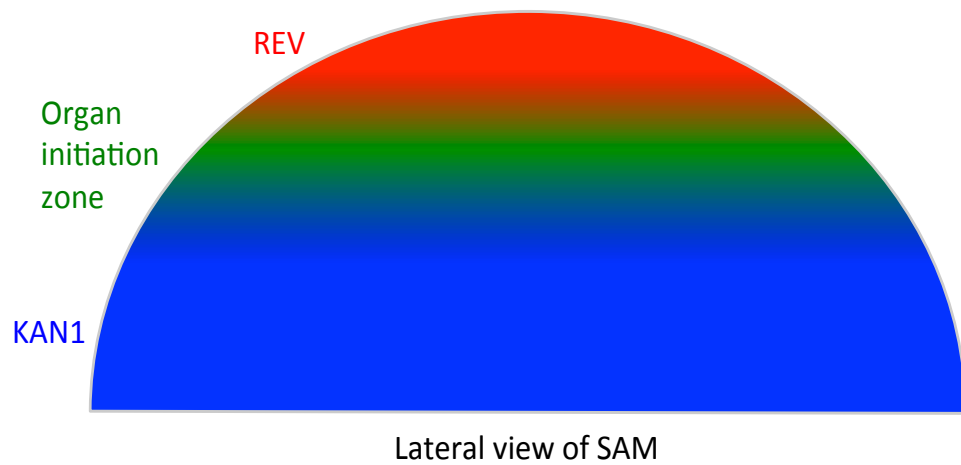


Figure 1.3. Key boundary markers of the SAM.

Lateral view of the SAM depicting KAN1 (blue) and REV (red), which straddle a ring of cells that are eligible for primordia initiation (Green).

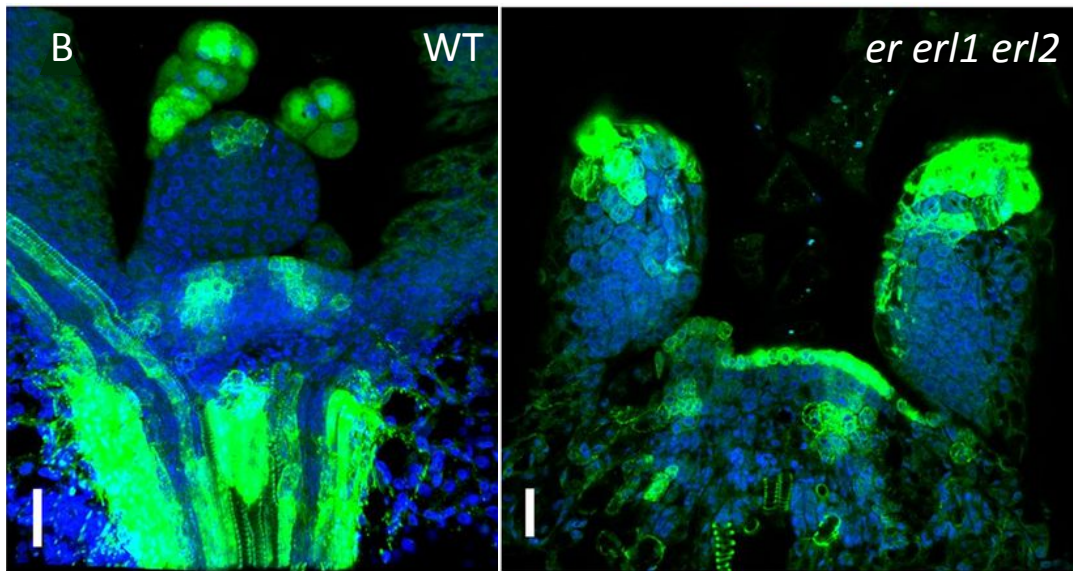
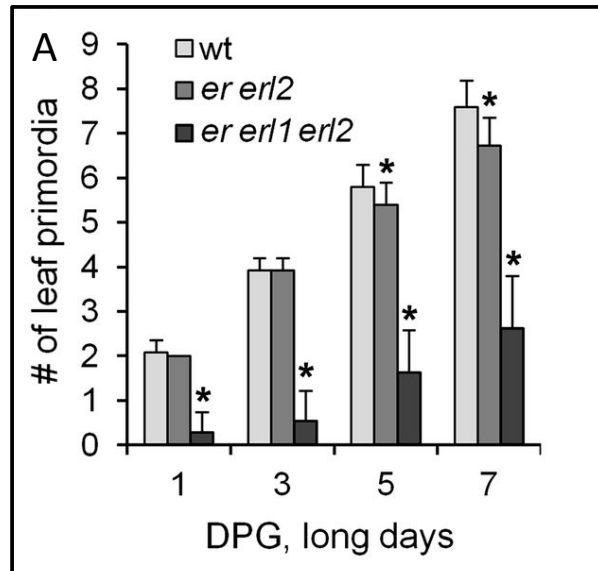


Figure 1.4. ERfs regulate leaf initiation and have altered auxin distribution.

A. Number of leaves initiated are decreased in *er erl1 erl2* compared to the wild type (wt) as determined by DIC microscopy of fixed samples. DPG = days post germination. Values are means $\pm$ SD. n = 22-25 plants. Values significantly different from the control are indicated by asterisks (P < 0.05). **B.** Auxin accumulation as shown by the auxin responsive *DR5rev::GFP* reporter in WT and *er erl1 erl2*. Auxin maxima are present in WT and ingress into the internal tissues. In *er erl1 erl2* auxin accumulation is elevated on the epidermal layer and is disjointed from the internal tissues. Adapted from Chen et al., 2013.

the hypocotyl, but is absent in *er erl1 erl2*. Real time qRT-PCR analysis indicates the altered *DR5rev::GFP* expression in *er erl1 erl2* does not correspond to significant changes in the key auxin biosynthesis gene *TAA1*, or the auxin biosynthesis regulators, *PLETHORA 3 (PLT3)*, and *PLT5* (Chen et al., 2013). Additionally, the translational reporter *TAA1-GFP* did not show any distinct differences between wild type and *er erl1 erl2* suggesting that while auxin responses are changed they may not be the result of altered auxin biosynthesis (Chen et al., 2013).

Do ERfs regulate auxin sensing? The auxin signaling pathway consists of the co-receptors TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX family (TIR1/AFB) and the Auxin/Indole-3-Acetic Acid (Aux/IAA) family (Figure 1.5). Aux/IAAs function as repressors that dimerize with ARF transcription factors to prevent transcriptional activity (Ulmasov et al., 1997). Aux/IAAs do not inhibit transcriptional activity directly. Instead, they recruit a family of co-repressors known as TOPLESS (TPL) that recruit chromatin-remodeling proteins to down-regulate transcription (Tiwari et al., 2004; Szemenyei et al., 2008). Auxin acts as a molecular glue that brings the TIR1/AFB and Aux/IAA co-receptors together (Tan et al., 2007). Once the co-receptors are in proximity, TIR1/AFB, which is part of an E-3 ubiquitin ligase complex, causes ubiquitination of Aux/IAA repressors and targets them to the proteasome for degradation (Gray et al., 2001; Dos Santos Maraschin et al., 2009). This subsequently frees ARFs to regulate transcription.

Real time qRT-PCR analysis of key components of the auxin signaling pathway, revealed that the *Aux/IAA* family genes, *IAA1* and *IAA19*, and *ARF5/MP (MONOPTEROS)* were down regulated in *er erl1 erl2* (Chen et al., 2013). Auxin has been shown to change the

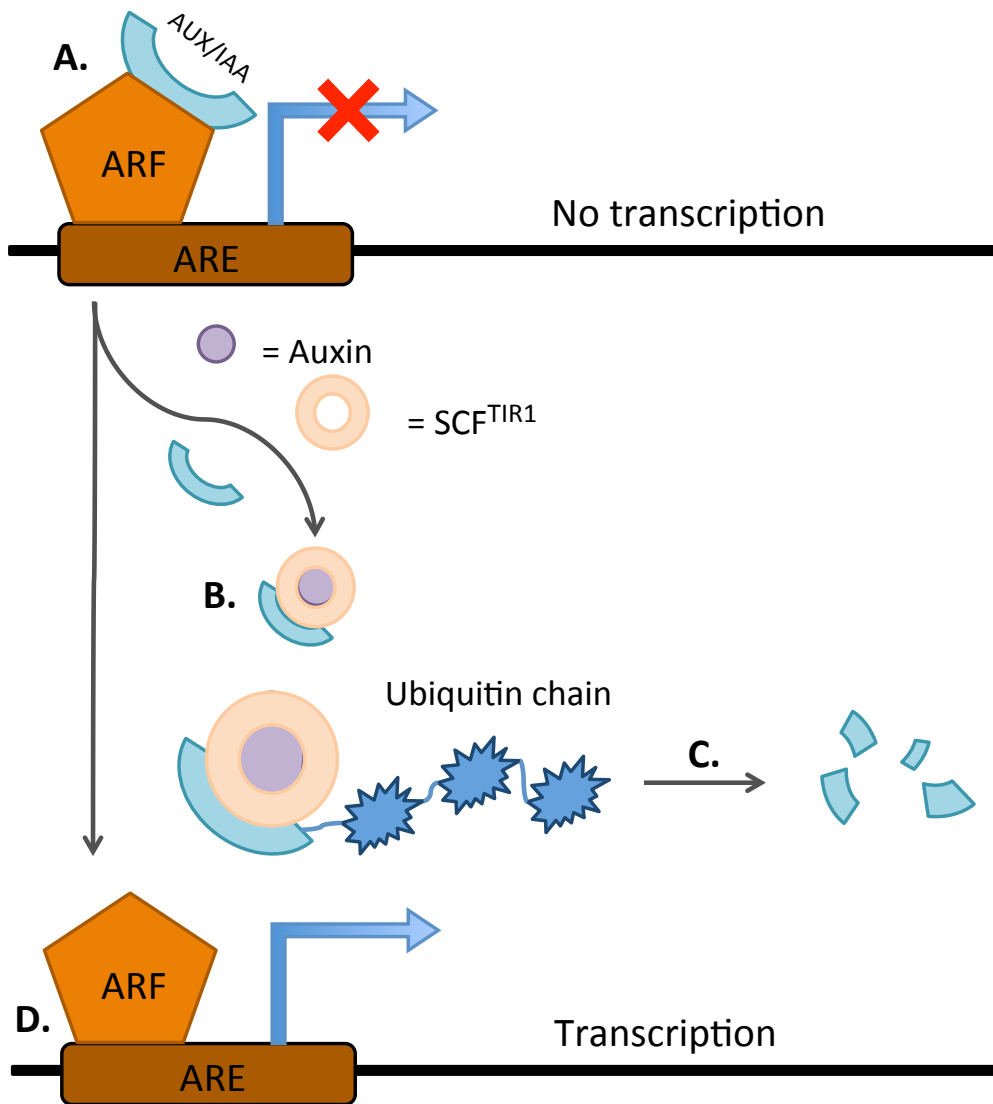


Figure 1.5. Auxin signaling pathway.

A. In the absence of auxin, Aux/IAA receptors in their unbound state repress auxin response factors (ARF). **B.** The E3 ubiquitin-ligase Skip/Cullin/F-Box containing complex (SCF) and TRANSPORT INHIBITOR RESPONSE 1 (TIR1), collectively SCF^{TIR1}, binds to the repressor Aux/IAA when exposed to auxin. **C.** Aux/IAA repressors are ubiquitinated and targeted to the proteasome for degradation. **D.** ARFs are free to promote the transcription of AREs, and an auxin response follows.

expression levels of *Aux/IAAs* and *ARFs*, which creates a complex feedback loop that is sensitive to its own signaling output (Tiwari et al., 2001; Wenzel et al., 2007; Lau et al., 2011). Therefore, it is unclear if ERf signaling is directly responsible for affecting the auxin responsive genes or if it is a consequence of altered auxin accumulation. Additionally, *IAA1* and *ARF5/MP* are expressed in leaves, which could explain their decreased expression in the leaf-deficient *er erl1 erl2* mutant (Wenzel et al., 2007; Lee et al., 2009). *WUS* is known to inhibit auxin signaling by promoting the expression of the co-repressor *TPL* (Szemenyei et al., 2008; Busch et al., 2010). Interestingly, despite an increase in *WUS* expression in *er erl1 erl2*, *TPL* expression was unchanged in *er erl1 erl2* (Chen et al., 2013). Finally, auxin sensing is occurring in *er erl1 erl2*, as the *DR5rev* promoter actively expresses GFP in the L1 layer of the SAM. While there are differences in expression of auxin related genes between wild type and *er erl1 erl2*, there is no clear evidence for disrupted auxin signaling.

Ectopic inducible expression of *ARF5/MP* on the SAM was shown to activate *PIN1* and polarize *PIN1* toward areas of high *MP* expression (Bhatia et al., 2016). *ARF5/MP* influences *PIN1* expression and polarity in both forming leaf vasculature (Wenzel et al., 2007), and in forming flower organs in inflorescence meristems (Bhatia et al., 2016). Despite the decreased *MP* expression in *er erl1 erl2*, there is no change in *PIN1* transcript expression levels or localization patterns (Chen et al., 2013). *PIN1* is normally expressed in the provasculature of initiating primordia (Heisler et al., 2005). However, *PIN1* protein localization is altered in *er erl1 erl2*. In *er erl1 erl2*, expression of the *PIN1*-GFP fusion protein was decreased in the vasculature of initiating leaf primordia compared to wild type (Chen et al., 2013). The L1 layer of the SAM in *er erl1 erl2* also has a decreased ratio of plasma membrane *PIN1* to internalized *PIN1*, especially in incipient primordia (Chen et al.,

2013) where PIN1 is responsible for accumulating auxin into peaks (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005). PIN1 functions in the plasma membrane, but internalized PIN1 has no known function (Naramoto, 2017; Béziat and Kleine-Vehn, 2018; Sauer and Kleine-Vehn, 2019).

Auxin abnormalities and leaf formation deficiencies are present in *er erl1 erl2*, but it is unclear if altered auxin distribution or altered auxin responses are causing the decreased organ formation. Current data detailing the relationship of ERfs with auxin driven organ initiation is correlative. *DR5rev::GFP* shows a surplus of auxin in the mutant *er erl1 erl2* SAM. If auxin is the limiting factor for leaf formation, why is leaf formation suppressed in the *erf* mutant? We have recently acquired data that shows leaf formation in *er erl1 erl2* is in fact limited by a deficient auxin response. We show that ERfs play a role in the response to auxin accumulation in the L1 layer of the SAM to appropriately signal initiation of organs. Finally, we provide evidence that the requirements for organ initiation are different for the vegetative meristem and the inflorescence meristem. These results are detailed in Chapter 3.

**Chapter 2: ERECTA family signaling constrains *CLAVATA 3* and
WUSCHEL to the center of the shoot apical meristem**

A version of this chapter was originally published as:

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The dissertation writer performed the majority of the laboratory work described below and contributed to writing the manuscript.

Abstract

Plants grow and produce organs throughout their entire life. Plants are sessile, and their body plans must be flexible to adjust to their environments. For aboveground organs, a reservoir of stem cells in the shoot apical meristem (SAM) constantly supplies cells for differentiation into organ primordia. The SAM must be tightly regulated to balance organ production with maintenance of the stem cell pool. A family of genes known as *ERECTA* family (ERfs) encodes three leucine-rich repeat receptor-like kinases (LRR-RLK) including *ERECTA* (*ER*), *ERECTA-LIKE 1* (*ERL1*), and *ERECTA-LIKE 2* (*ERL2*). ERf signaling regulates key processes in plant development. The meristematic regulators *WUSCHEL* (*WUS*) and *CLAVATA 3* (*CLV3*) make up a negative feedback loop to maintain the size of the SAM. The location of *WUS* and *CLV3* expression is critical for their function. Using genetic analysis, we show that ERf receptors and their EPIDERMAL PATTERNING FACTOR ligands (EPFL) synergistically with *CLV3* regulate the size of the SAM, and the *wus* mutation is epistatic to ERf signaling. We show that exogenous EPFL peptide treatment causes a decrease in *WUS* and *CLV3* transcript levels. ERf-EPFL restricts the lateral expression of *CLV3* and *WUS* and positions them to the center of the SAM. Taken together, we show ERf-EPFL signaling regulates lateral maintenance of the stem cell niche.

Introduction

Maintenance of stem cells in the shoot apical meristem (SAM) requires cellular signaling to ensure the correct balance of organ formation and stem cell conservation. This maintenance is regulated by the *WUSCHEL/CLAVATA 3 (WUS/CLV3)* feedback loop. One open question about *WUS* and *CLV3* stem cell maintenance is how the expression patterns of these meristematic genes are coordinated. The apical-basal localization of these genes is constrained by cytokinin and *HAIRY MERISTEM (HAM)*, but the signaling that constrains *WUS/CLV3* in the lateral direction is not known. Previously, it was shown that a family of three genes, *ERECTA*, *ERECTA-LIKE 1* and *ERECTA-LIKE 2 (ER, ERL1, ERL2)*, collectively called ERfs, regulates the SAM. ERfs encode leucine rich repeat receptors that bind *EPIDERMAL PATTERNING FACTOR (EPF)/EPF-LIKE (EPFL)* ligands. The loss of function mutant, *er erl1 erl2*, has a significantly wider SAM than wild type and is deficient in organ formation (Chen et al., 2013). The ligands of ERf that are responsible for SAM regulation have also been identified: *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* are expressed in the SAM, and the *epfl1 epfl2 epfl4 epfl6* mutant has identical SAM width and leaf formation as *er erl1 erl2* (Kosentka et al., 2019). However, the molecular mechanism behind ERf-EPFL regulation of the SAM is unknown. In this study, we show that ERf-EPFL signaling restricts *WUS* and *CLV3* expression in the lateral direction in the SAM.

Liang Zhang, a graduate student in the lab of Dr. Elena Shpak, previously examined the genetic interactions between ERfs and *CLV3* over multiple stages of vegetative growth (Zhang et al., 2021). SAM width was examined in wild type and the loss of function mutants *clv3*, *er erl1 erl2*, and *clv3 er erl1 erl2*. At 1 DPG, the SAM of *er erl1 erl2* is wider than both

wild type and *clv3*, indicating that ERfs play a considerable role in SAM maintenance in early embryogenesis. At 5 DPG, the *clv3* SAM width surpassed *er erl1 erl2*, indicating *CLV3* plays a larger role in SAM maintenance later in vegetative development. In the quadruple mutant *clv3 er erl1 erl2*, a massive meristem forms as early as 1 DPG and no leaves are initiated. In total, this data reveals a synergistic interaction between ERfs and *CLV3* in SAM maintenance.

CLV3 prevents overproliferation of the SAM by inhibiting *WUS* expression (Brand et al., 2000; Schoof et al., 2000; Müller et al., 2006). Loss of function *er erl1 erl2* also has significantly increased *WUS* expression (Uchida et al., 2012b; Chen et al., 2013). To determine how *WUS* expression is altered in *clv3 er erl1 erl2*, Liang Zhang used RT-qPCR to measure transcript levels in loss of function mutants. As expected, *clv3* and *er erl1 erl2* had increased *WUS* expression, with a slightly larger increase in the *clv3* mutant than in *er erl1 erl2*. The *clv3 er erl1 erl2* mutant had the most dramatic increase in *WUS* expression with a ~30-fold larger increase in compared to single mutant *clv3*. Taken together, these data indicate both *CLV3* and ERfs synergistically inhibit *WUS* expression.

SAM size is regulated by *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6*, and the collective loss of these genes conveys a SAM phenotype identical to *er erl1 erl2* (Kosentka et al., 2019). To investigate the genetic interaction of *EPFL* with *CLV3*, Liang Zhang examined the *clv3 epfl1 epfl2 epfl4 epfl6* mutant SAM. The quintuple mutant *clv3 epfl1 epfl2 epfl4 epfl6* mimics the dramatically enlarged SAM and the absence of leaves in the *clv3 er erl1 erl2* mutant, showing that these four ligands regulate SAM maintenance and organ initiation synergistically with *CLV3*.

To investigate the interactions between *WUS* and ERfs, Liang Zhang measured the SAM size of the loss of function mutants *wus*, *wus er erl1 erl2*, *wus clv3*, and *wus clv3 er erl1 erl2*. Despite the larger SAM size expected due to the *er erl1 erl2* and *clv3* mutations, the additional loss of *wus* prevented expansion of the SAM. In fact, *wus*, *wus clv3*, *wus er erl1 erl2*, and *wus clv3 er erl1 erl2* all had smaller meristems and were indistinguishable compared to wild type. This indicates the *wus* mutation is epistatic to *er erl1 erl2*.

Results

The following work was performed by Daniel DeGennaro and Dr. Elena Shpak (Daniel DeGennaro transformed the plants, evaluated the lines, performed microscopy, and image analysis while Dr. Elena Shpak designed and created the plasmids).

WUS and CLV3 are expanded laterally in the er erl1 erl2 mutant

The spatial expression pattern of *CLV3* and *WUS* is crucial to SAM maintenance. The loss of *HAM* signaling from the basal layers of the meristem disrupts the apical basal expression of *CLV3*. In the *ham1 ham2 ham3* mutant, there is a downward shift and expansion of the *CLV3* expression domain as seen by *proCLV3::GFP* (Engstrom et al., 2011; Zhou et al., 2018). In *er erl1 erl2*, SAM size is also increased. To investigate the effect of ERf signaling on the *CLV3* expression domain, *CLV3* native cis elements were used to drive the expression of the nuclear localized histone H2B-GFP fusion protein (*CLV3p:H2B-GFP:CLV3t*). This construct was transformed into wild type and *er erl1+/- erl2* mutants, and plants expressing the construct were analyzed in the T2 generation. *Er erl1+/- erl2* heterozygotes were transformed due to *er erl1 erl2* sterility, and we acquired transformants of both *er erl1 erl2*, and their siblings that segregated for *erl1* (*er erl1+/- erl2* and *er erl2*). *CLV3p:H2B-GFP:CLV3t* was analyzed in two independent transformations of

wild type, *er erl1 erl2* and the segregating siblings. Sequential line scanning using confocal microscopy was used to create high-resolution Z-stack images of the SAM one day post germination (Figure 2.1, A). Rotatable three-dimensional images were generated from the Z-stacks to allow observation from different angles.

CLV3 expression was not significantly different between wild type and the segregating siblings of *er erl1 erl2* (referred to as *er erl2*) (Figure 2.1, A-B), which is consistent with the identical meristem size and leaf initiation in wild type and *er erl2*. The *CLV3* reporter was observed in the L1 and L2 layers, and the upper two cell layers of the L3 layer in wild type and *er erl2*. When wild type and *er erl2* were viewed from above, it was evident that the *CLV3* reporter was expressed in an oval shape. The *CLV3* expression domain was slightly broader on the axis drawn between each of the boundaries of the cotyledons (width #1) (Figure 2.1, C). In *er erl1 erl2*, the reporter expression domain was dramatically larger (Figure 2.1, A). The apical view of *er erl1 erl2* shows increased expression area that is three times larger on average than wild type (Figure 2.1, B). The oval pattern of *CLV3* expression is still present in *er erl1 erl2*, but the long axis (width #1) and the short axis (width #2) of the triple mutant are more than two times larger than wild type. Expression of *CLV3* in the L1 layer is elongated in *er erl1 erl2*, and extends into the periphery of the SAM. The loss of *erf* signaling barely affected the height of *CLV3* expression, if at all. Collectively, these results indicate ERfs restrict the *CLV3* expression domain in the SAM in the lateral direction, but not vertically.

WUS expression levels are increased in the *er erl1 erl2* mutant (Uchida et al., 2012b; Chen et al., 2013). To observe the effect of ERf signaling on *WUS* expression, the *WUS*

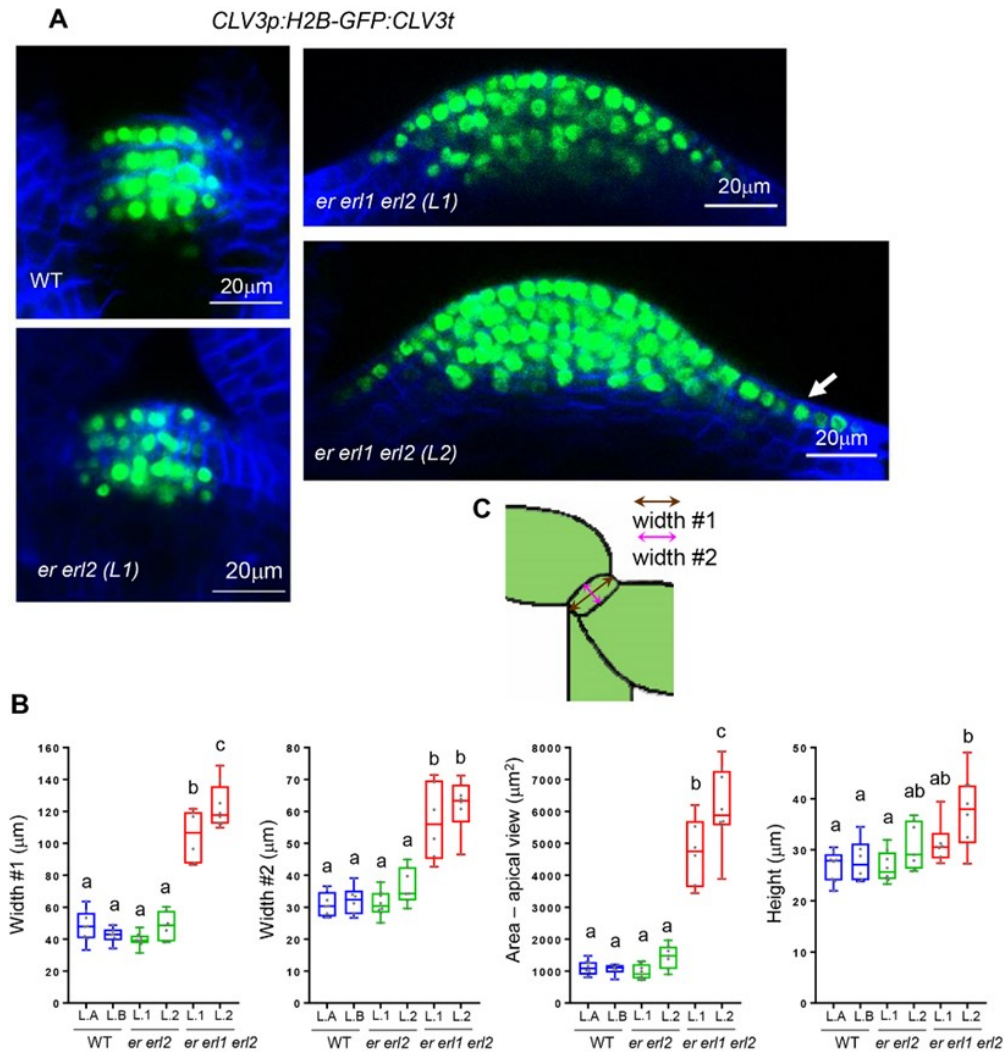


Figure 2.1. Expression of *CLV3* is expanded laterally in the *er erl1 erl2* mutants.

A. Confocal images of the SAM region of 1 DPG wild type (wt) and mutant seedlings transformed with *CLV3p::H2B-GFP::CLV3t* (green). The cell walls were stained with SR2200 (blue). The white arrow indicates extended expression of the reporter in the L1 layer. **B.** Measurements of *CLV3p::H2B-GFP::CLV3t* expression in the SAM of WT, *er erl1 erl2* mutants and the segregating siblings of *er erl1 erl2* (*er erl2*). L.A and L.B are two independent WT lines, L.1 and L.2 are two independent mutant lines. The median is indicated as a horizontal line between two boxes, upper and lower quartiles are represented by the boxes, and the vertical lines designate the maximum and the minimum. Grey dots represent individual data points. $n=6-8$. For multiple comparisons (one-way ANOVA with Tukey post-hoc test), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum P-value of <0.01 . **C.** Schematic of the seedling meristematic zone demonstrating how width #1 and width #2 of the SAM were measured in **B.** (as published in Zhang et al., 2021)

expression domain was observed and quantified similarly to *CLV3*. The *WUS* promoter was used to drive the expression of H2B-GFP fusion protein followed by the 35S terminator sequence (*WUSp:H2B-GFP:35St*). The construct was transformed into *er erl1+/- erl2*. Plants expressing the construct were analyzed in the T2 generation. Two independently transformed lines of *er erl1+/- erl2* were analyzed which segregated *er erl1 erl2*, *er erl1+/- erl2*, and *er erl2*. The *er erl1+/- erl2* and *er erl2* plants are referred to as *er erl2*. To observe *WUSp:H2B-GFP:35St* in the presence of functional ERF signaling, the segregating sibling of one line (L.1) was crossed with Columbia to produce the F1 generation with a wild type phenotype (referred to as wild type). One DPG seedlings were imaged using confocal microscopy to create Z-stacks and three-dimensional images of wild type, *er erl1 erl2*, and their segregating siblings (*er erl2* and *er erl1+/- erl2*) (Figure 2.2, A and B). In all three genotypes, *WUS* expression was absent from the L1 and L2 layer and present in the presumptive organizing center in the L3 layer (Figure 2.2, A). There was no significant difference between wild type and *er erl2*. When wild type and *er erl2* were viewed from above, *WUS* expression was oval in shape similar to *CLV3* expression, although *WUS* expression was approximately 50% of the area of *CLV3* (Figure 2.2, B). *WUS* expression in *er erl1 erl2* was still oval shaped, but it was considerably larger in area than in both the wild type and *er erl2*. However, the expansion only occurs laterally, and there was no difference in *WUS* domain height between wild type, *er erl2*, and *er erl1 erl2*. Taken together, the spatial characteristics of *WUS* expression in wild type and *er erl1 erl2* indicate ERfs restrict *WUS* to the center of the SAM laterally, but not vertically.

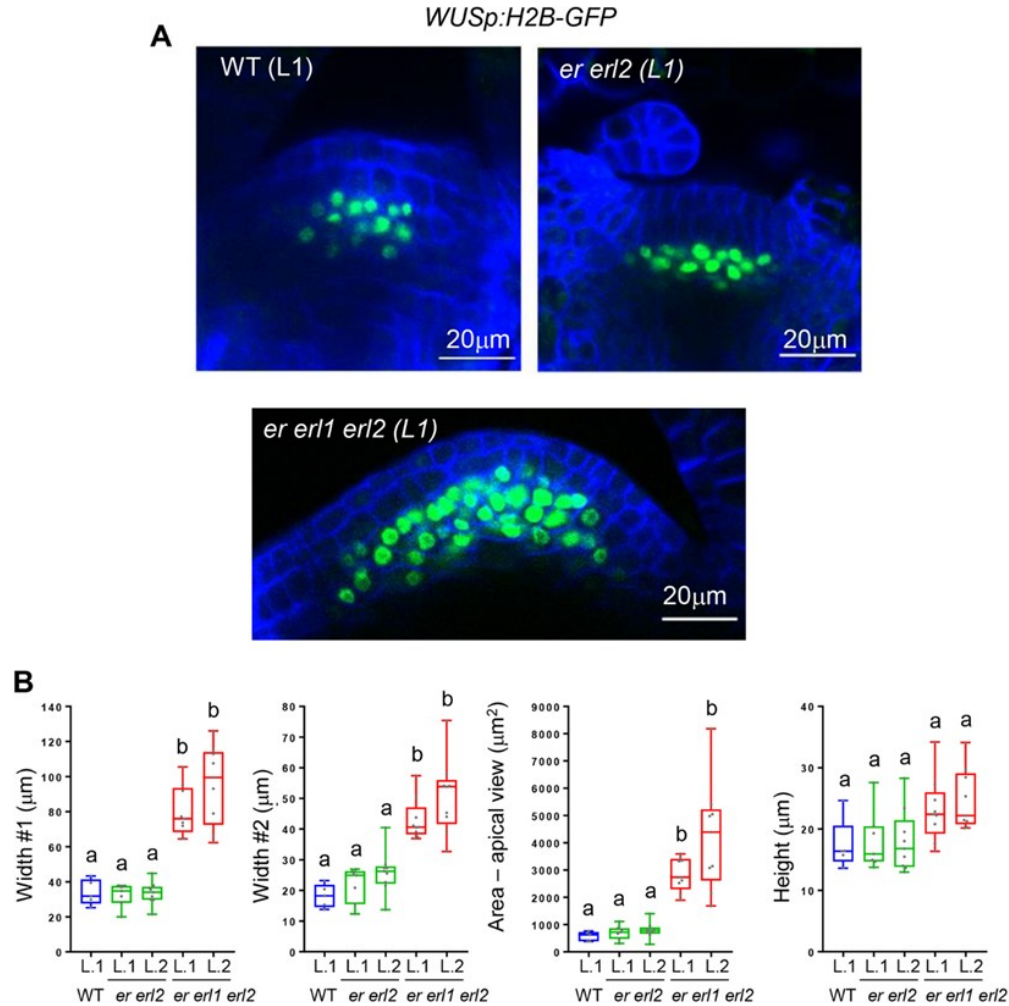


Figure 2.2. Expression of *WUS* is expanded laterally in the *er erl1 erl2* mutants.

A. Confocal images of the SAM region of 1 DPG seedlings transformed with *WUSp:H2B-GFP* (green). The cell walls were stained with SR2200 (blue). **B.** Measurements of *WUSp:H2B-GFP* expression in the SAM of *er erl1 erl2* mutants, their segregating siblings (*er erl2*) and F1 generation seedlings from the cross of the L.1 line with the wild type (WT). L.1 and L.2 are two independent lines. The median is indicated as a horizontal line between two boxes, upper and lower quartiles are represented by the boxes, and the vertical lines designate the maximum and the minimum. Grey dots represent individual data points. n=5-9. For multiple comparisons (one-way ANOVA with Tukey post-hoc test), lower case letters are used to label means, such that bars bearing different letters are statistically different from one another with a minimum P-value of <0.01. (as published in Zhang et al., 2021)

The following work was performed in collaboration between Daniel DeGennaro and Liang Zhang.

***WUS* and *CLV3* are directly targeted by ERF-EPFL signaling**

Expression domains of *WUS* and *CLV3* are increased in *er erl1 erl2*, suggesting that ERF-EPFL signaling restricts the two meristematic regulators to the center of the SAM. However, because *WUS* and *CLV3* form a negative feedback loop, it is unclear if ERF-EPFL signaling is targeting one or both of genes. Furthermore, it is not known if ERF-EPFL signaling directly targets *WUS* and *CLV3*. To determine if ERF-EPFL targets one or both of *WUS* and *CLV3*, RT-qPCR was performed on *clv3 epfl1 epfl2 epfl4 epfl6* mutant seedlings after application of a synthetic EPFL6 peptide (Figure 2.3, A-D). The quintuple mutant was used because the enlarged SAM allows for a higher yield of the weakly expressed *WUS* and *CLV3* transcripts. The *clv3-9* mutant that was used to create the quintuple mutant has a point mutation in the *CLV3* sequence that disrupts gene function but not its expression. *WUS* and *CLV3* expression in seedlings treated with 10 μ M EPFL6 ligand were compared to seedlings that were treated with buffer only (mock) (Figure 2.3, A and B). Measurements were taken after 1, 3, and 6 hours of treatment. *CLV3* expression decreased in the EPFL6 treated seedlings after 1 hour, and in as little as 3 hours of treatment, both *WUS* and *CLV3* expression showed a ~50% reduction. Extended ligand treatment did not result in further reduction. To determine if the effect of ERF-EPFL signaling on *WUS* and *CLV3* expression depends on translation, cycloheximide (CHX) was used to inhibit protein synthesis during a 3 hour EPFL6 treatment. Expression of *WUS* and *CLV3* had even further decreases compared to the same 3 hour EPFL6 treatment without CHX, hinting that there may be a buffering mechanism for *CLV3* and *WUS* that requires translation.

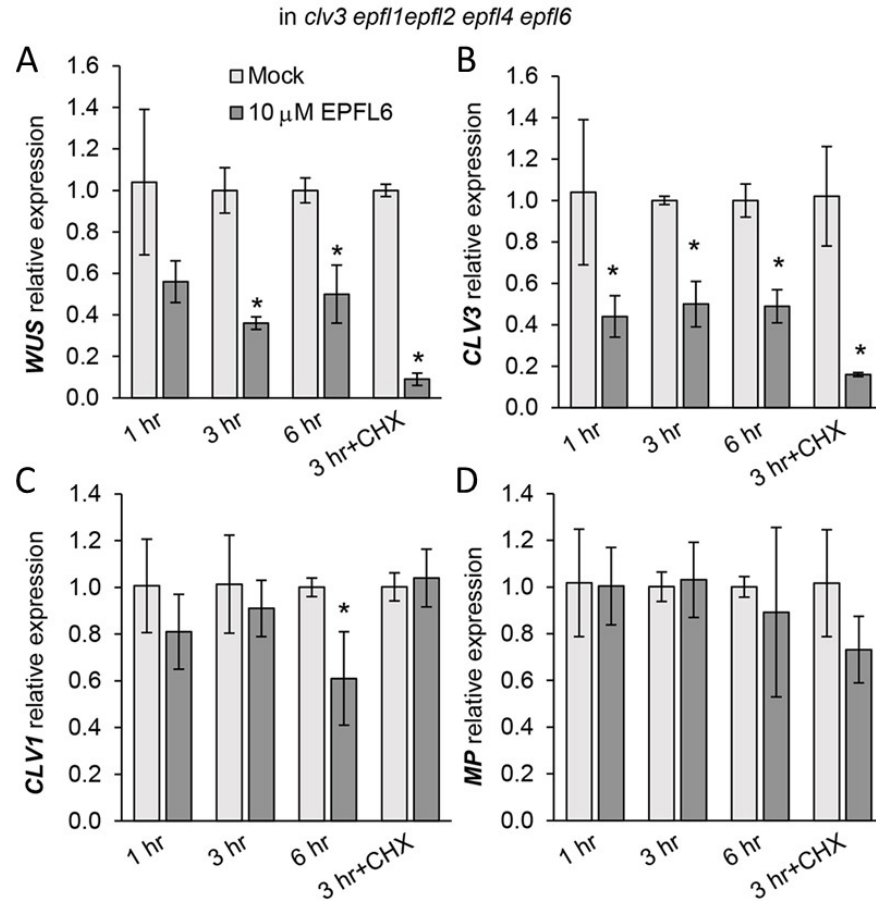


Figure 2.3. *WUS* and *CLV3* are direct targets of the ERF signaling pathway.

A-D. Relative expression levels of selected mRNAs in aboveground organs of 3 DPG *clv3 epfl1 epfl2 epfl4 epfl6* seedlings after treatment with 10 μ M EPFL6 peptide compared with mock treatment. Seedlings were treated with peptide for 1, 3, and 6 hrs. +CHX indicates treatment with 10 μ M cycloheximide. Data are mean $\pm$ S.D. *ACTIN2* was used as an internal control. * $P < 0.05$ (unpaired two-tailed Student's t-test). (as published in Zhang et al., 2021)

Two other genes with decreased expression in the *er erl1 erl2* mutant are *MONOPTEROS (MP)*, which encodes an auxin response factor, and *CLAVATA 1 (CLV1)*, which encodes the CLV3 receptor (Chen et al., 2013). To test whether the effect of EPFL6 signaling is unique to *WUS* and *CLV3*, we measured transcript levels of *MP* and *CLV1* in *clv3 epfl1 epfl2 epfl4 epfl6* mutants exposed to the EPFL6 peptide (Figure 2.3, C and D). No change was seen in either *MP* or *CLV1* expression after exposure to the EPFL6 ligand indicating that the decrease in *WUS* and *CLV3* is likely specific to those meristematic regulators. There was a decrease in *CLV1* expression after the 6 hour treatment (Figure 2.3, C). However, this effect was not seen in the 3 hour treatments, indicating this effect is likely indirect and complex. In sum, our data shows that ERf-EPFL signaling targets *WUS* and *CLV3* expression in the SAM and this is independent of protein translation.

Discussion

All plants must balance continual replenishment of cells used for organ growth and prevention of stem cell overproduction. ERf-EPFL signaling can now be included in the regulatory mechanisms that govern this balance. Prior to our findings, the molecular targets of ERf-EPFL signaling in SAM were unknown. To find them, we quantitatively and spatially characterized expression of the two meristematic regulators, *WUS* and *CLV3*. We found that expression patterns of *WUS* and *CLV3* transcriptional reporters were increased in the lateral direction, but not vertically. This is consistent with the phenotype of *er erl1 erl2* mutant SAM which expands primarily in the lateral direction in contrast to the *clv3* SAM which expands vertically (Mandel et al., 2016). The *EPFLs* responsible for SAM

maintenance are expressed in the periphery of the meristem, and ectopic expression of *EPFL1* by the peripheral zone promoter *KANADI*, can fully rescue the *epfl1 epfl2 epfl4 epfl6* enlarged SAM (Kosentka et al., 2019). ERfs are expressed ubiquitously in the SAM, but ectopic expression of *ER* under the *CLV3* promoter is sufficient to rescue SAM width (Kosentka et al., 2019). This is similar to leaf tooth elongation where *EPFL2* expression is excluded from the tip, but expressed in the bordering tissue, and *ER* expressed at the tip of the leaf tooth under the *DR5* promoter can rescue growth (Tameshige et al., 2016). Our data that shows *WUS* and *CLV3* are restricted by peripheral ERf-EPFL signaling suggests this pattern of border-to-centralized tissue signaling is conserved in multiple organs.

We also revealed through exogenous EPFL ligand treatment that *WUS* functions downstream of EPFL signaling independently of protein synthesis. Interestingly, *CLV3* a known inhibitor of *WUS* expression, decreased simultaneously with *WUS* upon the same EPFL peptide signal in the absence of protein synthesis. Taken together, ERf-EPFL signaling plays a dual role in meristematic regulation by inhibiting both *WUS* and *CLV3*.

The simultaneous decrease in transcript levels of *WUS* and *CLV3* in the presence of EPFL6 indicates that ERf-EPFL signaling regulates genetic factors that increase and decrease stem cell proliferation, respectively. The inhibition of *WUS* is the predominant function of ERfs. The rampant expression of *WUS* in *clv3 er erl1 erl2* indicates that the synergistic interaction between *CLV3* and *ERf* is due to their convergence on *WUS* expression. Differential expression patterns of *WUS* and *CLV3* may also contribute to the predominant negative regulation of *WUS*. In this study and others, *WUS* is basally located in the organizing center and *CLV3* is apically located up against the epidermis (Mayer et al.,

1998; Fletcher et al., 1999). The different locations of *WUS* and *CLV3* in relation to EPFL may result in a different functional regulatory effect that inhibits *WUS* more than *CLV3*, causing the larger SAM in loss of function *erf* or *epfl* mutants. Alternatively, each individual EPFL may activate ERF receptors in unique ways to differently target *WUS* or *CLV3*.

Our experimental data is in agreement with a recent computational model that investigated the individual inhibitory effect of ERF signaling on *WUS* or *CLV3*. The model predicted that if ERF signaling only inhibited *WUS* and not *CLV3*, there would be a widening of *CLV3* expression, and an increase in internal expression on the border of the organizing center (Liu et al., 2020). The internal *CLV3* expression on the border of the organizing center is not seen in wild type plants, and expression is restricted to the central zone. This supports the combined inhibitory effect of ERF signaling on both *CLV3* and *WUS*. Our experimental data agrees with this as the decrease in *WUS* and *CLV3* expression with ERF signaling activation occurs independently of protein translation showing that ERFs inhibit both meristematic regulators.

It has been proposed previously that ERF acts as a buffer for stem cell homeostasis through modulation of cytokinin signaling (Uchida et al., 2012b). However, the cycloheximide treatment does not allow for translation of secondary signaling molecules to inhibit *CLV3*. Instead, there is likely a downstream target of ERF in the form of an already present transcription factor, which changes *WUS* and *CLV3* expression. The MAP kinase cascade is known to function downstream of ERF in stomata development (Bergmann et al., 2004; Hara et al., 2007; Wang et al., 2007; Meng et al., 2012). Furthermore, a constitutively active form of the MAP kinase kinase kinase, YODA, can partially rescue *er erl1 erl2*

meristem size (Chen et al., 2013). However, the downstream transcriptional regulators in ERF-EPFL signaling that converge on *WUS* and *CLV3* in the SAM remain elusive.

EPFL1, *EPFL2*, *EPFL4*, and *EPFL6* are all expressed in the SAM and regulate SAM function (Kosentka et al., 2019). However, the true function of each individual ligand is not well understood. *EPFL4* and *EPFL6* have been shown to bind to the receptor domain of all three ERECTA receptors *in vitro* (Lin et al., 2017). More data is required to understand the biochemical interactions that exist between the individual ERF receptors and *EPFL1* and *EPFL2*. Additionally, individual *EPFLs* have overlapping yet distinct patterns in the SAM (Kosentka et al., 2019). The unique *EPFL* expression patterns would make it possible for *EPFLs* to regulate specific parts of the *CLV3* or *WUS* expression domains. In this study, *EPFL4* (data not shown) and *EPFL6* are the ligands that target *WUS* and *CLV3* expression, but this does not rule out *EPFL1* or *EPFL2*. More data is needed to assign individual functions to each *EPFL* in SAM regulation.

In conclusion, our data suggest that ERF-EPFL enables correct spatial expression of *WUS* and *CLV3* through inhibition of these meristematic regulators in the periphery of the SAM (Figure 2.4). ERF-EPFL regulates *WUS* and *CLV3* directly and independently of protein translation. Taken together, ERF-EPFL restricts *WUS* and *CLV3* expression to the center of the meristem to prevent a widening of the SAM and overproliferation of stem cells.

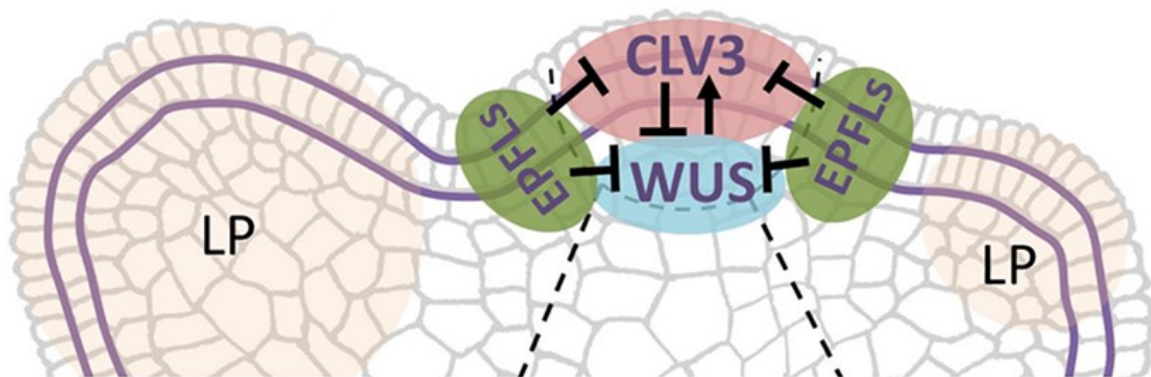


Figure 2.4. Model for the role of ERF signaling in regulation of shoot meristem maintenance.

EPFL signals originating from the periphery of the SAM activate the ERF signaling cascade that restricts the expression of *CLV3* and *WUS* to the center of the SAM. LP, leaf primordium. (as published in Zhang et al., 2021)

Materials and methods

Plant Materials and Growth Conditions

Columbia ecotype was used as the wild type. The following mutants used all are in the Columbia ecotype background and have been described previously: *er-105 erl1-2 erl2-1* (Shpak et al., 2004), *epfl1 epfl2 epfl4*, *epfl1 epfl2 epfl6* and *epfl1 epfl2 epfl4 epfl6* (Kosentka et al., 2019), *clv3-9* (Nimchuk et al., 2015) and *wus* null allele (SAIL_150_G06 (CS807292); (Sonoda et al., 2007)). The *clv3-9* mutant was crossed with *epfl1/+ epfl2 epfl4 epfl6* to generate *clv3-9 epfl1 epfl2 epfl4 epfl6* plants. The *epfl* mutations were genotyped as detailed previously (Kosentka et al., 2019). The nuclear localized *WUS* transcriptional reporter construct, *WUSp:H2B-GFP:35St* (*pESH746*), was created by fusing 4.5 kb of the *WUS* promoter sequence (Yadav et al., 2009) with *H2B-EGFP* followed by the 35S terminator. The nuclear localized *CLV3* transcriptional reporter construct, *CLV3p:H2B-GFP:CLV3t* construct (*pESH747*), was created by fusing 1.5 kb of the *CLV3* promoter sequence with *H2B-EGFP* followed by the 1.2 kb *CLV3* terminator. Both sequences were obtained by overlapping PCR and were placed into the *pPZP222* vector between the restriction sites BamHI and Sall. The amplification template for *H2B-EGFP* was a plasmid from the Z. Nimchuk lab (UNC Chapel Hill, USA). The constructs were evaluated through sequencing of amplified regions. Then *pESH746* and *pESH747* were transformed into an *Agrobacterium tumefaciens* strain GV3101/pMP90 via electroporation. The floral dip method was used to transform the constructs into wild type (Columbia) and *er erl1/+ erl2 Arabidopsis thaliana*. Gentamycin was used to select T1 transgenic plants. The T1 generation heterozygous *erl1/+* plants were identified with genotyping. Plant growth conditions were as previously

described (Kosentka et al., 2017) using long day, 18 h light/6 h dark cycle at 21°C. Seeds were stratified for 2 days at 4°C prior to germination. To analyze gene expression after EPFL6 treatment, *clv3 epfl1 epfl2 epfl4 epfl6* plants were grown on 1% sucrose Murashige and Skoog (MS) medium plates for 3 DPG (5 days total). 10 seedlings per biological replicate were transferred to 1 ml of liquid MS medium with 10 µM EPFL6. Peptide purification was described previously (Lin et al., 2017). EPFL6 was dissolved in 10 mM Bis-Tris, 100 mM NaCl (pH 6.0). A buffer solution of equal volume was added to the medium (8.7 µL) for a mock treatment. To determine the effect of EPFL6 with cycloheximide, seedlings were incubated for 10 min in MS medium with 10 µM cycloheximide and then 10 µM of EPFL6 was added to the treatment group. Three biological replicates consisting of ten seedlings each were used per treatment.

Microscopy

We used a Leica SP8 White Light Laser Confocal System to image *WUSp::H2B-GFP:35S* and *CLV3p::H2B-GFP:CLV3* expression domains. To monitor both the nuclear localized GFP and the cell wall stained SCRI Renaissance 2200 (SR2200) fluorescence (Musielak et al., 2015), we used sequential line scanning to generate z-stacks. GFP was excited using a 488-nm White Light Supercontinuum Laser. SR2200 was excited with a Diode 405nm "UV" laser. GFP and SR2200 fluorescence emission was collected with the HyD "Hybrid" Super Sensitivity SP Detector and PMT SP Detector. For better observation of GFP expression in the shoot meristematic region of live 1 day old seedlings, one cotyledon was removed before imaging. Vacuum infiltration was performed to stain internal cell layers of the

meristem, followed by a PBS buffer wash. SR2200 stain was created as detailed by Musielak et al with the exclusion of paraformaldehyde (Musiak et al., 2015).

Quantitative Measurements

The FIJI distribution of ImageJ was used for all quantitative image measurements (Schindelin et al., 2012). Single two-dimensional optical sections with a lateral view were used to measure the height of the center of the expression domain of *WUSp::H2B-GFP:35St* and *CLV3p:H2B-GFP:CLV3t* using the straight line tool. Three-dimensional images were used for all other measurements. All perimeter and area measurements were acquired by drawing a circle around the expression domain with an apical view of the meristem using the freehand measurement tool. Width 1 is the axis connecting the boundaries of cotyledons. Width 2 is perpendicular to width 1. All width 1 and width 2 measurements were acquired using an apical view using the straight-line tool.

Quantitative RT-PCR analysis

RNA isolation and cDNA synthesis were performed by Liang Zhang. Quantitative RT-PCR was performed by Daniel DeGennaro

Aerial tissues of 3 DPG seedlings were used to isolate total RNA using the Spectrum Plant RNA Isolation Kit (Sigma-Aldrich). Isolated RNA was treated with RNase-free RQ1 DNase (Promega). RNA quantity and purity was analyzed using a nanodrop. LunaScript™ RT SuperMix Kits (New England Biolabs) was used to synthesize complementary cDNA. SsoFast EvaGreen Supermix (Bio-Rad) was used for Quantitative PCR with CFX96 Touch Real-Time PCR Detection System (Bio-Rad) equipment. Three technical replicates of three biological replicates were used in a total volume of 10 µL per reaction with 4 µL of 10×, 20× or 40× diluted cDNA.

Cycling was performed under the following conditions: 3 min at 95°C; then 40 repeats of 10 s at 95°C, 10 s at 52°C for ACTIN2 and STM; 10 s at 55°C for WUS; 10 s at 57°C for MP; 10 s at 50°C for CLV1, and 10 s at 68°C, followed by the melt-curve analysis. Cycling conditions for CLV3 were slightly different with 3 min at 95°C; then 40 cycles of 95°C for 10 s and 60°C for 10 s, followed by the melt-curve analysis. Primers for ACTIN2, STM, WUS, MP (Chen et al., 2013), CLV3 (Chiu et al., 2007), and CLV1 (Nimchuk et al., 2015) were described previously. Relative quantification by the $2^{-\Delta\Delta CT}$ algorithm was used to calculate the fold difference in gene expression.

Chapter 3: ERfs regulate auxin response in the shoot apical meristem to promote leaf initiation

Chapter 3 represents unpublished work by Daniel DeGennaro, Liang Zhang, Ming-Kun Chen, and Elena Shpak.

The dissertation writer performed the majority of the laboratory work.

Abstract

Organ initiation and growth occurs throughout the entire life of a plant until senescence. Plants must adapt to environmental pressures, such as light, to survive. Because plants are sessile, this is accomplished through changes in plant architecture. A structure at the top of the plant called the shoot apical meristem (SAM), supplies stem cells for aboveground organs, such as leaves and stem. The plant hormone auxin is a master regulator of organ initiation. The polarly localized auxin transport protein, PIN1, concentrates auxin into peaks in the L1 layer of the SAM in a process called polar auxin transport (PAT). Auxin peaks mark where incipient organ primordia will form. A family of genes known as *ERECTA* family (ERfs) encodes three leucine-rich repeat receptor-like kinases (LRR-RLK): *ERECTA* (*ER*), *ERECTA-LIKE 1* (*ERL1*), and *ERECTA-LIKE 2* (*ERL2*). ERf signaling regulates key processes in plant development including organ initiation. Mutants deficient in all three *erfs* have dramatically decreased leaf formation, and this correlates with altered auxin signaling. Using the auxin responsive reporter, *DR5rev::GFP*, we show that the *er erl1 erl2* mutant has elevated auxin signaling in the L1 layer, but this is not sufficient to initiate leaves. Elevating auxin levels in the L1 layer of the *er erl1 erl2* SAM with either exogenous auxin or by auxin efflux inhibition partially rescues leaf initiation, indicating the threshold required for leaf initiation is elevated in the mutant. The PIN polarity regulator, PID, synergistically regulates organ formation with ERfs. Leaf initiation is absent in the *pid er erl1 erl2* mutant due to altered auxin accumulation and decreased auxin response. Finally, we reveal that while ERfs inhibit auxin accumulation in the inflorescence meristem, their role in promoting auxin responses is not as essential for organ initiation as it is in the vegetative meristem. Taken together, we show that ERfs stimulate auxin response in the vegetative SAM to promote organ formation.

Introduction

Aerial organ formation in different species of plants occurs at varying rates and positions to optimize light exposure to specific environments (Sarlikioti et al., 2011). In all plants, leaves and flowers initiate from the shoot apical meristem (SAM). A ring of cells in the peripheral zone initiates lateral organs at regular intervals known as a phyllotactic pattern. Leaves initiate at a fixed distance from the apex of the SAM. The molecular basis for the initiation zone around the SAM is not fully understood. One hypothesis is that genes mark the presumptive adaxial and abaxial sides of incipient primordia, and the place where they meet represents a ring of competent cells that can form primordia (Emery et al., 2003; Caggiano et al., 2017; Heisler and Byrne, 2020). In *Arabidopsis*, the first two true leaves form at a 180° angle and perpendicular to the cotyledons (Irish and Sussex, 1992; Medford et al., 1992). The following primordia initiate 137.5° away from the previous leaf to allow the greatest possible distance between nearby organs (Clarke et al., 1999). The continuous ring of cells in the initiation zone requires an activation signal to enable phyllotaxy. The first molecular marker of incipient primordia in the SAM in the L1 layer of the initiation zone is the plant hormone auxin (Benková et al., 2003; Heisler et al., 2005; Smith et al., 2006; Heisler and Byrne, 2020). The auxin gradients, or peaks, regulate the specific phyllotaxy and shape of organ primordia. The pattern of the auxin peaks modulates the size and shape of leaf primordia. Wider primordia initiate when auxin is applied to the SAM at higher concentrations (Reinhardt et al., 2000).

Auxin peaks are formed by polarly localized transport proteins in the plasma membrane, which enable auxin efflux through polar auxin transport (PAT). PAT regulates

phyllotaxy by depleting auxin from the area surrounding the peak (Reinhardt et al., 2003). Multiple auxin efflux transporters exist, but PIN1 is the primary transporter involved in organ formation in the SAM (Vernoux et al., 2000; Reinhardt et al., 2003; Kierzkowski et al., 2013). It is thought that PIN1 promotes auxin peak formation, and auxin concentrations control PIN1 polarization in a positive feedback loop. The feedback loop helps to polarize PIN1 toward auxin peaks in the L1 layer to reinforce auxin accumulation into maxima and deplete auxin from the surrounding tissues (Reinhardt et al., 2003). The orientation of PIN1 is directed through its phosphorylation by multiple families of protein kinases (Sauer and Kleine-Vehn, 2019). The PINOID (PID) kinase plays a key role in directing PIN1 to the apical side of cells in the L1 layer by phosphorylating its hydrophilic loop. This promotes PAT to the incipient primordia and enhances auxin peaks (Friml et al., 2004; Dhonukshe et al., 2010).

Once auxin accumulates into peaks in the L1 layer, auxin also appears in the lower tissues beneath the maximum (Benková et al., 2003). As auxin accumulation in the lower tissues coincides with PIN1 expression, it was proposed that PAT causes the ingress of auxin from the upper to the lower tissues (Reinhardt et al., 2003; Smith et al., 2006). However, new data suggests that the canalization of auxin to the lower tissues of the SAM does not require PIN1 and may be the result of diffusion (Kierzkowski et al., 2013; Ravichandran et al., 2020).

The *ERECTA* family genes (ERfs), which include *ERECTA*, *ERL1*, and *ERL2*, have previously been shown to promote leaf initiation and phyllotaxy (Chen et al., 2013; Kosentka et al., 2019). ERfs encode leucine rich repeat receptor-like kinases (Shpak et al.,

2004). These kinases are embedded in the plasma membrane and bind EPF/EPFL (epidermal patterning factor/EPF-like) family ligands (Hara et al., 2007; Shpak, 2013; Lin et al., 2017). Loss of function *er erl1 erl2* and *epfl1 epfl2 epfl4 epfl6* mutants have dramatically decreased leaf initiation (Chen et al., 2013; Kosentka et al., 2019). The normal divergence angle of leaves is completely lost in *er erl1 erl2*. These leaf formation deficiencies in the mutant correspond to altered auxin accumulation (Chen et al., 2013). The auxin responsive reporter *DR5rev::GFP* indicates auxin signaling is increased in the L1 layer of *er erl1 erl2* compared to wild type, and individual auxin maxima cannot form. The auxin signaling defects are also present in the internal layers of the SAM. The procambial cells of organ primordia and the mature vasculature in the leaves and the hypocotyl have decreased *DR5rev::GFP* expression. PIN1 expression is also altered in *er erl1 erl2* and correlates with altered auxin signaling. The *er erl1 erl2* SAM has increased accumulation of PIN1 in the L1 layer and decreased accumulation in the internal tissues (Chen et al., 2013).

Auxin abnormalities are evident in *er erl1 erl2*, and leaf formation deficiencies can conveniently be attributed to altered auxin signaling. However, this data is correlative, and it is unclear if altered auxin signaling is causing decreased organ formation. The feedback loop between auxin accumulation and PIN1 expression and polarization makes it difficult to understand the role of ERfs in auxin transport. In this work, we investigated the role of ERfs in leaf initiation, and the relationship between ERfs and auxin. We have acquired data that shows leaf formation in *er erl1 erl2* is limited by auxin. We show that *er erl1 erl2* can accumulate and sense auxin in the L1 layer, but it is not a sufficient signal for organ initiation. Furthermore, there are complex genetic interactions in organ initiation between ERfs and the auxin transport related genes, *PIN1* and *PID*. Finally, we provide evidence that

the genetic requirements for auxin driven organ initiation are different in the vegetative meristem and the inflorescence meristem.

Results

Leaf initiation in *er erl1 erl2* is limited by auxin availability

In previous studies, *er erl1 erl2* mutants displayed decreased leaf formation (Uchida et al., 2012c; Chen et al., 2013). Auxin responses are also disrupted in *er erl1 erl2* (Chen et al., 2013). However, there are other significant defects present in the *er erl1 erl2* SAM, including increased stem cell proliferation (Chen et al., 2013; Zhang et al., 2021). To determine if decreased leaf formation in *er erl1 erl2* is the result of auxin abnormalities, we perturbed auxin levels in the mutant (Figure 3.1, A and B). First, we used the auxin biosynthesis inhibitor L-Kynurenine (Kyn), a competitive inhibitor of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAA1/TAR) enzymes, which catalyze the main step in the auxin biosynthesis pathway (Stepanova et al., 2008; Tao et al., 2008; He et al., 2011). Wild type plants showed a decrease in leaf organ formation when grown on increasing concentrations of Kyn at both 2 days post germination (DPG) and 5 DPG (Figure 3.1, A). The effect of Kyn on leaf formation in wild type was expected as auxin biosynthesis is required for leaf formation (Cheng et al., 2007a; Stepanova et al., 2008). When *er erl1 erl2* mutants were grown with Kyn, there was also a loss of leaf formation at 2 DPG and 5 DPG. The effect was more dramatic in *er erl1 erl2* plants, which were unable to form leaves at high concentrations of the auxin

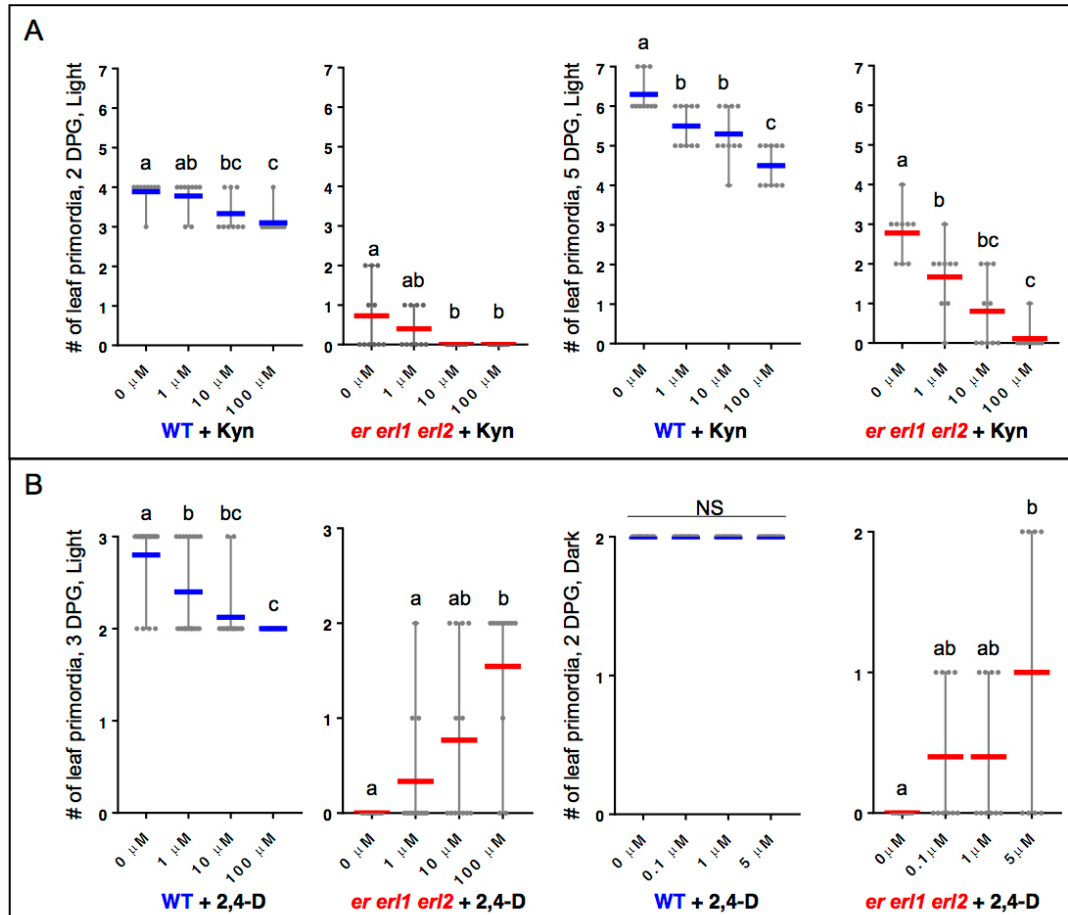


Figure 3.1. Leaf initiation in the *er erl1 erl2* mutant is limited by auxin availability. **A.** Inhibition of auxin biosynthesis by kynurenine (Kyn) inhibits leaf initiation in both wild type (WT) and *er erl1 erl2*. **B.** Exogenous auxin (2,4D) promotes leaf initiation in *er erl1 erl2* but not in the WT. **A, B.** The rate of leaf primordia initiation was determined by DIC microscopy of fixed samples. Seedlings were grown in light or dark as indicated. DPG = days post germination. The mean is indicated as a thick horizontal bar. Blue bars are WT and red bars are *er erl1 erl2*. The vertical lines designate the maximum and the minimum. Each grey point represents number of leaves from one individual plant. Different lowercase letters above bars indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test. NS = not significant. $n=9-20$.

biosynthesis inhibitor at both stages measured. This indicates that *er erl1 erl2* requires auxin to form leaves and is highly sensitive to decreased auxin levels.

Next we grew wild type and *er erl1 erl2* plants on media supplemented with the synthetic auxin analog 2,4-Dichlorophenoxyacetic acid (2,4-D) to increase auxin levels (Figure 3.1, B). In wild type plants, leaf number decreased with increasing concentrations of 2,4-D at 3 DPG. Interestingly, leaf formation in *er erl1 erl2* was partially rescued with increased auxin levels. Light is known to activate auxin biosynthesis (Bhalerao et al., 2002). Etiolated seedlings have a smaller pool of auxin and are highly sensitive to auxin perturbations. In dark conditions, wild type seedlings invariably form two leaves, regardless of the concentration of 2,4-D applied. This indicates robust leaf formation of the first two true leaves developed by wild type plants. Dark grown *er erl1 erl2* seedlings had improved leaf formation with increased concentrations of exogenous auxin. Taken together, this data indicates *er erl1 erl2* mutants are deficient in leaf formation due to limitations in auxin levels or responses.

Decreasing auxin efflux partially rescues leaf initiation in *er erl1 erl2*

Auxin efflux driven by PIN1 transport proteins play a crucial role in organ formation by generating auxin peaks in the SAM (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Cheng et al., 2007a; Traas and Monéger, 2010; Qi et al., 2014; Xiong and Jiao, 2019). To test genetic interactions between ERF and *PIN1*, we generated a *pin1 er erl1 erl2* quadruple loss of function mutant and compared the number of leaves formed to *pin1* and *er erl1 erl2* in 5 DPG seedlings (Figure 3.2, A). As reported previously, *pin1* had less leaves

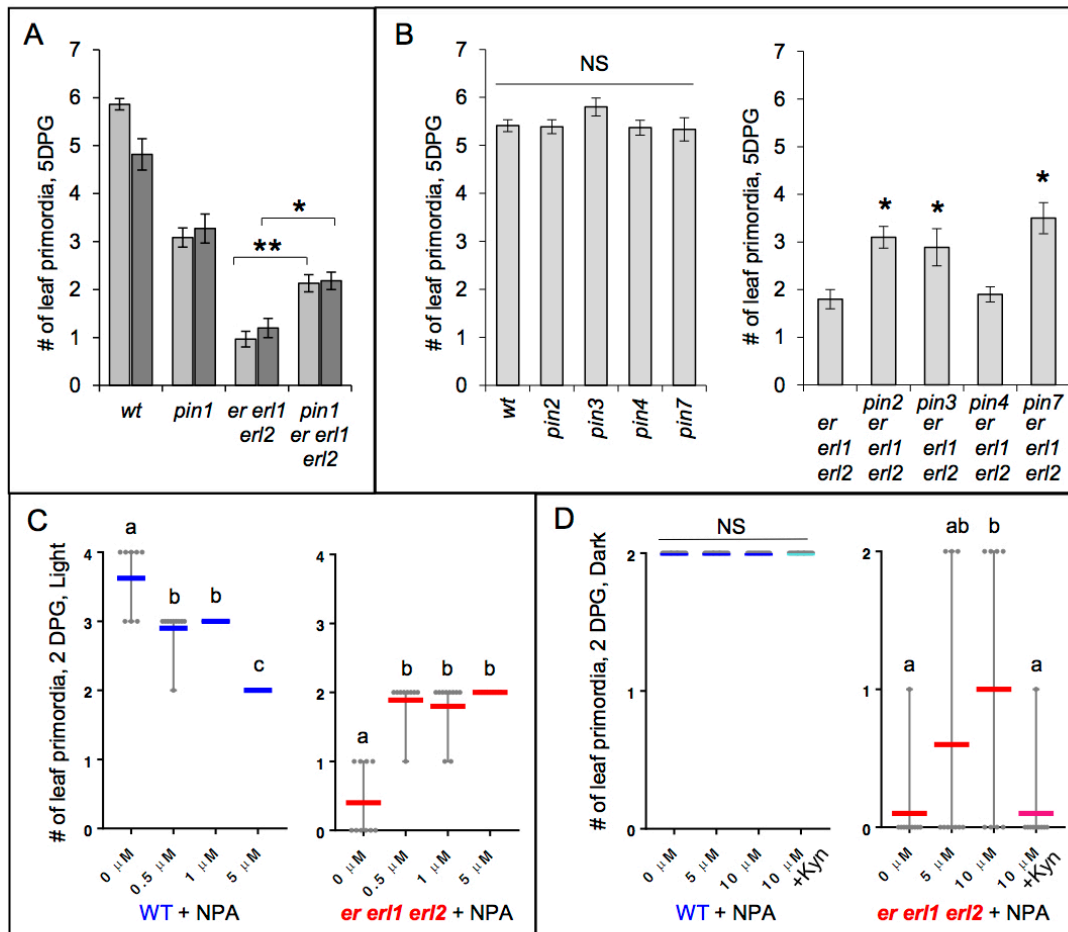


Figure 3.2. Decreased auxin transport partially rescues leaf initiation in *er erl1 erl2*.

A. The *pin1* mutation partially rescues leaf initiation defects of the *er erl1 erl2* mutant. Data are mean $\pm$ S.E. Two independent experiments were performed. Experiment #1 in light grey $n=23-30$; experiment #2 in dark grey $n=10-11$; Asterisks above bars indicate significant difference at $p<0.005$ (*) and $p<0.0005$ (**) as measured by unpaired two-tailed Student's t-test. **B.** While *pin2*, *pin3*, *pin4* and *pin7* mutations do not change leaf initiation on their own, *pin2*, *pin3*, and *pin7* partially rescue leaf initiation of *er erl1 erl2* $n=10-20$. An asterisk above a bar indicates significant difference at $p<0.01$ compared to the wt as measured by unpaired two-tailed Student's t-test. NS – no statistical difference. **A and B.** Seedlings were grown on solid MS media. Data are mean $\pm$ S.E. **C and D.** Inhibition of auxin transport with NPA promotes leaf initiation in *er erl1 erl2* while it inhibits leaf initiation in the WT seedlings in light and in dark. NPA cannot promote leaf initiation in *er erl1 erl2* seedlings in the presence of 10 μ M kynurenine (+Kyn) (turquoise or magenta). The rate of leaf primordia initiation was determined by DIC microscopy of fixed samples. Seedlings were grown in liquid MS media in light or dark as indicated. DPG = days post germination. The mean is indicated as a thick horizontal bar. Blue is WT and red is *er erl1 erl2*. The vertical lines designate the maximum and the minimum. Each grey point represents number of leaves from one individual plant. Different lowercase letters above bars indicate significant difference at $p<0.05$, as determined by one-way ANOVA with Tukey's post test. $n=8-20$.

compared to wild type (Guenot et al., 2012), but leaf formation was not reduced as drastically as in *er erl1 erl2*. Surprisingly, the quadruple mutant had more leaves than *er erl1 erl2*. The loss of *pin1*, a key promoter of leaf formation was not expected to improve leaf formation in *er erl1 erl2*, so we verified the result with two independent experiments (Figure 3.2, A, white and dark gray bars).

PIN1 is one member of the PIN-FORMED family of auxin efflux transporters (Sauer and Kleine-Vehn, 2019). To determine the effect of the other PIN-FORMED efflux transporters involved in PAT, we examined leaf formation in single mutants: *pin2*, *pin3*, *pin4*, and *pin7*. *PIN1* is the most important in leaf initiation as all other single *pin* mutants initiated the same number of leaves as wild type (Figure 3.2, B). To investigate the relationship between ERf and these PIN transporters, we generated quadruple *pin er erl1 erl2* mutants with *pin2*, *pin3*, *pin4*, and *pin7*. Leaf formation was improved in *er erl1 erl2* mutants with the additional loss of *pin2*, *pin3*, and *pin7*, but not *pin4*. The *PINs* that genetically interact with ERf – *PIN1*, *PIN2*, *PIN3*, and *PIN7* – are all expressed in either the vegetative SAM, or leaves (Guenot et al., 2012). *PIN4* is only weakly expressed in the epidermis of mature leaves, which may be why the loss of *pin4* did not alter *er erl1 erl2* leaf formation.

To further investigate the interaction between ERf regulated leaf initiation and auxin efflux, we utilized the auxin efflux inhibitor, N-1-Naphthylphthalamic Acid (NPA) (Teale and Palme, 2017). We grew wild type and *er erl1 erl2* plants with media supplemented with increasing concentrations of NPA and counted the number of leaves formed in 2 DPG plants (Figure 3.2, C). Wild type formed fewer leaves when exposed to

NPA, which corroborates previous results that auxin efflux is required for organ formation (Reinhardt et al., 2000). When *er erl1 erl2* was grown with the same concentrations of NPA that decreased leaf formation in wild type, there was improved leaf initiation. To verify the unexpected result of partially rescued leaf initiation in *er erl1 erl2* with the loss of auxin efflux, we grew wild type and *er erl1 erl2* seedlings in the dark with NPA supplemented media (Figure 3.2, D). Wild type leaf initiation was not altered. This indicates that initiation of the first two true leaves may occur prior to germination. In *er erl1 erl2*, leaf formation was again improved with inhibited auxin efflux. However, the partial rescue was not observed when we grew *er erl1 erl2* with both NPA and Kyn, indicating that the increased leaf formation in *er erl1 erl2* is dependent on the availability of auxin. In sum, our data indicates that the decreased leaf initiation in *er erl1 erl2* can be partially rescued by inhibiting auxin efflux through both genetic and pharmaceutical methods.

Increasing auxin on the L1 layer of the SAM rescues leaf initiation in *er erl1 erl2*

Previous results demonstrated that *er erl1 erl2* has abnormal auxin distribution. The auxin responsive *DR5rev::GFP* indicated that *er erl1 erl2* seedlings had elevated, sheet-like auxin accumulation in the L1 layer of the SAM (Chen et al., 2013). However, decreasing auxin levels using Kyn in younger seedlings did not improve leaf formation, but exogenous auxin was beneficial (Figure 3.1, A and B). This indicates that the decreased leaf formation in *er erl1 erl2* is not the result of excess auxin accumulation. To further understand the auxin defect in the *er erl1 erl2* SAM, we used confocal microscopy to examine *er erl1 erl2* seedlings expressing *DR5rev::GFP* at less than 1 DPG when the first two true leaves initiated

(Figure 3.3). Auxin peaks preceded leaf primordia formation in both wild type and in *er erl1 erl2* (Figure 3.3, A and B). In contrast to older vegetative stages, 1 DPG *er erl1 erl2* was able to form auxin peaks with distinct maxima and minima (Figure 3.3, B). The peaks in *er erl1 erl2* were sometimes patchy, and occasionally did not coincide with emergence of a primordium bump (Figure 3.3 C). At 1 DPG, wild type had already begun formation of the third primordium, but *er erl1 erl2* had only initiated formation of one leaf. Auxin accumulation in wild type leaf primordia was narrow, and ingressed downward into the lower tissues to form the presumptive vasculature as reported previously (Heisler et al., 2005). In contrast, *er erl1 erl2* had elevated, broader *DR5rev::GFP* expression, and the signal was restricted to the epidermis of the leaf.

Although *DR5rev::GFP* expression was abnormal and elevated in young *er erl1 erl2* plants, peaks still formed. It remained unclear why *er erl1 erl2* could not effectively form leaves. We inferred there could be at least two explanations for decreased leaf initiation in *er erl1 erl2*: either the L1 layer of the SAM cannot efficiently respond to auxin, or the absence of auxin signaling in the internal tissues is the main obstacle. NPA causes a redistribution of auxin in the vegetative SAM (Caggiano et al., 2017). In wild type *Arabidopsis*, tomato, and *Zea mays* this redistribution is unfavorable for leaf formation (Reinhardt et al., 2000; Scanlon, 2003). However, *er erl1 erl2* can initiate more leaves when treated with NPA. Does NPA alter auxin accumulation in the L1 layer, in internal tissues, or in both? To better understand why NPA treatment partially rescues *er erl1 erl2* leaf formation, we imaged and quantified *DR5rev::GFP* expression in *er erl1 erl2* with and without 5 μ M NPA treatment (Figure 3.4, A-C). To accurately compare auxin responses in

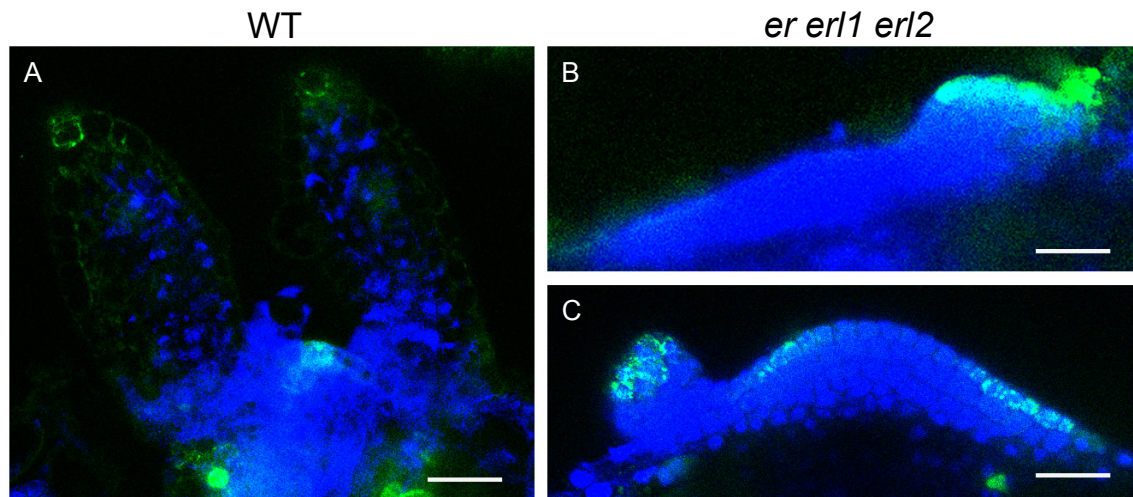


Figure 3.3. Auxin signaling is altered at early stages of seedling development in *er erl1 erl2*.

A-C. Confocal micrographs depicting auxin localization in the SAM of WT and *er erl1 erl2* in 1 DPG seedlings using the *DR5rev::GFP* auxin responsive reporter. **A.** In WT, *DR5rev::GFP* is expressed in the distal tips of the leaf primordia. Incipient primordia show expression in punctate maxima and signal ingresses downward into the lower tissues. **B.** In *er erl1 erl2*, only one leaf has formed, and expression is elevated and restricted to the epidermal layers of the emerging primordia. **C.** Expression in *er erl1 erl2* is elevated, sometimes patchy, and does not always coincide with primordia formation. **A-C.** Green = *DR5rev::GFP* expression. Blue = DAPI. Scale bar = 25 μ m. A and B are maximum projections of multiple optical sections, and C is a single optical section.

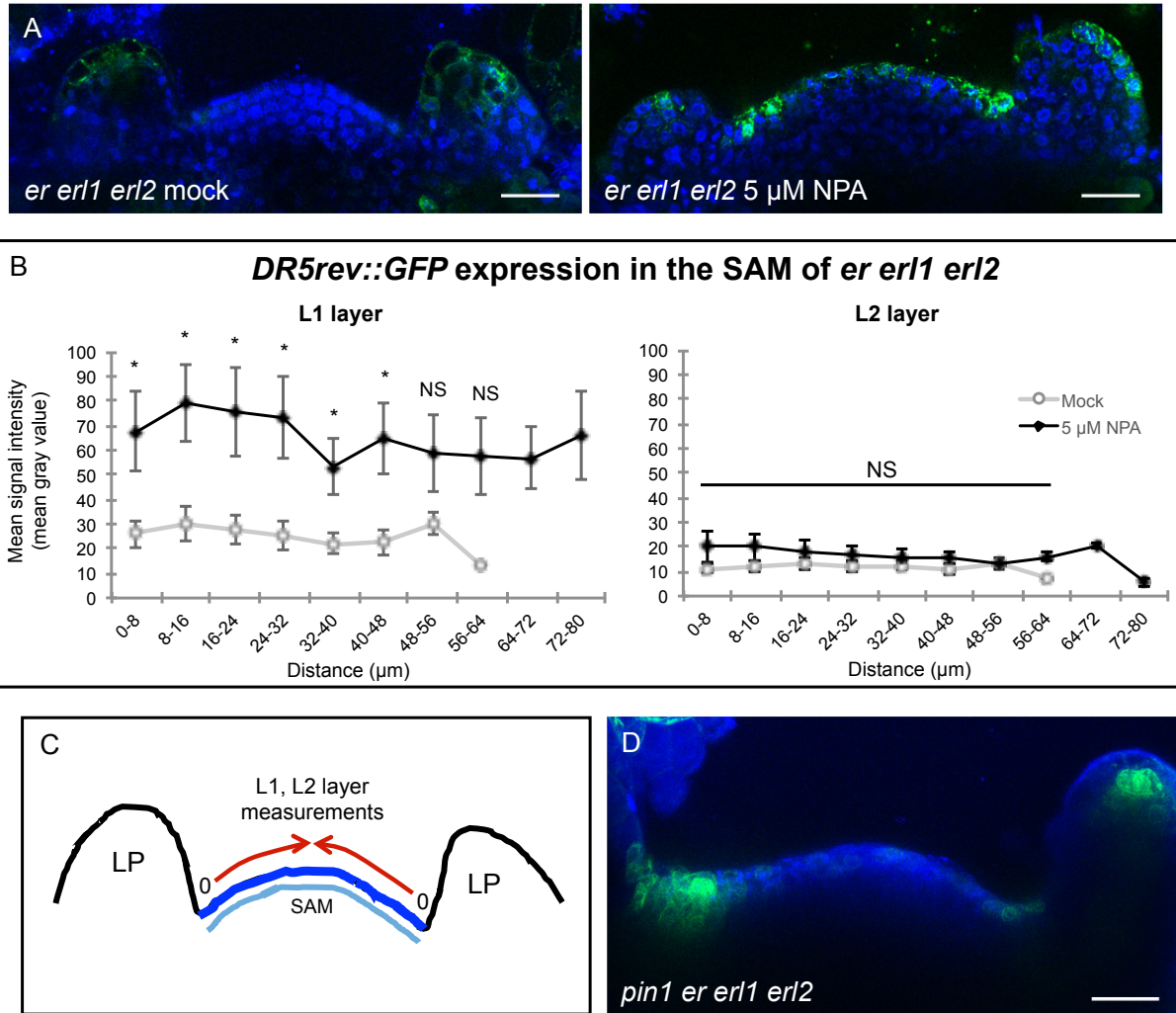


Figure 3.4. Inhibiting auxin efflux increases auxin accumulation in the L1 layer of *er erl1 erl2*

A. Optical sections of 1 DPG mock and NPA treated *er erl1 erl2* seedlings expressing *DR5rev::GFP*. Auxin accumulation is elevated on the L1 layer of *er erl1 erl2* in the presence of the auxin efflux inhibitor NPA (right) compared to mock (left). **B.** Quantitative measurements of the L1 layer (left) and the L2 layer (right) of the *er erl1 erl2* SAM. The presence of NPA increases the *DR5rev::GFP* signal in the L1 layer of *er erl1 erl2*, but there is no significant difference on the L2 layer. Data points are mean signal intensity $\pm$ S.E over the distance indicated on the x-axis. White circle = mock. Black diamond = 5 μ M NPA. Asterisks above data points indicate significant difference at $p < 0.05$ as measured by unpaired two-tailed Student's t-test. NS = not significant. **C.** Diagram of quantitative measurement process shown in B. The L2 layer measurements are slightly shorter due to the tight radius of the L2 layer in the dome-shaped SAM. Red arrows = direction of measurement from leaf primordia (zero) to center of SAM. Blue = L1 layer measurement. Turquoise = L2 layer measurement. LP = leaf primordia. **D.** Representative confocal micrograph of 1 DPG *pin1 er erl1 erl2* expressing *DR5rev::GFP* with elevated auxin accumulation on the L1 layer. **A and D.** Blue = DAPI. Green = *DR5rev::GFP*. Scale bar = 25 μ m.

mock and NPA, we only evaluated 1 DPG seedlings that had initiated two leaves, because leaves become auxin sources when they emerge (Cheng et al., 2007a). We measured GFP signal intensity of the L1 and L2 layers using optical sections of the SAM. Measurements were acquired starting at the boundary of the adaxial side of the leaf and the SAM (0 μ m) in either the L1 or L2 layers and moved inward to the center of the SAM (Figure 3.4, C). L2 layer measurements yielded slightly shorter distances due to the smaller radius of the lower cell layer of the dome shaped meristem. NPA treatment slightly increased the width of the *er erl1 erl2* SAM (Figure 3.4, B). This is not specific to *er erl1 erl2* as a similar response was previously observed in wild type (Zhang et al., 2020). The *DR5rev::GFP* signal was often absent from the center of the meristem in both mock and NPA treated *er erl1 erl2* (Figure 3.4, A and B), which is consistent with previous results that the SAM is less sensitive to auxin in the center (Vernoux et al., 2011). However, it was evident that the signal in the L1 layer close to the forming leaf primordia of NPA treated *er erl1 erl2* seedlings was significantly brighter than mock. The difference in *DR5rev::GFP* signal did not extend downward into the L2 layer, as no significant changes in signal were seen in the NPA treated seedlings compared to mock. The elevated signal indicates that auxin signaling is increased exclusively in the L1 layer of *er erl1 erl2* when exposed to an auxin efflux inhibitor, and this correlates with increased leaf formation (Figure 3.2, C and D).

We hypothesized that decreasing auxin efflux in *er erl1 erl2* promotes auxin accumulation on the epidermis of the SAM to partially rescue leaf formation. To test our hypothesis, we used an alternative method to reduce auxin efflux by eliminating *pin1* from *er erl1 erl2*. In a previous study, *pin1* was shown to have an increased auxin accumulation in the vegetative SAM (Guenot et al., 2012). We expressed *DR5rev::GFP* in *pin1 er erl1 erl2*

quadruple mutant seedlings, and imaged them using fluorescent confocal microscopy. Similar to *er erl1 erl2* NPA treated seedlings, *pin1 er erl1 erl2* showed elevated auxin accumulation in the L1 layer of the SAM at the adaxial leaf junction compared to *er erl1 erl2* (Figure 3.4, A ‘mock’ and D). The increase of auxin in the L1 layer corresponds with the partial rescue of leaf initiation in *pin1 er erl1 erl2* compared with *er erl1 erl2* (Figure 3.2, A). Taken together, our data supports the hypothesis that inhibition of auxin efflux promotes auxin accumulation in the L1 layer of the *er erl1 erl2* SAM and improves leaf initiation.

ERf and *PID* synergistically promote initiation of cotyledons and leaves

ERfs promote leaf initiation, and the *er erl1 erl2* mutant leaf initiation can be improved by inhibiting auxin efflux. The polar localization on the plasma membrane of PIN efflux transporters is regulated by the PID kinase (Friml et al., 2004; Dhonukshe et al., 2010). Loss of function *pid* mutants greatly resemble the *pin1* phenotype (Benjamins et al., 2001). We wanted to test if the genetic interactions between *PID* and ERf were similar to the interactions between *PIN1* and ERf. To test this, we generated quadruple *pid er erl1 erl2* mutants using a strong *pid-3* allele.

Two cotyledons invariably form in wild type plants (Figure 3.5, A and Table B). In *er erl1 erl2*, two cotyledons form in 94 percent of seedlings, and seedlings occasionally initiate one or three cotyledons, indicating ERf weakly regulates cotyledon initiation. Loss of function *pid* mutants initiate three cotyledons in 46 percent of seedlings, but sometimes initiate a single cotyledon. *Pid* mutants do initiate two cotyledons, but many are bent or

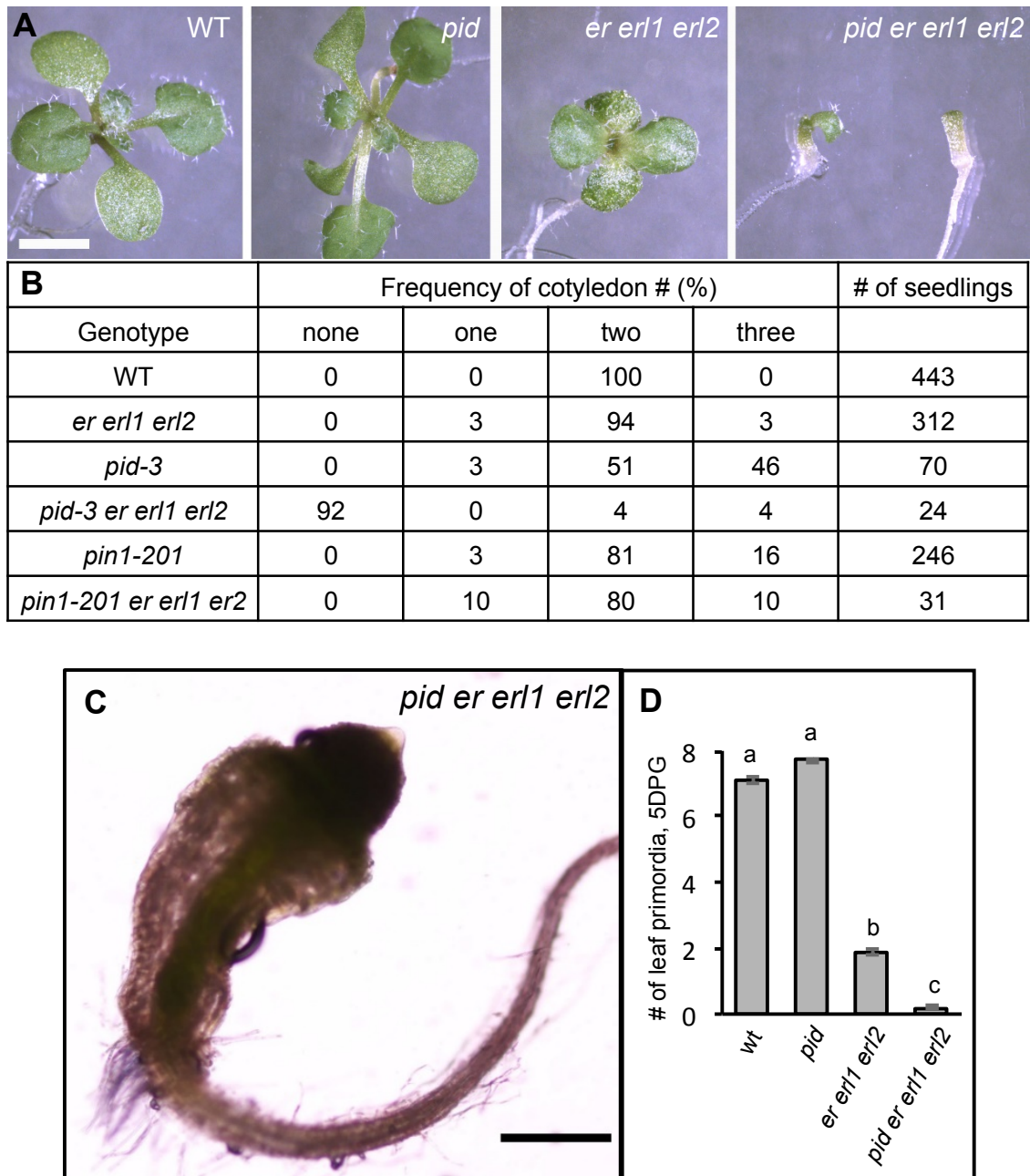


Figure 3.5. ERfs and *PID* synergistically promote initiation of cotyledons and leaves.

A. Representative images of 13 DPG seedlings demonstrate reduced number of cotyledons and leaves in *pid er erl1 erl2*. Scale bar = 2 mm. **B.** Comparison of cotyledon number in selected mutants versus WT. *Pid* and ERfs strongly, and synergistically interact in cotyledon initiation, but *PIN1* and ERfs weakly interact. **C.** Representative image of a one-month-old *pid er erl1 erl2* plant demonstrates the presence of a shoot apical meristem, and an absence of cotyledon and leaves. Scale bar = 0.5 mm. **D.** Comparison of formed leaf primordia in WT and mutants using 5 DPG seedlings. Data are mean $\pm$ S.E; n=10-15. Different lowercase letters above bars indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test.

asymmetrical, as previously reported (Bennett et al., 1995). In *pid er erl1 erl2* mutants, we observed a striking loss of all cotyledon initiation in 92 percent of seedlings. Because *PID* is a regulator of the *PIN1* auxin transporter, we also characterized the genetic interaction between *pin1* and *erfs* in cotyledon initiation (Figure 3.5, Table B). The loss of *pin1* alters cotyledon initiation, but the effect is weaker than the loss of *pid* with 19 percent of *pin1* seedlings having an abnormal number of cotyledons. The additional loss of *er erl1 erl2* from *pin1* mutants also decreases cotyledon initiation. The *pin1* mutation has a much weaker genetic interaction with *erf* than *pid* in embryonic organ development. The number of cotyledons in *pin1 er erl1 erl2* is only slightly changed compared to *pin1*. In contrast to *pid er erl1 erl2*, one or more cotyledons always initiate in *pin1 er erl1 erl2*, and two cotyledons initiate in ~80 percent of seedlings. Collectively, this data shows *PID* and *ERfs* synergistically promote cotyledon initiation, and *PIN1* has only an ancillary role. The redundancy of *PIN1*, *PIN3*, *PIN4*, and *PIN7* during embryogenesis was previously shown (Friml et al., 2003). The subtle defects in cotyledon initiation in *pin1 er erl1 erl2* compared to *pid er erl1 erl2* may be due to *PIN* redundancy.

In addition to the absence of embryonic organ initiation in the SAM, *pid er erl1 erl2* also has severe post-embryonic organ initiation deficiencies. The SAM is left exposed in plants as old as one month and is easily identifiable by its lack of pigmentation (Figure 3.5, C). Greater than 1 month old *pid er erl1 erl2* seedlings did eventually produce a stem-like organ, but it was extremely short. To characterize the genetic interactions between *ERf* and *PID* in leaf formation, we counted the number of leaves formed in 5 DPG *pid er er1 erl2* (Figure 3.5, D). Compared to wild type, *pid* formed approximately the same number of leaves, sometimes more, although the difference was not statistically significant

($P=0.07394$ in unpaired two-tailed Student's *t*-test). However, the quadruple mutant *pid er erl1 erl2* was almost always unable to form leaves. Sporadic leaf formation did occur, but only in 2 of 15 seedlings examined. Taken together, this data shows a synergistic interaction between ERf and *PID* in leaf initiation.

Decreasing PAT in the *er erl1 erl2* background improves leaf initiation. Surprisingly, the loss of *pid* has an opposite effect on *er erl1 erl2*. To understand why ERfs and *PID* synergistically regulate cotyledon formation, we observed auxin signaling in the cotyledon primordia in embryos. Both *pid* and *er erl1 erl2* are sterile and cannot form seeds. We circumvented this by examining embryos of the heterozygous, seed producing plants *pid*^{+/-}, *er erl1*^{+/-} *erl2*, and *pid*^{+/-} *er erl1*^{+/-} *erl2*; all of which expressed *DR5rev::GFP*. We imaged embryos in the late heart and early torpedo stage using fluorescent confocal microscopy (Figure 3.6). The wild type had a strong signal in the hypophysis, and in strands in the forming primordia marking the preprocambium of the cotyledons. The signal was also slightly elevated in auxin maxima at the tips of the primordia. The *er erl1 erl2* triple mutant embryos were identifiable by shorter cotyledon primordia (Chen and Shpak, 2014). The signal in the hypophysis of *er erl1 erl2* was the same as in wild type. Cotyledon expression in the *er erl1 erl2* mutant was like wild type, but the maxima at the tips were variable and sometimes brighter and broader. This was not evident in previous work using epifluorescent microscopy (Chen and Shpak, 2014), but confocal microscopy showed these details. Most often, the forming vasculature of *er erl1 erl2* had weaker signal than wild type. This is consistent with previous results that show decreased *DR5rev::GFP* signal in the vasculature of leaves and hypocotyl (Chen et al., 2013). The *pid* mutant embryos were identifiable by abnormal number or disproportionate size of cotyledons initiated (Bennett

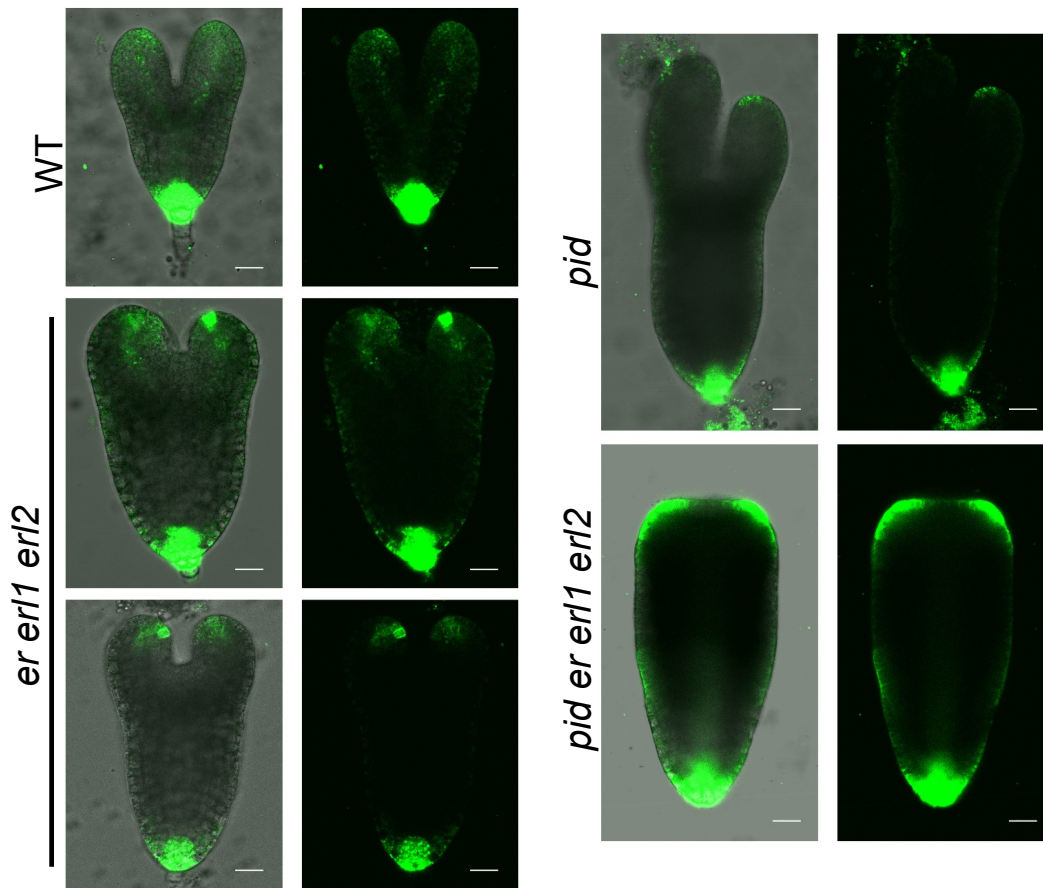


Figure 3.6. ERfs and *PID* synergistically promote auxin signaling during cotyledon initiation.

Optical sections of WT, *er erl1 erl2*, *pid*, and *pid er erl1 erl2 er erl1 erl2* embryos expressing *DR5rev::GFP*. Each genotype has a DIC image overlaid with corresponding fluorescent image (left) and the fluorescent image alone (right). In WT, *DR5rev::GFP* expression is elevated at the tips of cotyledon primordia, and there is clear expression in the forming vasculature of the internal tissues. The *er erl1 erl2* mutant is similar to wild type, but expression is increased and there is variation in the forming vasculature expression. The *pid* mutant forms broad peaks in the epidermis of the cotyledons, and the internal expression is absent. Cotyledons do not form in *pid er erl1 erl2*, but auxin signaling is elevated on the flank surrounding the SAM and is absent in the internal tissues. Green = *DR5rev::GFP*. Scale bar = 20 μ m.

et al., 1995). We observed weak, less focused auxin maxima in the cotyledon primordia, and the signal was dramatically reduced in the developing vasculature of the *pid* mutant, as reported previously (Treml et al., 2005). In contrast to *pid*, the quadruple *pid er erl1 erl2* mutant had extremely strong *DR5rev::GFP* expression at the flanks surrounding the SAM in the L1 layer where the cotyledons should have initiated. No signal was present in the internal tissues, as all of it was restricted to the epidermal tissues. In the *pid er erl1 erl2* embryo, *DR5rev::GFP* indicates auxin signaling is present where presumptive cotyledons should be in the periphery of the SAM. To investigate post-embryonic organ development in *pid er erl1 erl2* mutants, we observed auxin signaling in the vegetative SAM of 3 DPG plants (Figure 3.7). Even after 3 DPG, auxin signaling was absent from internal tissues and still elevated in the *pid er erl1 erl2* mutant where leaves should have initiated. Taken together, our data indicates that auxin accumulates and is perceived in the apex of *pid er erl1 erl2*, but the signal is not translated into an appropriate response for cotyledon or leaf initiation. In *er erl1 erl2*, the primary reason for reduced leaf initiation is the inability to respond to auxin. In *pid er erl1 erl2*, the decrease in auxin accumulation in internal tissues due to *pid* and *er erl1 erl2* mutations further exacerbates organ initiation and causes a catastrophic loss of organ formation.

Our work shows that leaf initiation in *er erl1 erl2* is limited by auxin levels (Figure 3.1), and auxin accumulation on the L1 layer can improve *er erl1 erl2* leaf initiation (Figure 3.4). We wanted to test this phenomenon on the extreme organ deficiency phenotype of *pid er erl1 erl2*. We grew *pid er erl1 erl2* on media supplemented with 10 μ M 2,4-D together with a mock control group. After three days of exposure to 2,4-D, we used DIC microscopy

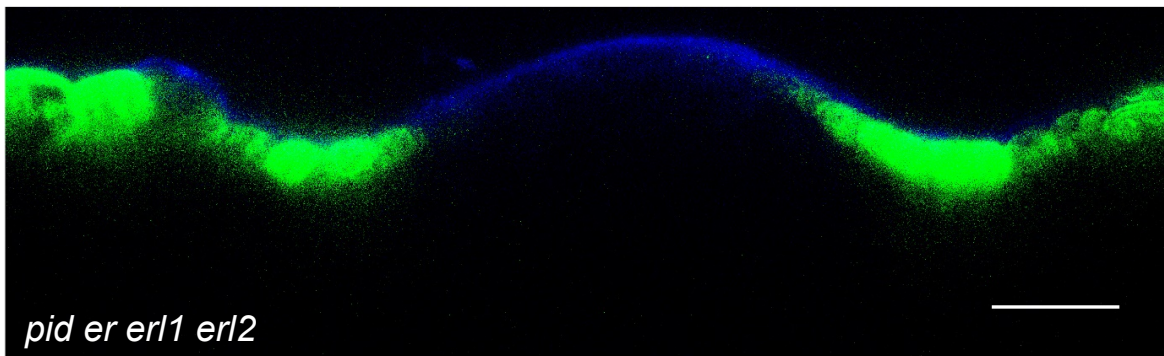


Figure 3.7. ERfs and *PID* synergistically promote auxin signaling in the SAM.
 The *DR5rev::GFP* reporter indicates auxin signaling is present in the periphery of the vegetative SAM of the 3 day post germination *pid er erl1 erl2* seedling, but leaves cannot initiate. Green = *DR5rev::GFP*. Blue = DAPI. Scale bar = 25 μ m.

to observe the SAM (Figure 3.8, A). In the mock treatment, no cotyledons or leaf primordia initiated. When grown with 2,4-D, *pid er erl1 erl2* formed a ring-like outgrowth of tissue where the presumptive cotyledons should initiate. The *pid er erl1 erl2* seedlings left to grow on 2,4-D media were stunted, and the organ primordia from the SAM could not elongate. This may be due to the inhibitory effect of auxin at elevated concentrations (Thimann, 1938). We then transferred both the mock and 2,4-D *pid er erl1 erl2* seedlings to standard media with no chemical treatment at 13 DPG. The mock treated seedlings were consistently unable to initiate organs from the SAM at 33 DPG (Figure 3.8, B). However, the 2,4-D treated plants moved to standard media formed callus-like growths at the SAM region. While stunted in their elongation, the growths had leaf-like qualities, and formed trichomes. The outgrowths initiated in concentric rings around the SAM, are reminiscent of the ring-like primordial tissue surrounding the SAM in 3 DPG seedlings (Figure 3.8, A). This suggests the lack of organ differentiation in the *pid er erl1 erl2* SAM is due to limited auxin response.

In sum, our data indicates that PID and ERf synergistically promote initiation of cotyledons and leaves. We also show that the loss of organ formation in the quadruple *pid er erl1 erl2* is primarily due to auxin deficiencies.

Different roles of ERfs in leaf and flower initiation

Until this point, we have examined auxin and leaf initiation in the vegetative SAM. Auxin drives the process of flower organ initiation in the inflorescence meristem similarly to leaf initiation in the vegetative meristem with some exceptions. Just like leaf primordia,

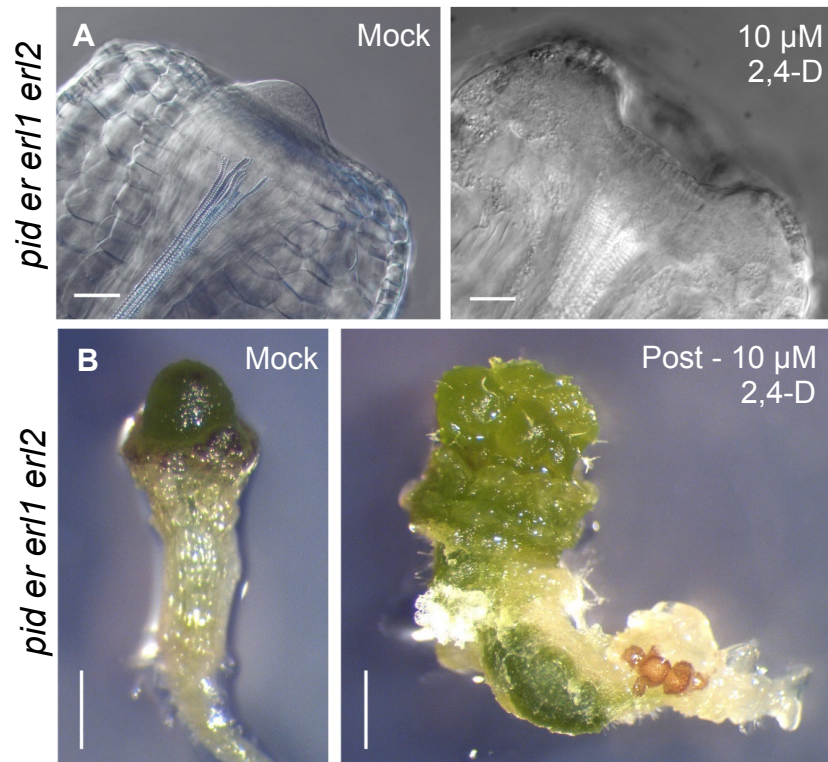


Figure 3.8. Aboveground tissue differentiation in *pid er erl1 erl2* is partially rescued by auxin.

A. Primordia tissue initiates in 3 DPG *pid er erl1 erl2* in a ring-like pattern when germinated on 2,4-D supplemented media. Scale bar = 50 μm. **B.** Callus tissue with leaf-like qualities initiate in a ring-like pattern in *pid er erl1 erl2* after transfer to standard media following 16 days of 2,4-D treatment. The meristematic region forms bulging green organs with trichomes. Plants shown are 33 DPG. Transfer took place at 13 DPG. Scale bar = 1 mm.

auxin peaks precede flower primordia initiation, but the peaks for flowers are driven exclusively by PIN1 (Guenot et al., 2012; Kierzkowski et al., 2013). The loss of function *pin1* mutant cannot initiate organs from the inflorescence meristem, but leaves still form in the vegetative meristem (Okada et al., 1991; Guenot et al., 2012). The *er erl1 erl2* triple mutant initiates organs from the inflorescence meristem, but the vegetative meristem is dramatically decreased in initiation rate. To address the contribution of ERfs to flower initiation, we studied the genetic interactions between *PIN1* and ERf in the inflorescence meristem.

We observed the mature inflorescences of *pin1*, *pin1 er erl2*, and *pin1 er erl1 erl2* (Figure 3.9, A). The naked, pin shaped inflorescence meristem was observed as expected in *pin1* mutants. Surprisingly, the additional loss of *erfs* partially rescued organ formation. The triple mutant *pin1 er erl2* formed small, aberrant, carpeloid protrusions all along the inflorescence stem. *Pin1 er erl2* was sterile, and formed compact, gnarled, carpeloid inflorescences closer to the apex of the plant. The quadruple mutant *pin1 er erl1 erl2* also had partially rescued organ formation. When *erf* signaling is completely eliminated from *pin1*, randomly positioned carpeloid organs are formed. These carpeloid protrusions were covered in papilla, especially at the apex of the inflorescence meristem. This data indicates that ERf and *PIN1* have a functional interaction in the inflorescence SAM.

The naked *pin1* inflorescence phenotype is attributed to the absence of auxin peaks in the inflorescence meristem (Guenot et al., 2012). Our data indicates that ERfs regulate auxin in the vegetative meristem. The unique functional interaction that ERfs have with *PIN1* implies that ERfs may also regulate auxin in the inflorescence meristem. To explore

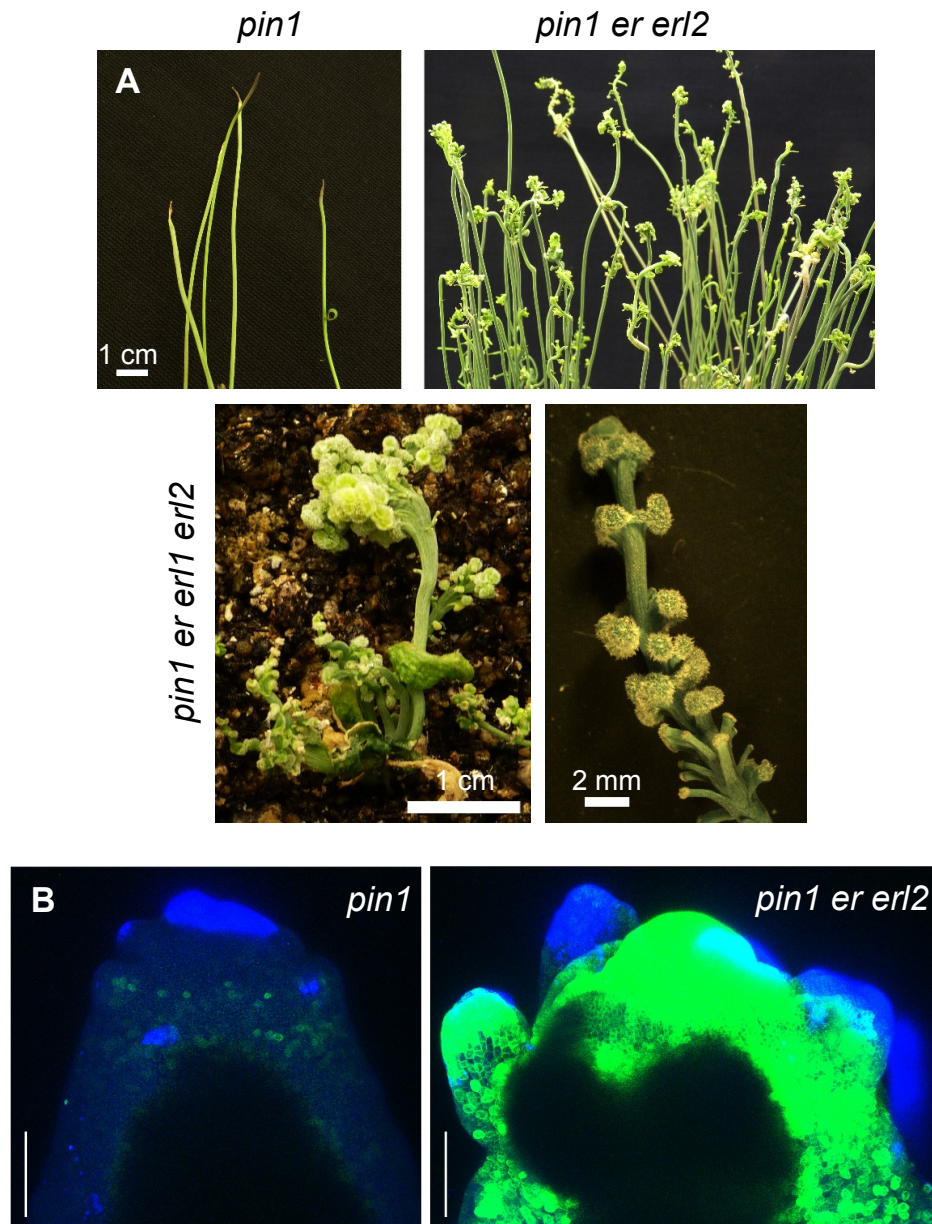


Figure 3.9. Downregulation of ERf signaling in the *pin1* mutant partially rescues organ initiation and increases auxin signaling in the epidermis of the inflorescence meristem.

A. The *er erl2* mutations partially rescue flower organ initiation defects observed in *pin1*. The *pin1 er erl1 erl2* mutant is an extreme dwarf which is able to form numerous randomly positioned carpeloid organs. **B.** Auxin signaling distribution in the inflorescence SAM of *pin1* and *pin1 er erl2* mutants, as determined by the activity of the synthetic auxin response element *DR5rev::GFP*. Auxin signaling in the *pin1* mutant is elevated with the loss of *erf* signaling. Scale bar = 100 μ m.

the effect of ERfs on auxin during flower formation, we expressed *DR5rev::GFP* in *pin1* and *pin1 er erl2* and observed the mature inflorescence meristem (Figure 3.9, B). The *pin1* mutant inflorescence had an extremely diffuse signal, apart from some cells sparsely arranged around the flank of the inflorescence SAM. No auxin peaks were observed. The down regulation of *erf* signaling in the *pin1* SAM caused dramatically altered auxin accumulation. *DR5rev::GFP* expression was elevated all over the epidermis of the *pin1 er erl2* apex. Aberrant organ primordia initiated, and the *DR5rev::GFP* signal in the primordia was also elevated. The more elongated inflorescence primordia were, the more the signal was increased at the distal tip compared to the proximal portion. This pattern was also apparent in the primary inflorescence meristem. The apex of the inflorescence meristem had the highest signal, and the cells in the flanks decreased in signal the farther away from the apex they were. Taken together, the loss of *erfs* leads to increased auxin accumulation on the *pin1* inflorescence meristem, which correlates with improved organ initiation.

We also examined the transition from vegetative rosette leaf growth to inflorescence bolting in *er erl1 erl2* and *pin1 er erl1 erl2* (Figure 3.10, A). The *pin1 er erl1 erl2* (~47 DPG) plant bolted later than *er erl1 erl2* (~33 DPG). The flower-like organs produced by both plants were sterile and deformed. Additionally, we examined the mature plant inflorescence stem height, as ERfs and PIN1 are known to promote stem length (Shpak et al., 2004; Jones et al., 2005). Compared to wild type, *pin1* and *er erl1 erl2* both had decreased stem height (Figure 3.10, B). The triple mutant *er erl1 erl2* was shorter than *pin1* indicating ERf signaling is more important for stem elongation. The effect of losing both *pin1* and *erf* caused severe dwarfism to an even greater extent than the combined decrease in height caused by individual *pin1* or *erf* mutations (Figure 3.10, B and C). This



Figure 3.10. *PIN1* and ERfs synergistically promote inflorescence growth.

A. Representative images of *er erl1 erl2* and *pin1 er erl1 erl2*. The *pin1 er erl1 erl2* takes longer to bolt. Scale bar = 5 mm. **B.** Average length of the main stem at maturity. Data are mean $\pm$ SD, $n=8-30$. Different lowercase letters above bars indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test. **C.** Representative images of two-month-old plants. *Quad* = *pin1 er erl1 erl2*. Two *quad* plants are depicted. Pictures are at the same magnification. Scale bar = 3 cm.

indicates that ERf and PIN1 synergistically regulate height. Our current and previous work (Chen et al., 2013) suggests ERfs promote auxin signaling in the internal tissues. PIN1 promotes auxin transport through the vasculature of stem segments (Okada et al., 1991; Bennett et al., 2016). We therefore speculate that the loss of internode elongation in *pin1 er erl1 erl2* is due to auxin defects in internal tissues.

Discussion

The relationship between auxin and ERfs has been investigated previously in *Arabidopsis* in other organs besides the SAM. The *erf* and *epfl2* mutants have reduced leaf teeth and broadened auxin maxima at the emerging serrations compared to wild type (Tameshige et al., 2016). ERfs are strongly expressed in the newly elongating leaf teeth, especially at the distal tip of the leaf, and *EPFL2* expression is excluded from the elongating serration. Serration outgrowth can be rescued in *erf* mutants by expressing *ERECTA* at the tip of the leaf tooth, and *epfl2* mutant leaf teeth can be rescued with *EPFL2* expression in the sinus tissue surrounding the teeth. A similar phenomenon was seen in ovule initiation. *EPFL2* is expressed in the border between ovules in developing pistils, and *ERL1* and *ERL2* are expressed throughout the septum, especially at the tips of the emerging ovules (Kawamoto et al., 2020). Mutant *epfl2* plants have variable distances between the expression domains of *DORNROSCHEN* (*DRN*), an auxin responsive gene, in forming ovules compared to wild type. This corresponds to ovules forming in irregular pattern. In the vegetative SAM, *er erl1 erl2* mutants have elevated auxin signaling in the L1 layer, and this correlates with decreased leaf initiation and altered phyllotaxy (Chen et

al., 2013). These studies present a model where ERf signaling negatively regulates auxin signaling at the border region to promote new tissue outgrowth.

We examined the SAM and showed that ERfs negatively regulate auxin accumulation in organ initiation in multiple stages of development. The *er erl1 erl2* embryo has variable, elevated auxin maxima in newly initiated cotyledon primordia. Mutants deficient in *er erl1 erl2* signaling have elevated, patchy signaling in the L1 layer of the vegetative SAM. The *pin1* mutant cannot accumulate auxin in the inflorescence meristem, but the additional loss of *erf* signaling causes a dramatic increase in auxin signaling. Our data supports a model where ERfs negatively regulate auxin accumulation.

We also showed that while ERfs negatively regulate auxin accumulation, they are also required to adequately translate auxin signal into a physiological response (Figure 3.11, A). We revealed that *er erl1 erl2* has decreased leaf initiation due to decreased L1 layer auxin response, despite its ability to perceive auxin, as indicated by the auxin responsive construct *DR5rev::GFP* (Figure 3.11, B). Leaf initiation in *er erl1 erl2* can be improved by elevating auxin levels in the L1 layer with either exogenous auxin or inhibition of auxin efflux (Figure 3.11, C), indicating the necessary auxin accumulation threshold for leaf initiation is increased in *er erl1 erl2*. Additionally, decreasing auxin levels with the auxin biosynthesis inhibitor Kyn severely inhibited leaf initiation in *er erl1 erl2* (Figure 3.11, D). The *pid* mutation, which causes diffuse auxin peaks and decreased auxin accumulation in the internal tissues, triggers aborted organ initiation when combined with *er erl1 erl2*. Interestingly, *DR5rev::GFP* expression is elevated where cotyledons and leaves should initiate in the *pid er erl1 erl2* SAM, indicating the quadruple

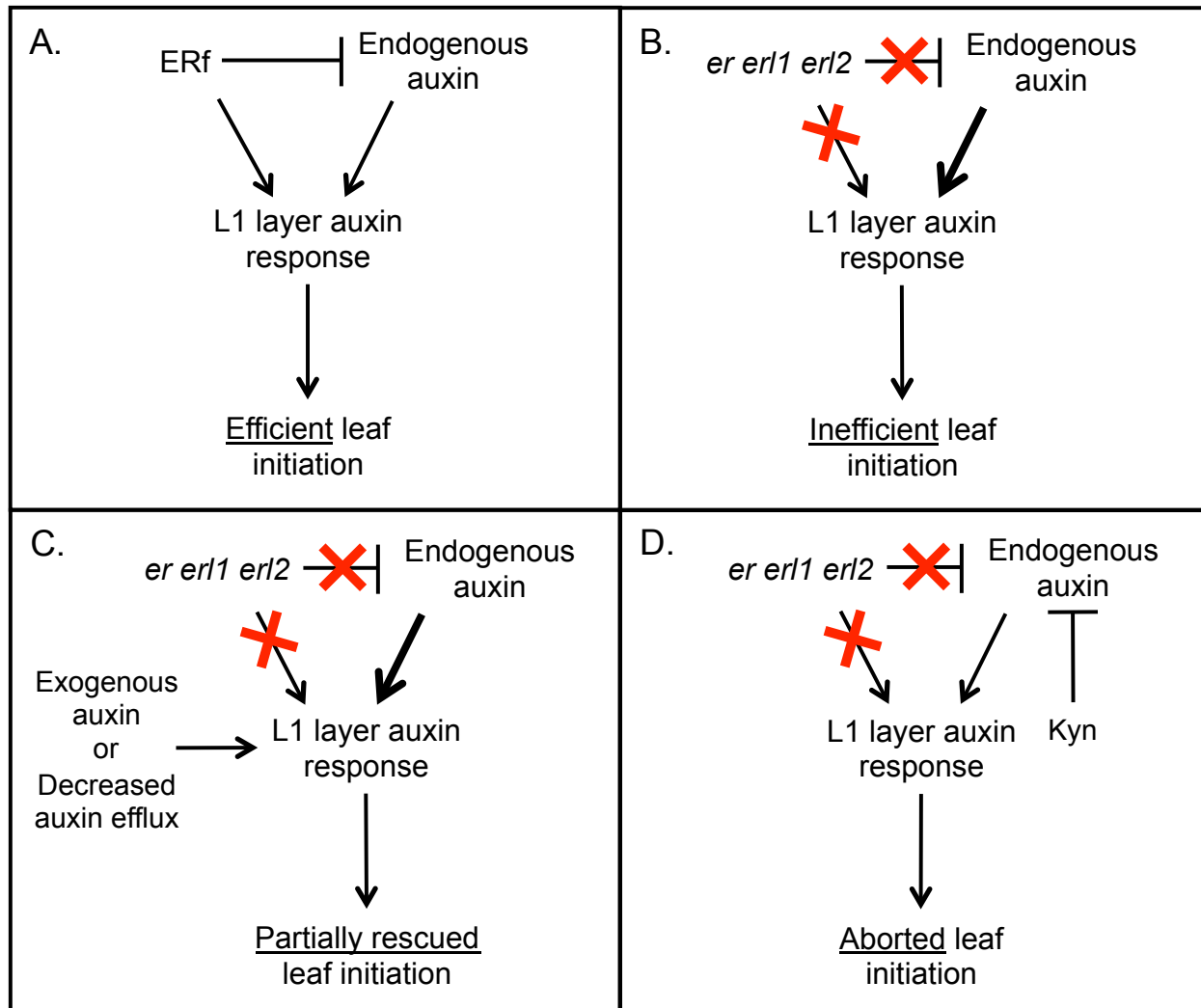


Figure 3.11. ERfs have a dual role in auxin regulation.

A. Functional ERfs inhibit endogenous auxin accumulation and promote L1 layer auxin response in the SAM to efficiently initiate leaf formation. **B.** Loss of all three *erfs* causes decreased L1 layer auxin response and elevated auxin accumulation, which prevents efficient leaf initiation. **C.** Inefficient leaf initiation in *er erl1 erl2* is due to inadequate L1 layer auxin responses and can be partially rescued by elevating auxin accumulation in the L1 layer through exogenous auxin or decreased auxin efflux. **D.** The *er erl1 erl2* mutant with inadequate auxin response in the L1 layer is further exacerbated by decreasing levels with the auxin biosynthesis inhibitor, Kynurenine (Kyn). Organ formation is decreased, and leaves cannot form.

mutant is capable of auxin perception, but is not able to translate auxin sensing into an organ initiation response.

A favorable redistribution of auxin occurs in *er erl1 erl2* mutants treated with NPA that elevates auxin signaling in the L1 layer and rescues leaf initiation. In wild type plants treated with NPA, leaf initiation decreased. A redistribution of auxin to the epidermal layers in NPA treated wild type plants was previously observed in the first two true leaves (Qi et al., 2014). Wild type plants have less auxin accumulation in the L1 layer than *er erl1 erl2* mutants, and rely primarily on auxin transport to accumulate auxin into distinct maxima on the L1 layer to reach the appropriate threshold to initiate leaf formation. When auxin transport is lost, wild type plants accumulate auxin to the L1 layer, but auxin levels are too low to form leaves efficiently. Conversely, *er erl1 erl2* has plenty of auxin in the L1 layer of the SAM. The primary limitation for organ initiation in *er erl1 erl2* is the inability to translate L1 layer auxin to a physiological response. Inhibiting auxin efflux in *er erl1 erl2* with either NPA or the loss of *pin1* redistributes excess auxin to the L1 layer of the vegetative SAM. This allows *er erl1 erl2* plants to reach the required threshold for leaf initiation.

Earlier work shows increased expression of *PIN1pro:PIN1-GFP* in the L1 layer in *er erl1 erl2* seedlings (Chen et al., 2013). Auxin gradients in the SAM polarize PIN1 and promote PIN1 expression in a positive feedback loop (Reinhardt et al., 2003; Sauer et al., 2006; Bhatia et al., 2016). With this understanding, the increased PIN1 expression may be a side effect of the buildup of auxin accumulation due to the inability of *er erl1 erl2* to negatively regulate auxin signaling. Therefore, inhibiting auxin efflux may have a twofold benefit to promote leaf initiation in *er erl1 erl2*. The first is elevating auxin accumulation in the L1 layer. The second is

throttling the unrestricted secretion of auxin due to elevated PIN1 expression that occurs after the auxin-PIN1 feedback loop increases PIN1 accumulation.

It is curious that *PID*, a regulator of *PIN* polarity, exhibits a dramatically different interaction with ERF signaling than *PIN1*. While loss of *pin1* improves leaf initiation in *er erl1 erl2*, the combined loss *pid* and *erf* completely abolishes all organ initiation from the SAM. Auxin accumulation in the *pid* mutant is altered in both the epidermal tissues and the internal tissues. Either one or both of these auxin accumulation defects are intensifying the defects of *er erl1 erl2*. Our data cannot determine if the aborted organ formation in *pid er erl1 erl2* is the result of the loss of *pid* causing a decrease in internal auxin accumulation or altered auxin responses in the epidermal tissues.

The *pid er erl1 erl2* mutant bears a striking resemblance to other *pid* mutant combinations, but there are distinct differences within each. The double mutant *pid pin1* is unable to generate cotyledons due to the loss of a crucial auxin efflux transporter which promotes auxin accumulation into peaks to reach the appropriate threshold for cotyledon initiation (Furutani et al., 2004). The loss of *erf* signaling has the opposite effect on PIN1 with elevated expression in the L1 layer of the SAM showing different effects of ERF and *PID* signaling on PIN1 (Chen et al., 2013). The *pid yuc1 yuc4* mutant fails to initiate cotyledons indicating auxin biosynthesis is necessary to achieve adequate auxin levels for organogenesis (Cheng et al., 2007b). In contrast, *DR5rev::GFP* is clearly active where organs should initiate in *pid er erl1 erl2* in response to endogenous auxin production. The *ENHANCER OF PINOID (ENP)/MACCHIBOU 4/(MAB4)* encodes a scaffold protein that was proposed to prevent the lateral diffusion of PIN1 on the plasma membrane to promote auxin peaks (Glanc et al., 2021). Loss of function *pid*

enp/mab4 mutants also cannot initiate cotyledons and have weak, or entirely absent auxin accumulation in the shoot (Trembl et al., 2005). In contrast, *pid er erl1 erl2* mutant has strongly elevated *DR5rev::GFP* expression indicating auxin is present and can be perceived, but there is still no organ initiation response. Interestingly, despite the already strong *DR5rev::GFP* expression in *pid er erl1 erl2*, the quadruple mutant benefits from exogenous auxin, which can partially improve organ initiation. This reaffirms that the severe phenotype is in fact the result of auxin defects due to the synergistic effect of *pid* and *er erl1 erl2* mutations. Therefore, all of these mutants that lack cotyledons are the result of auxin deficiencies, but *pid er erl1 erl2* has no issues accumulating auxin. The *pid er erl1 erl2* mutant is instead unable to convert auxin signal into a viable physiological response.

Prior to this study, enhancing auxin levels was shown to rescue some of the *erf* mutant phenotypes. Loss of function *er* reduced plant height and shortened pedicels can be rescued by up regulation of *YUCCA5*, a member of an enzyme family responsible for the final conversion of indole-3-pyruvic acid (IPyA) to form indole-3-acetic acid (IAA) (Woodward et al., 2005). The shortened hypocotyl of *er erl1 erl2* can be improved by low concentrations of exogenous auxin (Qu et al., 2017). It is possible that ERfs regulate auxin biosynthesis, but there is currently no direct link between ERfs and auxin biosynthesis. The improved organ elongation with increased auxin biosynthesis could be due to the result of elevated auxin levels compensating for the inability of *er erl1 erl2* to respond to auxin. More work is required to determine if ERfs regulate auxin biosynthesis, or if altered auxin biosynthesis is a side effect of the inability of *er erl1 erl2* to respond to auxin.

Additionally, we show that ERfs play a different role in organ initiation when the plant transitions to flowering. Auxin accumulation into peaks is required to reach a necessary auxin threshold to initiate flowers in the inflorescence meristem. The loss of *pin1* causes a complete loss of flower initiation because auxin maxima cannot form and these maxima are required to reach the auxin threshold to form flowers in the inflorescence meristem (Guenot et al., 2012). In the vegetative meristem, the loss of *pin1* prevents the formation of auxin peaks in the L1 layer, but leaves can still initiate because auxin levels are higher than in the inflorescence meristem, and accumulation of auxin into peaks is not as important for leaf formation. The dual role of ERfs inhibits auxin accumulation and promotes auxin responses. This has different effects on the inflorescence meristem versus the vegetative meristem. In the vegetative meristem, the *er erl1 erl2* mutant cannot respond effectively to auxin, which dramatically decreases organ initiation because auxin levels in the L1 layer are crucial for leaf formation. In the inflorescence meristem the loss of *erfs* still disrupts auxin responses, as flowers are deformed and sterile. However, the increased auxin signal is enough to initiate organs, even in the *pin1* background. In summary, ERfs are more important for organ initiation in the vegetative meristem because they sensitize the SAM to auxin to initiate organs, but they are secondary in the inflorescence meristem because there are other pathways that can promote sensitivity to auxin at later developmental stages.

In conclusion, ERfs promote organ initiation in an auxin dependent manner. The *er erl1 erl2* mutant is capable of sensing auxin, but leaf initiation is limited by decreased auxin responses in the L1 layer of the SAM. The *er erl1 erl2* mutant is capable of auxin perception but is unable to translate the signal to a viable leaf initiation response. This makes the *erf* mutant

more sensitive to auxin perturbations. Further decreasing auxin signaling by decreasing auxin biosynthesis or eliminating *pid* causes a catastrophic loss of organ initiation. A strong genetic interaction exists between ERfs and *PID* to promote leaf and cotyledons, indicating that both auxin distribution and the adequate response to auxin are required for organ initiation in the SAM. Future work should address the mechanism by which ERf signaling translates auxin perception into physiological responses.

Materials and methods

Plant materials

The *Arabidopsis thaliana* ecotype Columbia was used as the wild type. The *ER* family mutants have been described previously (Torii et al., 1996; Shpak et al., 2004). The mutants *pin1-201* (De Rybel et al., 2009), *pin2/eir1-1* (Roman et al., 1995), *pin3-5* (Žádníková et al., 2010) (SALK/ABRC), *pin4-3* (Friml et al., 2002), *pin7-2* (Friml et al., 2003) were generated by external laboratories. The higher order *er pin* family mutant plants were generated by crossing *pin1-201*, *pin2/eir1-1*, *pin3-5*, *pin4-3*, *pin7-2* to *er erl1+/- erl2*. The *er erl1 erl2* plants containing the additional *pin* mutation were identified in the F2. The plants named were identified by genotyping. Verification of the T-DNA insert through genotyping was performed using the following primers: *pin1-201* – *pin1-5* (5-GTCCCTCATTTCTTCAAGTACTGTT-3), *pin1-6* (5-ACCACCACAACATTAACATCATCATTAT-3), LBB1 (5-CGTGGACCGCTTGCTGCAACT-3), *pin3-5* – *pin3* (5-CCCATCCCCAAAAGTAGAGTG-3), *pin3.rc* (5-GGAAGTGGTGGAGAGGGAAAAG-3), LBB1 (5-

CGTGGACCGCTTGCTGCAACT-3), *pin4-3* – *pin4.984* (5-CAACGCCGTTAAATATGG-3), *pin4.1837.rc* (5-TTCCCACTACAATTATTCC-3), *En91R* (5-TGCAGCAAAACCCACACTTTTACTTC-3), *pin7-2* – *pin7* (5-TTTACTTGAACAATGGCCACAC-3), *pin7.rc* (5-GGTAAAGGAAGTGCCTAACGG3-3), *LBB1.3* (5-ATTTTGCCGATTTCGGAAC-3). The *er erl1 erl2 pin2* plants were identifiable through agravitropic roots as seen when grown vertically for three to five days and rotating the plate. Stomata clusters were identifiable in *er erl1 erl2*. To genotype *erl1-2*, we used the primers *erl1g3659* (5-GAGCTTGGACATATAATC-3), *erl1g4411.rc* (5-CCGGAGAGATTGTTGAAGG-3), and *JL202* (5-CATTTTATAATAACGCTGCGGACATCTAC-3). To genotype *erl2-1*, we used the primers *erl2g2166* (5-GCCTATTCCACCAATACTTG-3) and *ertj3182.rc* (5-ACAAATCTGAGAGAGTTAATGCAAAGCAG-3) or *JL202* and *ertj3182.rc*. The *pid-3* mutant is described earlier (Bennett et al., 1995). The *pid er erl1 erl2* mutant was generated by crossing *pid+/-* with *er erl1+/- erl2*. The *er erl1 erl2* plants containing the *pid* mutation were identified in the F2. *Er*, *erl1*, and *erl2* were selected through genotyping. The *pid-3* mutant is the result of a point mutation, and is identifiable through genotyping and sequencing of the product. The *pid-3* mutation was genotyped using the following primers: *RT-PID-c783F* (5-TTATGCCGCCGAAGTTCTAGTG-3), *PID6* (5-GACGAGGAAGATTCAACGGCT-3).

The *DR5rev::GFP* transgenic plant is in the Columbia background and is described previously (Benková et al., 2003; Friml et al., 2003). To generate the *er erl1 erl2*, *pin1*, and *pid* mutants expressing *DR5rev::GFP*; *er erl1+/- erl2*, *pid+/-*, or *pin1+/-* were crossed to *DR5rev::GFP*. The mutants listed containing *DR5rev::GFP* signal were identified in the F2 generation by observing GFP fluorescence and phenotype.

To generate the *pid er erl1 erl2 DR5rev::GFP* and *pin1 er erl1 erl2 DR5rev::GFP* plants, *pid+/- DR5rev::GFP* or *pin1+/- DR5rev::GFP* were crossed with *er erl1+/- erl2 DR5rev::GFP*. Plants were selected in the F2 by observing the phenotype.

Growth conditions

Plants were grown as previously described (Kosentka et al., 2017) under an 18-h-light/6-h-dark (long days) cycle at 21°C.

For analysis of leaf and cotyledon counting in *pin er erl1 erl2* and *pid er erl1 erl2*, seedlings were grown on modified Murashige and Skoog (MS) medium plates supplemented with 1% (w/v) sucrose. For confocal microscopy analysis, plants were grown with the addition of NPA added to the media as a stock solution dissolved in DMSO.

For analysis of leaf initiation using NPA, L-Kyn, and 2,4-D, plants were grown in liquid MS medium supplemented with 1% (w/v) sucrose. The chemicals listed were added as a stock solution dissolved in DMSO. Plants were grown with slight agitation under long day conditions, or in the dark as specified in the main text.

For the *pid er erl1 erl2* 2,4-D treatment, *pid er erl1 erl2* mutants were germinated and grown on modified MS medium plates supplemented with 10 µM 2,4-D or the mock with an equal volume of DMSO. DIC images were taken using 3 DPG plants. At 13 DPG, the same experimental replicates were moved to standard MS medium plates without 2,4-D along with the mock. The seedlings selected for transfer were chosen for their absence of cotyledons. Post-2,4-D treated *pid er erl1 erl2* plant was imaged after 30 days (33 DPG).

Analysis of plant height was performed using wild type, *pin1*, *erl1 erl2*, and *pin1 erl1 erl2* plants grown to maturity on soil.

For all experiments, seeds were stratified for 2 days at 4°C.

Microscopy

Leaf number counts were performed by fixing 2, 3, and 5 DPG seedlings overnight in ethanol:acetic acid [9:1 (v/v)]. Seedlings were rehydrated with an ethanol series to 30% (v/v) ethanol and cleared in chloral hydrate solution to observe the shoot apex. For the chloral hydrate:water:glycerol 8:1:1 (w/v/v) solution, the pH was adjusted to a pH of ~4.2 using 10 mM KOH to prevent degradation of tissues. Chloral hydrate (Sigma-Aldrich) can vary in acidity between batches. Test KOH concentration experimentally. DIC microscopy was performed as described previously (Chen et al., 2013).

Cotyledon counts were performed using 10x magnification goggles on 5-10 DPG plants and selected according to phenotype.

One month old *pid erl1 erl2* and *pin1 erl1 erl2* plants were imaged using a Leica MZ6 modular stereomicroscope (Leica Microsystems) with a Leica MC190 HD Microscope Camera (Leica Microsystems).

Confocal micrographs of the *DR5rev::GFP* expressing plants were made using the Leica SP8 White Light Laser Confocal microscope. A 488-nm White Light Supercontinuum Laser (Leica Microsystems) was used to excite the EGFP fluorophore. A 'UV' 405 nm diode laser (Leica Microsystems) was used to excite 4',6-diamino-phenylindole (DAPI) fluorescence.

The HyD 'Hybrid' Super Sensitivity SP Detector (Leica Microsystems) and the PMT SP Detector (Leica Microsystems) were used to collect emission of EGFP and DAPI, respectively. Z-stacks made with sequential line scanning were used to evaluate 3D images and select the most representative optical section. One cotyledon was dissected prior to imaging to expose the SAM of 1- day old live seedlings. A 1000x DAPI stock solution was prepared by dissolving 5 mg per 1 mL of PBS buffer. Samples were incubated in 1 mL of 1x staining solution with 1% Triton X-100 under vacuum for ~1-2 min at room temperature, and further incubated for ~7-8 min with gentle rocking. Seedlings were washed with 1× PBS buffer to remove excess dye, and imaged.

Inflorescence meristems were imaged by excising the shoot apex of *DR5rev::GFP* expressing plants and using sequential line scanning to create a Z-stack. The Fiji image-processing package was used to generate the final Z-project.

Quantitative microscopy measurements

The Fiji image-processing package was used for all quantitative image measurements. The center optical sections of 6-8 shoot meristems in mock and NPA treated *erl1 erl2* seedlings were chosen by finding the optical section with GFP minima. Using Fiji image processing software, the freehand tool was used to draw a line across the L1 cell layer of the SAM starting from one adaxial leaf junction and finishing at the opposite adaxial leaf junction. A plot profile measuring the mean pixel intensity was generated for each seedling measured. The data was transferred to Microsoft Excel where the data points were binned into 8 µm increments where 0 µm marks adaxial leaf junction and greater than 0 µm marks

a point closer to the center of the SAM. The average pixel intensity value for each 8 μm increment of an individual seedling was calculated. Average pixel intensity for all seedlings were averaged together at each 8 μm segment to form a single data point. Standard error was calculated for mock and 10 μM NPA treated seedlings and the statistical differences were determined using an unpaired two-tailed Student's t-test with a p-value threshold of greater than 0.05.

Chapter 4: Conclusion

Organ initiation and stem cell maintenance are fundamental processes in plant development. ERECTA family genes are key regulators of these fundamental processes and are conserved among all land plants. Phylogenetic analysis suggests ERECTA family genes are ancient and appeared early in land plant evolution (Villagarcia et al., 2012). The elucidation of the molecular role of ERECTA family signaling in organ formation and stem cell maintenance presented in this work contributes to our understanding of all land plant development.

In chapter two, we identify the molecular role of ERF-EPFL in SAM maintenance. We used genetic analysis to reveal a complex relationship between ERF-EPFL and two critical meristematic genes, *WUS* and *CLV3*. Mutants deficient in ERF signaling with the loss of *erl1 erl2* or *epfl1 epfl2 epfl4 epfl6* have enlarged meristems. ERF-EPFL and *CLV3* synergistically regulate SAM size. The combined loss of *erf* signaling and *clv3* causes severe stem cell overproliferation. On the other hand, the *wus* mutation is epistatic to *erfs*. The loss of *wus* function depletes the stem cell pool, regardless of the presence or absence of ERF signaling. We analyzed transcript levels of *WUS* and *CLV3* in the mutants deficient in *erf* signaling and found that both *WUS* and *CLV3* expression was increased. We showed that this effect is the direct result of the absence of *erf-epfl* signaling. Exogenous EPFL4 and EPFL6 applied to *epfl* mutants decreased *WUS* and *CLV3* expression, independently of protein translation. Finally, we analyzed localization of *WUS* and *CLV3* expression in the SAM in wild type and the *erl1 erl2* mutant. Loss of *erf* signaling causes a significant expansion of the *WUS* and *CLV3* expression domains. Altogether, this demonstrates that ERFs restrict the lateral expression of *WUS* and *CLV3* to regulate SAM size and prevent stem cell overproliferation.

Future work should address the final downstream targets of ERfs that regulate SAM function. When stimulated, ERfs activate a MAPK phosphorylation cascade that phosphorylates downstream targets (Bergmann et al., 2004; Wang et al., 2007; Meng et al., 2012; Chen et al., 2013). Our data suggests that a decrease in *WUS* and *CLV3* transcript levels occurs simultaneously after ERf activation. For this outcome to occur, there is likely a transcription factor that ERf signaling targets to rapidly alter gene expression. It is unclear whether the target of ERfs is a single transcription factor that regulates both *WUS* and *CLV3*, or multiple transcription factors that individually modulate *WUS* or *CLV3*. Identification of the transcription factor or transcription factors downstream of ERf signaling would reveal a new avenue, and ultimately provide insight into new ways to manipulate stem cell activity in plants.

In chapter three we provide evidence that ERfs regulate auxin responses in the SAM to promote leaf initiation. The *er erl1 erl2* mutant initiates leaves at a reduced rate. We used the auxin responsive *DR5rev::GFP* reporter to show the *er erl1 erl2* mutant has elevated auxin signaling in the L1 layer, but is unable to efficiently to initiate leaf formation, which suggests ERfs promote auxin responses. Increasing auxin levels in the *er erl1 erl2* mutant can rescue leaf initiation. Two methods of auxin efflux inhibition, NPA or the *pin1* mutation, can elevate auxin response on the L1 layer to promote leaf initiation in *er erl1 erl2*. We used quantitative confocal microscopy to show that NPA elevates auxin responses exclusively on the L1 layer and not the internal tissues to rescue leaf initiation. This suggests that even though the *er erl1 erl2* mutant can accumulate and sense auxin in the L1 layer, the threshold to respond and initiate leaf formation is higher in the mutant, indicating ERfs are required to translate auxin signal into a physiological response of organ initiation.

Lowering auxin levels in the *er erl1 erl2* mutant with an auxin biosynthesis inhibitor causes a dramatic decrease in leaf initiation. The *pid* mutant has diffuse auxin peaks in the epidermis and decreased auxin in the internal tissues. The combination of *pid* and *er erl1 erl2* mutations causes a catastrophic loss of organ initiation. Despite strong auxin signaling where organs should initiate in *pid er erl1 erl2*, elevating auxin levels in the mutant causes differentiation of tissue in the meristematic region, indicating that the quadruple mutant has defective auxin responses. Taken together, this shows that the combined loss of *pid* and the inability to respond to auxin signaling due to the loss of *erfs* eliminates organ formation in the SAM.

Using mutants deficient in *erf* signaling, we revealed a difference in organ initiation between the inflorescence meristem and the vegetative meristem. In the inflorescence meristem, the loss of *pin1* prevents auxin accumulation, which eliminates flower formation. The additional loss of *erf* signaling causes a dramatic elevation of auxin signaling in the epidermis. This allows the inflorescence meristem to initiate primordia. In contrast, the vegetative meristem requires ERf function to enhance responses to auxin. The increase in auxin signaling that occurs with the loss of *erfs* is not sufficient for leaf initiation in the vegetative meristem and leaves cannot form efficiently. In sum, while ERfs function similarly in the vegetative and inflorescence meristems, the inflorescence meristem does not require ERfs to promote auxin responses to initiate flower formation, but the vegetative meristem requires ERfs to adequately respond to auxin to initiate leaves.

Different plant tissues respond to the same auxin concentration to varying degrees. For example, the roots of plants can be inhibited by the same auxin concentration that

promotes shoot elongation (Thimann, 1938). Even within the small pool of cells in the SAM, auxin signaling is modulated by variation in expression patterns of the auxin signaling components AUXIN RESPONSE FACTOR (ARF) transcription factors and Aux/IAA repressors (Vernoux et al., 2011). The *er erl1 erl2* mutant is unable to translate auxin signal to an auxin response, suggesting that ERfs may play a role in modulating sensitivity to auxin. More data is required to determine the molecular mechanism that ERfs may use to regulate the response to auxin. Future work should investigate the possibility of an auxin signaling effector that may exist downstream of ERf signaling.

Auxin promotes the differentiation of cells in the periphery of the SAM (Benková et al., 2003; Heisler et al., 2005). In *er erl1 erl2*, there is an increase in auxin accumulation in the L1 layer of SAM (Chen et al., 2013). Additionally, the stem cell inhibitor *CLV3* is more widely expressed in *er erl1 erl2*, even across the L1 layer. It is noteworthy that auxin, which causes differentiation of cells, and *CLV3*, which prevents overproliferation of stem cells, are both regulated by ERf-EPFL signaling. It is possible that these two pathways of ERf-auxin signaling and ERf-*CLV3* intersect in the regulation of organ formation. The synergistic relationship of these two pathways in organ formation is evident in *clv3 er erl1 erl2* and *clv3 epfl1 epfl2 epfl4 epfl6* which are unable to form organs. The loss of auxin regulation due to the absence of *erf-epfl* signaling, coupled with the loss of stem cell regulation due to the absence of *erf-epfl* signaling and *clv3* are major disruptions to organ initiation, which result in the loss of organ formation. Future work should address the role of ERfs in the interplay between stem cell maintenance and organ initiation.

In conclusion, our investigation adds to the portfolio of molecular processes governed by ERfs. The production of organs and the function of the stem cell niche are of the upmost importance in plant development and architecture. A greater understanding of these fundamental processes in plants should be used to enhance crop plants. In future studies, our results will help engineer plant architecture to improve organ initiation and stem cell maintenance to optimize growth in a specific environment for the purpose of more efficient food yield or survival.

References

- Abrash EB, Davies KA, Bergmann DC** (2011) Generation of signaling specificity in Arabidopsis by spatially restricted buffering of ligand-receptor interactions. *Plant Cell* **23**: 2864-2879
- Bainbridge K, Guyomarc'h S, Bayer E, Swarup R, Bennett M, Mandel T, Kuhlemeier C** (2008) Auxin influx carriers stabilize phyllotactic patterning. *Genes & Development* **22**: 810-823
- Barbez E, Kubeš M, Rolčík J, Béziat C, Pěňčík A, Wang B, Rosquete MR, Zhu J, Dobrev PI, Lee Y, Zažímalová E, Petrášek J, Geisler M, Friml J, Kleine-Vehn J** (2012) A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* **485**: 119-122
- Barton MK, Poethig RS** (1993) Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the shoot meristemless mutant. *Development* **119**: 823-831
- Benjamins R, Quint A, Weijers D, Hooykaas P, Offringa R** (2001) The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development* **128**: 4057-4067
- Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J** (2003) Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell* **115**: 591-602
- Bennett SRM, Alvarez J, Bossinger G, Smyth DR** (1995) Morphogenesis in pinoid mutants of *Arabidopsis thaliana*. *The Plant Journal* **8**: 505-520
- Bennett T, Hines G, van Rongen M, Waldie T, Sawchuk MG, Scarpella E, Ljung K, Leyser O** (2016) Connective Auxin Transport in the Shoot Facilitates Communication between Shoot Apices. *PLOS Biology* **14**: e1002446
- Bergmann DC, Lukowitz W, Somerville CR** (2004) Stomatal Development and Pattern Controlled by a MAPKK Kinase. *Science* **304**: 1494-1497
- Béziat C, Barbez E, Feraru MI, Lucyshyn D, Kleine-Vehn J** (2017) Light triggers PILS-dependent reduction in nuclear auxin signalling for growth transition. *Nature Plants* **3**: 17105
- Béziat C, Kleine-Vehn J** (2018) The Road to Auxin-Dependent Growth Repression and Promotion in Apical Hooks. *Current Biology* **28**: R519-R525
- Bhalerao RP, Eklöf J, Ljung K, Marchant A, Bennett M, Sandberg G** (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. *The Plant Journal* **29**: 325-332
- Bhatia N, Bozorg B, Larsson A, Ohno C, Jönsson H, Heisler MG** (2016) Auxin Acts through MONOPTEROS to Regulate Plant Cell Polarity and Pattern Phyllotaxis. *Current biology : CB* **26**: 3202-3208
- Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R** (2000) Dependence of Stem Cell Fate in *Arabidopsis* on a Feedback Loop Regulated by CLV3 Activity. *Science* **289**: 617-619
- Busch W, Miotk A, Ariel FD, Zhao Z, Forner J, Daum G, Suzaki T, Schuster C, Schultheiss SJ, Leibfried A, Haubeiß S, Ha N, Chan RL, Lohmann JU** (2010) Transcriptional Control of a Plant Stem Cell Niche. *Developmental Cell* **18**: 841-853
- Caggiano MP, Yu X, Bhatia N, Larsson A, Ram H, Ohno CK, Sappl P, Meyerowitz EM, Jönsson H, Heisler MG** (2017) Cell type boundaries organize plant development. *eLife* **6**: e27421
- Chen L, Wu Z, Hou S** (2020) SPEECHLESS Speaks Loudly in Stomatal Development. *Frontiers in plant science* **11**: 114-114
- Chen M-K, Shpak ED** (2014) ERECTA family genes regulate development of cotyledons during embryogenesis. *FEBS Letters* **588**: 3912-3917
- Chen M-K, Wilson RL, Palme K, Ditengou FA, Shpak ED** (2013) ERECTA Family Genes Regulate Auxin Transport in the Shoot Apical Meristem and Forming Leaf Primordia. *Plant Physiology* **162**: 1978-1991

- Cheng Y, Dai X, Zhao Y** (2007a) Auxin Synthesized by the YUCCA Flavin Monooxygenases Is Essential for Embryogenesis and Leaf Formation in Arabidopsis. *The Plant Cell* **19**: 2430-2439
- Cheng Y, Qin G, Dai X, Zhao Y** (2007b) NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in Arabidopsis. *Proceedings of the National Academy of Sciences* **104**: 18825-18829
- Chickarmane VS, Gordon SP, Tarr PT, Heisler MG, Meyerowitz EM** (2012) Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing Arabidopsis shoot meristem. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 4002-4007
- Chiu W-H, Chandler J, Cnops G, Van Lijsebettens M, Werr W** (2007) Mutations in the TORNADO2 gene affect cellular decisions in the peripheral zone of the shoot apical meristem of Arabidopsis thaliana. *Plant Molecular Biology* **63**: 731-744
- Cho M, Cho H-T** (2013) The function of ABCB transporters in auxin transport. *Plant signaling & behavior* **8**: e22990-e22990
- Clark SE, Running MP, Meyerowitz EM** (1993) CLAVATA1, a regulator of meristem and flower development in Arabidopsis. *Development* **119**: 397-418
- Clark SE, Williams RW, Meyerowitz EM** (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**: 575-585
- Clarke JH, Tack D, Findlay K, Van Montagu M, Van Lijsebettens M** (1999) The SERRATE locus controls the formation of the early juvenile leaves and phase length in Arabidopsis. *The Plant Journal* **20**: 493-501
- Daum G, Medzihradszky A, Suzaki T, Lohmann JU** (2014) A mechanistic framework for noncell autonomous stem cell induction in Arabidopsis. *Proceedings of the National Academy of Sciences* **111**: 14619-14624
- De Rybel B, Audenaert D, Vert G, Rozhon W, Mayerhofer J, Peelman F, Coutuer S, Denayer T, Jansen L, Nguyen L, Vanhoutte I, Beemster GT, Vleminckx K, Jonak C, Chory J, Inzé D, Russinova E, Beeckman T** (2009) Chemical inhibition of a subset of Arabidopsis thaliana GSK3-like kinases activates brassinosteroid signaling. *Chem Biol* **16**: 594-604
- DeYoung BJ, Bickle KL, Schrage KJ, Muskett P, Patel K, Clark SE** (2006) The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. *The Plant Journal* **45**: 1-16
- Dhonukshe P, Huang F, Galvan-Ampudia CS, Mähönen AP, Kleine-Vehn J, Xu J, Quint A, Prasad K, Friml J, Scheres B, Offringa R** (2010) Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling. *Development* **137**: 3245-3255
- Ding Z, Wang B, Moreno I, Dupláková N, Simon S, Carraro N, Reemmer J, Pěnčík A, Chen X, Tejos R, Skůpa P, Pollmann S, Mravec J, Petrášek J, Zažímalová E, Honys D, Rolčík J, Murphy A, Orellana A, Geisler M, Friml J** (2012) ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in Arabidopsis. *Nature Communications* **3**: 941
- Doerner P** (2003) Plant Meristems: A Merry-Go-Round of Signals Review. *Current Biology* **13**: R368-R374
- Dos Santos Maraschin F, Memelink J, Offringa R** (2009) Auxin-induced, SCFTIR1-mediated poly-ubiquitination marks AUX/IAA proteins for degradation. *The Plant Journal* **59**: 100-109
- Douady S, Couder Y** (1996) Phyllotaxis as a Dynamical Self Organizing Process Part II: The Spontaneous Formation of a Periodicity and the Coexistence of Spiral and Whorled Patterns. *Journal of Theoretical Biology* **178**: 275-294

- El-Lithy ME, Clerkx EJM, Ruys GJ, Koornneef M, Vreugdenhil D** (2004) Quantitative trait locus analysis of growth-related traits in a new Arabidopsis recombinant inbred population. *Plant physiology* **135**: 444-458
- Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL** (2003) Radial Patterning of Arabidopsis Shoots by Class III HD-ZIP and KANADI Genes. *Current Biology* **13**: 1768-1774
- Endo H, Torii KU** (2019) Stomatal Development and Perspectives toward Agricultural Improvement. *Cold Spring Harbor Perspectives in Biology* **11**
- Engineer CB, Ghassemian M, Anderson JC, Peck SC, Hu H, Schroeder JI** (2014) Carbonic anhydrases, EPF2 and a novel protease mediate CO₂ control of stomatal development. *Nature* **513**: 246-250
- Engstrom EM, Andersen CM, Gumulak-Smith J, Hu J, Orlova E, Sozzani R, Bowman JL** (2011) Arabidopsis Homologs of the Petunia HAIRY MERISTEM Gene Are Required for Maintenance of Shoot and Root Indeterminacy. *Plant Physiology* **155**: 735-750
- Fan M, Wang M, Bai MY** (2016) Diverse roles of SERK family genes in plant growth, development and defense response. *Sci China Life Sci* **59**: 889-896
- Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM** (1999) Signaling of Cell Fate Decisions by CLAVATA3 in Arabidopsis Shoot Meristems. *Science* **283**: 1911-1914
- Friml J, Benková E, Blilou I, Wisniewska J, Hamann T, Ljung K, Woody S, Sandberg G, Scheres B, Jürgens G, Palme K** (2002) AtPIN4 Mediates Sink-Driven Auxin Gradients and Root Patterning in Arabidopsis. *Cell* **108**: 661-673
- Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G** (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* **426**: 147-153
- Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O, Benjamins R, Ouwerkerk PBF, Ljung K, Sandberg G, Hooykaas PJJ, Palme K, Offringa R** (2004) A PINOID-Dependent Binary Switch in Apical-Basal PIN Polar Targeting Directs Auxin Efflux. *Science* **306**: 862-865
- Furner IJ, Pumfrey JE** (1992) Cell fate in the shoot apical meristem of Arabidopsis thaliana. *Development* **115**: 755-764
- Furutani M, Vernoux T, Traas J, Kato T, Tasaka M, Aida M** (2004) PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. *Development* **131**: 5021-5030
- Gallois J-L, Woodward C, Reddy GV, Sablowski R** (2002) Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in Arabidopsis. *Development* **129**: 3207-3217
- Gälweiler L, Guan C, Müller A, Wisman E, Mendgen K, Yephremov A, Palme K** (1998) Regulation of Polar Auxin Transport by AtPIN1 in Arabidopsis Vascular Tissue. *Science* **282**: 2226-2230
- Glanc M, Van Gelderen K, Hoermayer L, Tan S, Naramoto S, Zhang X, Domjan D, Včelařová L, Hauschild R, Johnson A, de Koning E, van Dop M, Rademacher E, Janson S, Wei X, Molnár G, Fendrych M, De Rybel B, Offringa R, Friml J** (2021) AGC kinases and MAB4/MEL proteins maintain PIN polarity by limiting lateral diffusion in plant cells. *Current Biology* **31**: 1918-1930.e1915
- Goldsmith MH** (1967) Movement of pulses of labeled auxin in corn coleoptiles. *Plant physiology* **42**: 258-263
- Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM** (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. *Proceedings of the National Academy of Sciences* **106**: 16529-16534
- Gray WM, Kepinski S, Rouse D, Leyser O, Estelle M** (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. *Nature* **414**: 271-276

- Guenot B, Bayer E, Kierzkowski D, Smith RS, Mandel T, Žádníková P, Benková E, Kuhlemeier C** (2012) PIN1-Independent Leaf Initiation in Arabidopsis. *Plant Physiology* **159**: 1501-1510
- Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T** (2007) The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. *Genes & development* **21**: 1720-1725
- Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T** (2009) Epidermal Cell Density is Autoregulated via a Secretory Peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis Leaves. *Plant and Cell Physiology* **50**: 1019-1031
- He W, Brumos J, Li H, Ji Y, Ke M, Gong X, Zeng Q, Li W, Zhang X, An F, Wen X, Li P, Chu J, Sun X, Yan C, Yan N, Xie DY, Raikhel N, Yang Z, Stepanova AN, Alonso JM, Guo H** (2011) A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in Arabidopsis. *Plant Cell* **23**: 3944-3960
- Heisler MG, Byrne ME** (2020) Progress in understanding the role of auxin in lateral organ development in plants. *Current Opinion in Plant Biology* **53**: 73-79
- Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM** (2005) Patterns of Auxin Transport and Gene Expression during Primordium Development Revealed by Live Imaging of the Arabidopsis Inflorescence Meristem. *Current Biology* **15**: 1899-1911
- Hofmeister W** (1868) Allgemeine morphologie der Gewächse. Engelmann
- Hu C, Zhu Y, Cui Y, Cheng K, Liang W, Wei Z, Zhu M, Yin H, Zeng L, Xiao Y, Lv M, Yi J, Hou S, He K, Li J, Gou X** (2018) A group of receptor kinases are essential for CLAVATA signalling to maintain stem cell homeostasis. *Nature Plants* **4**: 205-211
- Hunt L, Gray JE** (2009) The Signaling Peptide EPF2 Controls Asymmetric Cell Divisions during Stomatal Development. *Current Biology* **19**: 864-869
- Irish VF, Sussex IM** (1992) A fate map of the Arabidopsis embryonic shoot apical meristem. *Development* **115**: 745-753
- Izhaki A, Bowman JL** (2007) KANADI and Class III HD-Zip Gene Families Regulate Embryo Patterning and Modulate Auxin Flow during Embryogenesis in Arabidopsis. *The Plant Cell* **19**: 495-508
- Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M** (2005) KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. *Curr Biol* **15**: 1560-1565
- Jeong S, Trotochaud AE, Clark SE** (1999) The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**: 1925-1934
- Jones SE, DeMeo JS, Davies NW, Noonan SE, Ross JJ** (2005) Stems of the Arabidopsis pin1-1 mutant are not deficient in free indole-3-acetic acid. *Planta* **222**: 530-534
- Kasahara H** (2016) Current aspects of auxin biosynthesis in plants. *Biosci Biotechnol Biochem* **80**: 34-42
- Kawamoto N, Del Carpio DP, Hofmann A, Mizuta Y, Kurihara D, Higashiyama T, Uchida N, Torii KU, Colombo L, Groth G, Simon R** (2020) A Peptide Pair Coordinates Regular Ovule Initiation Patterns with Seed Number and Fruit Size. *Current Biology* **30**: 4352-4361.e4354
- Kayes JM, Clark SE** (1998) CLAVATA2, a regulator of meristem and organ development in Arabidopsis. *Development* **125**: 3843-3851
- Kerstetter RA, Bollman K, Taylor RA, Bomblies K, Poethig RS** (2001) KANADI regulates organ polarity in Arabidopsis. *Nature* **411**: 706-709
- Kierzkowski D, Lenhard M, Smith R, Kuhlemeier C** (2013) Interaction between Meristem Tissue Layers Controls Phyllotaxis. *Developmental Cell* **26**: 616-628

- Kinoshita A, Betsuyaku S, Osakabe Y, Mizuno S, Nagawa S, Stahl Y, Simon R, Yamaguchi-Shinozaki K, Fukuda H, Sawa S** (2010) RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis. *Development* **137**: 3911-3920
- Kondo T, Kajita R, Miyazaki A, Hokoyama M, Nakamura-Miura T, Mizuno S, Masuda Y, Irie K, Tanaka Y, Takada S, Kakimoto T, Sakagami Y** (2009) Stomatal Density is Controlled by a Mesophyll-Derived Signaling Molecule. *Plant and Cell Physiology* **51**: 1-8
- Kosentka PZ, Overholt A, Maradiaga R, Mitoubsi O, Shpak ED** (2019) EPFL Signals in the Boundary Region of the SAM Restrict Its Size and Promote Leaf Initiation. *Plant Physiology* **179**: 265-279
- Kosentka PZ, Zhang L, Simon YA, Satpathy B, Maradiaga R, Mitoubsi O, Shpak ED** (2017) Identification of critical functional residues of receptor-like kinase ERECTA. *Journal of experimental botany* **68**: 1507-1518
- Křeček P, Skůpa P, Libus J, Naramoto S, Tejos R, Friml J, Zažímalová E** (2009) The PIN-FORMED (PIN) protein family of auxin transporters. *Genome Biology* **10**: 249
- Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J** (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**: 652-655
- Laibach F, Mai G** (1936) Über die künstliche Erzeugung von Bildungsabweichungen bei Pflanzen. Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen **134**: 200-206
- Lampard GR, MacAlister CA, Bergmann DC** (2008) Arabidopsis Stomatal Initiation Is Controlled by MAPK-Mediated Regulation of the bHLH SPEECHLESS. *Science* **322**: 1113-1116
- Landrein B, Refahi Y, Besnard F, Hervieux N, Mirabet V, Boudaoud A, Vernoux T, Hamant O** (2015) Meristem size contributes to the robustness of phyllotaxis in Arabidopsis. *Journal of Experimental Botany* **66**: 1317-1324
- Lau S, Slane D, Herud O, Kong J, Jürgens G** (2012) Early Embryogenesis in Flowering Plants: Setting Up the Basic Body Pattern. *Annual Review of Plant Biology* **63**: 483-506
- Lau S, Smet ID, Kolb M, Meinhardt H, Jürgens G** (2011) Auxin triggers a genetic switch. *Nature Cell Biology* **13**: 611-615
- Laux T, Mayer KF, Berger J, Jürgens G** (1996) The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* **122**: 87-96
- Lease KA, Lau NY, Schuster RA, Torii KU, Walker JC** (2001) Receptor serine/threonine protein kinases in signalling: analysis of the erecta receptor-like kinase of Arabidopsis thaliana. *New Phytologist* **151**: 133-143
- Lee DJ, Park JW, Lee HW, Kim J** (2009) Genome-wide analysis of the auxin-responsive transcriptome downstream of *iaa1* and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. *Journal of Experimental Botany* **60**: 3935-3957
- Lee JS, Hnilova M, Maes M, Lin YC, Putarjunan A, Han SK, Avila J, Torii KU** (2015) Competitive binding of antagonistic peptides fine-tunes stomatal patterning. *Nature* **522**: 439-443
- Lee JS, Kuroha T, Hnilova M, Khatayevich D, Kanaoka MM, McAbee JM, Sarikaya M, Tamerler C, Torii KU** (2012) Direct interaction of ligand-receptor pairs specifying stomatal patterning. *Genes Dev* **26**: 126-136
- Leibfried A, To JPC, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU** (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* **438**: 1172-1175
- Lenhard M, Jürgens G, Laux T** (2002) The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. *Development* **129**: 3195-3206

- Lin G, Zhang L, Han Z, Yang X, Liu W, Li E, Chang J, Qi Y, Shpak ED, Chai J** (2017) A receptor-like protein acts as a specificity switch for the regulation of stomatal development. *Genes & development* **31**: 927-938
- Lindsay DL, Sawhney VK, Bonham-Smith PC** (2006) Cytokinin-induced changes in CLAVATA1 and WUSCHEL expression temporally coincide with altered floral development in Arabidopsis. *Plant Science* **170**: 1111-1117
- Liu Z, Shpak ED, Hong T** (2020) A mathematical model for understanding synergistic regulations and paradoxical feedbacks in the shoot apical meristem. *Computational and Structural Biotechnology Journal* **18**: 3877-3889
- Long JA, Moan EI, Medford JI, Barton MK** (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature* **379**: 66-69
- Mandel T, Candela H, Landau U, Asis L, Zelinger E, Carles CC, Williams LE** (2016) Differential regulation of meristem size, morphology and organization by the ERECTA, CLAVATA and class III HD-ZIP pathways. *Development* **143**: 1612-1622
- Marcotrigiano M, Bernatzky R** (1995) Arrangement of cell layers in the shoot apical meristems of periclinal chimeras influences cell fate. *The Plant Journal* **7**: 193-202
- Mayer KFX, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T** (1998) Role of WUSCHEL in Regulating Stem Cell Fate in the Arabidopsis Shoot Meristem. *Cell* **95**: 805-815
- McConnell JR, Emery J, Eshed Y, Bao N, Bowman J, Barton MK** (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature* **411**: 709-713
- Medford JI, Behringer FJ, Callos JD, Feldmann KA** (1992) Normal and Abnormal Development in the Arabidopsis Vegetative Shoot Apex. *The Plant Cell* **4**: 631-643
- Meng X, Chen X, Mang H, Liu C, Yu X, Gao X, Torii Keiko U, He P, Shan L** (2015) Differential Function of Arabidopsis SERK Family Receptor-like Kinases in Stomatal Patterning. *Current Biology* **25**: 2361-2372
- Meng X, Wang H, He Y, Liu Y, Walker JC, Torii KU, Zhang S** (2012) A MAPK Cascade Downstream of ERECTA Receptor-Like Protein Kinase Regulates Arabidopsis Inflorescence Architecture by Promoting Localized Cell Proliferation. *The Plant Cell* **24**: 4948-4960
- Miyashima S, Sebastian J, Lee J-Y, Helariutta Y** (2013) Stem cell function during plant vascular development. *The EMBO Journal* **32**: 178-193
- Mravec J, Kubeš M, Bielach A, Gaykova V, Petrášek J, Skůpa P, Chand S, Benková E, Zažímalová E, Friml J** (2008) Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. *Development* **135**: 3345-3354
- Mravec J, Skůpa P, Bailly A, Hoyerová K, Křeček P, Bielach A, Petrášek J, Zhang J, Gaykova V, Stierhof Y-D, Dobrev PI, Schwarzerová K, Rolčík J, Seifertová D, Luschnig C, Benková E, Zažímalová E, Geisler M, Friml J** (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**: 1136-1140
- Müller R, Bleckmann A, Simon R** (2008) The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell* **20**: 934-946
- Müller R, Borghi L, Kwiatkowska D, Laufs P, Simon Rd** (2006) Dynamic and Compensatory Responses of Arabidopsis Shoot and Floral Meristems to CLV3 Signaling. *The Plant Cell* **18**: 1188-1198
- Musielak TJ, Schenkel L, Kolb M, Henschen A, Bayer M** (2015) A simple and versatile cell wall staining protocol to study plant reproduction. *Plant Reproduction* **28**: 161-169
- Naramoto S** (2017) Polar transport in plants mediated by membrane transporters: focus on mechanisms of polar auxin transport. *Current Opinion in Plant Biology* **40**: 8-14
- Nimchuk ZL, Zhou Y, Tarr PT, Peterson BA, Meyerowitz EM** (2015) Plant stem cell maintenance by transcriptional cross-regulation of related receptor kinases. *Development* **142**: 1043-1049

- Ohki S, Takeuchi M, Mori M** (2011) The NMR structure of stomagen reveals the basis of stomatal density regulation by plant peptide hormones. *Nature Communications* **2**: 512
- Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y** (1991) Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. *The Plant Cell* **3**: 677-684
- Otsuga D, DeGuzman B, Prigge MJ, Drews GN, Clark SE** (2001) REVOLUTA regulates meristem initiation at lateral positions. *The Plant Journal* **25**: 223-236
- Ottoline Leyser HM, Furner IJ** (1992) Characterisation of three shoot apical meristem mutants of *Arabidopsis thaliana*. *Development* **116**: 397-403
- Péret B, Swarup K, Ferguson A, Seth M, Yang Y, Dhondt S, James N, Casimiro I, Perry P, Syed A, Yang H, Reemmer J, Venison E, Howells C, Perez-Amador MA, Yun J, Alonso J, Beemster GTS, Laplace L, Murphy A, Bennett MJ, Nielsen E, Swarup R** (2012) AUX/LAX Genes Encode a Family of Auxin Influx Transporters That Perform Distinct Functions during Arabidopsis Development. *The Plant Cell* **24**: 2874-2885
- Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A** (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proceedings of the National Academy of Sciences* **98**: 11812-11817
- Poethig S** (1989) Genetic mosaics and cell lineage analysis in plants. *Trends in Genetics* **5**: 273-277
- Qi J, Wang Y, Yu T, Cunha A, Wu B, Vernoux T, Meyerowitz E, Jiao Y** (2014) Auxin depletion from leaf primordia contributes to organ patterning. *Proceedings of the National Academy of Sciences* **111**: 18769-18774
- Qu X, Zhao Z, Tian Z** (2017) ERECTA Regulates Cell Elongation by Activating Auxin Biosynthesis in *Arabidopsis thaliana*. *Frontiers in Plant Science* **8**
- Ravichandran SJ, Linh NM, Scarpella E** (2020) The canalization hypothesis – challenges and alternatives. *New Phytologist* **227**: 1051-1059
- Reddy GV, Heisler MG, Ehrhardt DW, Meyerowitz EM** (2004) Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of *Arabidopsis thaliana*. *Development* **131**: 4225-4237
- Rédei GP** (1992) A heuristic glance at the past of Arabidopsis genetics. *In Methods in Arabidopsis Research*. WORLD SCIENTIFIC, pp 1-15
- Reinhardt D, Mandel T, Kuhlemeier C** (2000) Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* **12**: 507-518
- Reinhardt D, Pesce E-R, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlemeier C** (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* **426**: 255-260
- Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR** (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. *Genetics* **139**: 1393-1409
- Rychel AL, Peterson KM, Torii KU** (2010) Plant twitter: ligands under 140 amino acids enforcing stomatal patterning. *Journal of Plant Research* **123**: 275-280
- Sager RE, Lee J-Y** (2018) Plasmodesmata at a glance. *Journal of Cell Science* **131**: jcs209346
- Sarlikioti V, de Visser PH, Buck-Sorlin GH, Marcelis LF** (2011) How plant architecture affects light absorption and photosynthesis in tomato: towards an ideotype for plant architecture using a functional-structural plant model. *Ann Bot* **108**: 1065-1073
- Satina S, Blakeslee AF, Avery AG** (1940) Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *American Journal of Botany* **27**: 895-905
- Sauer M, Balla J, Luschnig C, Wiśniewska J, Reinöhl V, Friml J, Benková E** (2006) Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. *Genes & Development* **20**: 2902-2911

- Sauer M, Kleine-Vehn J** (2019) PIN-FORMED and PIN-LIKES auxin transport facilitators. *Development* **146**: dev168088
- Scanlon MJ** (2003) The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. *Plant physiology* **133**: 597-605
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A** (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676-682
- Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T** (2000) The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* **100**: 635-644
- Scofield S, Dewitte W, Murray JA** (2014) STM sustains stem cell function in the Arabidopsis shoot apical meristem and controls KNOX gene expression independently of the transcriptional repressor AS1. *Plant signaling & behavior* **9**: e28934
- Shi B, Vernoux T** (2019) Chapter Four - Patterning at the shoot apical meristem and phyllotaxis. *In* U Grossniklaus, ed, *Current Topics in Developmental Biology*, Vol 131. Academic Press, pp 81-107
- Shimada T, Sugano SS, Hara-Nishimura I** (2011) Positive and negative peptide signals control stomatal density. *Cell Mol Life Sci* **68**: 2081-2088
- Shpak ED** (2013) Diverse Roles of ERECTA Family Genes in Plant Development. *Journal of Integrative Plant Biology* **55**: 1238-1250
- Shpak ED, Berthiaume CT, Hill EJ, Torii KU** (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. *Development* **131**: 1491-1501
- Shpak ED, McAbee JM, Pillitteri LJ, Torii KU** (2005) Stomatal Patterning and Differentiation by Synergistic Interactions of Receptor Kinases. *Science* **309**: 290-293
- Simon S, Skůpa P, Viaene T, Zwiewka M, Tejos R, Klíma P, Čarná M, Rolčák J, De Rycke R, Moreno I, Dobrev PI, Orellana A, Zažímalová E, Friml J** (2016) PIN6 auxin transporter at endoplasmic reticulum and plasma membrane mediates auxin homeostasis and organogenesis in Arabidopsis. *New Phytologist* **211**: 65-74
- Smith LG, Greene B, Veit B, Hake S** (1992) A dominant mutation in the maize homeobox gene, Knotted-1, causes its ectopic expression in leaf cells with altered fates. *Development* **116**: 21-30
- Smith RS, Guyomarc'h S, Mandel T, Reinhardt D, Kuhlemeier C, Prusinkiewicz P** (2006) A plausible model of phyllotaxis. *Proc Natl Acad Sci* **103**: 1301-1306
- Snow M, Snow GRS** (1962) A theory of the regulation of phyllotaxis based on *Lupinus albus*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **244**: 483-513
- Snow M, Snow R** (1937) Auxin and Leaf Formation. *New Phytologist* **36**: 1-18
- Sonoda Y, Yao S-G, Sako K, Sato T, Kato W, Ohto M-a, Ichikawa T, Matsui M, Yamaguchi J, Ikeda A** (2007) SHA1, a novel RING finger protein, functions in shoot apical meristem maintenance in Arabidopsis. *The Plant Journal* **50**: 586-596
- Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Dolezal K, Schlereth A, Jürgens G, Alonso JM** (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* **133**: 177-191
- Stuurman J, Jäggi F, Kuhlemeier C** (2002) Shoot meristem maintenance is controlled by a GRAS-gene mediated signal from differentiating cells. *Genes Dev* **16**: 2213-2218
- Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Hara-Nishimura I** (2010) Stomagen positively regulates stomatal density in Arabidopsis. *Nature* **463**: 241-244

- Szemenyei H, Hannon M, Long JA** (2008) TOPLESS Mediates Auxin-Dependent Transcriptional Repression During Arabidopsis Embryogenesis. *Science* **319**: 1384-1386
- Takata N, Yokota K, Ohki S, Mori M, Taniguchi T, Kurita M** (2013) Evolutionary Relationship and Structural Characterization of the EPF/EPFL Gene Family. *PLOS ONE* **8**: e65183
- Tameshige T, Okamoto S, Lee JS, Aida M, Tasaka M, Torii KU, Uchida N** (2016) A Secreted Peptide and Its Receptors Shape the Auxin Response Pattern and Leaf Margin Morphogenesis. *Curr Biol* **26**: 2478-2485
- Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N** (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640-645
- Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballaré CL, Sandberg G, Noel JP, Chory J** (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* **133**: 164-176
- Teale W, Palme K** (2017) Naphthylphthalamic acid and the mechanism of polar auxin transport. *Journal of Experimental Botany* **69**: 303-312
- Thimann KV** (1938) Hormones and the Analysis of Growth. *Plant physiology* **13**: 437-449
- Tiwari SB, Hagen G, Guilfoyle TJ** (2004) Aux/IAA Proteins Contain a Potent Transcriptional Repression Domain. *The Plant Cell* **16**: 533-543
- Tiwari SB, Wang XJ, Hagen G, Guilfoyle TJ** (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *The Plant cell* **13**: 2809-2822
- To JPC, Haberer G, Ferreira FJ, Deruère J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ** (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *The Plant cell* **16**: 658-671
- Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y** (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *The Plant Cell* **8**: 735-746
- Traas J, Monéger F** (2010) Systems Biology of Organ Initiation at the Shoot Apex. *Plant Physiology* **152**: 420-427
- Treml BS, Winderl S, Radykewicz R, Herz M, Schweizer Gn, Hutzler P, Glawischnig E, Ruiz RnAT** (2005) The gene ENHANCER OF PINOID controls cotyledon development in the Arabidopsis embryo. *Development* **132**: 4063-4074
- Truskina J, Vernoux T** (2018) The growth of a stable stationary structure: coordinating cell behavior and patterning at the shoot apical meristem. *Current Opinion in Plant Biology* **41**: 83-88
- Uchida N, Lee JS, Horst RJ, Lai H-H, Kajita R, Kakimoto T, Tasaka M, Torii KU** (2012a) Regulation of inflorescence architecture by intertissue layer ligand-receptor communication between endodermis and phloem. *Proceedings of the National Academy of Sciences* **109**: 6337-6342
- Uchida N, Shimada M, Tasaka M** (2012b) ERECTA-Family Receptor Kinases Regulate Stem Cell Homeostasis via Buffering its Cytokinin Responsiveness in the Shoot Apical Meristem. *Plant and Cell Physiology* **54**: 343-351
- Uchida N, Shimada M, Tasaka M** (2012c) Modulation of the balance between stem cell proliferation and consumption by ERECTA-family genes. *Plant Signaling & Behavior* **7**: 1506-1508
- Uchida N, Tasaka M** (2013) Regulation of plant vascular stem cells by endodermis-derived EPFL-family peptide hormones and phloem-expressed ERECTA-family receptor kinases. *Journal of Experimental Botany* **64**: 5335-5343
- Uchida N, Torii KU** (2019) Stem cells within the shoot apical meristem: identity, arrangement and communication. *Cell Mol Life Sci* **76**: 1067-1080

- Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ** (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**: 1963-1971
- Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, Das P, Larrieu A, Wells D, Guédon Y, Armitage L, Picard F, Guyomarc'h S, Cellier C, Parry G, Koumproglou R, Doonan JH, Estelle M, Godin C, Kepinski S, Bennett M, De Veylder L, Traas J** (2011) The auxin signalling network translates dynamic input into robust patterning at the shoot apex. *Molecular Systems Biology* **7**: 508
- Vernoux T, Kronenberger J, Grandjean O, Laufs P, Traas J** (2000) PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development* **127**: 5157-5165
- Villagarcia H, Morin A-C, Shpak ED, Khodakovskaya MV** (2012) Modification of tomato growth by expression of truncated ERECTA protein from *Arabidopsis thaliana*. *Journal of Experimental Botany* **63**: 6493-6504
- Vochting H** (1898) *Über Organbildung im Pflanzenreich*.
- Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S** (2007) Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in *Arabidopsis*. *The Plant Cell* **19**: 63-73
- Wenzel CL, Schuetz M, Yu Q, Mattsson J** (2007) Dynamics of MONOPTEROS and PIN-FORMED1 expression during leaf vein pattern formation in *Arabidopsis thaliana*. *The Plant Journal* **49**: 387-398
- Wilkens S** (2015) Structure and mechanism of ABC transporters. *F1000prime reports* **7**: 14-14
- Wilkins MB, Martin M** (1967) Dependence of Basipetal Polar Transport of Auxin upon Aerobic Metabolism 1. *Plant Physiology* **42**: 831-839
- Woodward C, Bemis SM, Hill EJ, Sawa S, Koshiba T, Torii KU** (2005) Interaction of Auxin and ERECTA in Elaborating *Arabidopsis* Inflorescence Architecture Revealed by the Activation Tagging of a New Member of the YUCCA Family Putative Flavin Monooxygenases. *Plant Physiology* **139**: 192-203
- Xiong Y, Jiao Y** (2019) The Diverse Roles of Auxin in Regulating Leaf Development. *Plants (Basel, Switzerland)* **8**: 243
- Yadav RK, Girke T, Pasala S, Xie M, Reddy GV** (2009) Gene expression map of the *Arabidopsis* shoot apical meristem stem cell niche. *Proceedings of the National Academy of Sciences* **106**: 4941-4946
- Yadav RK, Perales M, Gruel J, Girke T, Jönsson H, Reddy GV** (2011) WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex. *Genes Dev* **25**: 2025-2030
- Yadav S, Kumar H, Yadav RK** (2020) Local auxin biosynthesis promotes shoot patterning and stem cell differentiation in *Arabidopsis* shoot apex. *bioRxiv*: 819342
- Yanai O, Shani E, Dolezal K, Tarkowski P, Sablowski R, Sandberg G, Samach A, Ori N** (2005) *Arabidopsis* KNOX1 Proteins Activate Cytokinin Biosynthesis. *Current Biology* **15**: 1566-1571
- Žádníková P, Petrášek J, Marhavý P, Raz V, Vandenbussche F, Ding Z, Schwarzerová K, Morita MT, Tasaka M, Hejátko J, Van Der Straeten D, Friml J, Benková E** (2010) Role of PIN-mediated auxin efflux in apical hook development of *Arabidopsis thaliana*. *Development* **137**: 607-617
- Zhang L, DeGennaro D, Lin G, Chai J, Shpak ED** (2021) ERECTA family signaling constrains CLAVATA3 and WUSCHEL to the center of the shoot apical meristem. *Development* **148**
- Zhang WJ, Zhai LM, Yu HX, Peng J, Wang SS, Zhang XS, Su YH, Tang LP** (2020) The BIG gene controls size of shoot apical meristems in *Arabidopsis thaliana*. *Plant Cell Reports* **39**: 543-552

- Zhou Y, Liu X, Engstrom EM, Nimchuk ZL, Pruneda-Paz JL, Tarr PT, Yan A, Kay SA, Meyerowitz EM** (2015) Control of plant stem cell function by conserved interacting transcriptional regulators. *Nature* **517**: 377-380
- Zhou Y, Yan A, Han H, Li T, Geng Y, Liu X, Meyerowitz EM** (2018) HAIRY MERISTEM with WUSCHEL confines CLAVATA3 expression to the outer apical meristem layers. *Science* **361**: 502-506
- Zoulas N, Harrison EL, Casson SA, Gray JE** (2018) Molecular control of stomatal development. *The Biochemical journal* **475**: 441-454

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