SOCIAL NETWORKS OF RECEPTOR-LIKE KINASES REGULATE CELL IDENTITY IN *ARABIDOPSIS THALIANA*.

by

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EXPLANATION OF THE DISSERTATION FORMAT

This work is in accordance with the Manual of Thesis and Dissertations of The University of Arizona and in accordance with the policies of The University of Arizona Graduate Program of Molecular and Cellular Biology. This work is presented in two chapters and two appendices. The first chapter is a review of the literature of the current studies and outlines questions that still remain. Sections of this chapter include excerpts from a published book chapter for which I am a primary author and excerpts from a published review article for which I am also an author. The second chapter contains the two present studies including one published manuscript and another manuscript in preparation.

Contributions to the work:

Introduction: Pages 11-12, 18, 24, 26-38 are modified from a book chapter (in press) for which I am a primary author.

   Pages 13, 24-25 are modified from a published review article for which I am an author.

   The remaining sections of this introduction are my own work.

Present study:

Appendix A: Manuscript in progress is my own work.

Appendix B: Published manuscript for which I am the primary author.
ABSTRACT

Receptor-like kinases (RLKs) make up one of the largest gene families in Arabidopsis thaliana. These genes are required for various biological processes, including response to biotic stress, cell elongation, cell proliferation, and cell fate patterning. An emerging theme in Arabidopsis and other plants is that networks of RLKs are required to regulate a specific process throughout development involving spatial and temporal regulation of transcription factors. However, there are still many RLKs (>50%) with no known function.

Several RLKs regulate epidermal development by contributing to early embryonic epidermal maintenance or to epidermal differentiation. In my first analysis, I characterize the role of two related RLKs GASSHO1 (GSO1) and GSO2 in epidermal differentiation. gso1 gso2 double mutants initially form an epidermis during embryogenesis, but analysis of post-embryonic root development indicates the mis-expression of epidermal-specific genes. Three previously characterized RLKs that are involved in epidermal development are also involved in meristem maintenance. In order to decipher the RLK gene networks controlling epidermal development and meristem maintenance, it is necessary to identify additional RLKs involved in both of these processes. I further identified roles for GSO1 and GSO2 in maintaining root growth and root apical meristem (RAM) activity. A future goal will be to elucidate the networks of RLKs, including GSO1 and GSO2 in regulating epidermal and RAM development.

The development of the vasculature in plants is controlled by a vascular meristem, the procambium. Oriented cell divisions from the procambium produce phloem, to the
periphery, and xylem, to the center of the plant. In a reverse genetic screen to determine
to roles of the remaining RLKs with unknown function, we identified the RLK XYLEM
INTERMIXED WITH PHLOEM1 (XIP1) that is required for vascular development. We
show XIP1 is required for regulating the differentiation of the phloem and for the
organization of xylem vessel elements. Our analysis indicates that XIP1 is part of a
vascular meristem network, further emphasizing the importance of social networks of
RLKs regulating a specific process in development.
CHAPTER 1: INTRODUCTION

Origin of cellular identities

Pattern formation in both plants and animals requires precise coordination of cell proliferation and differentiation, leading to complex arrangements of cells in space and time. One important question is how do cells know their specific identity? Two different models have been suggested; 1) cell fate identities are passed on to their progeny by the segregation of proteins or RNAs through a lineage-based mechanism and 2) the identity of a cell is based on its spatial location, suggesting a position-dependent mechanism. The lineage mechanism for cell fate determination is based on the principle that asymmetric segregation of intrinsic factors is the sole requirement for differentiation, whereas position-dependent factors require extrinsic signals to coordinate cell fate.

In plants, particularly in Arabidopsis thaliana, nearly invariant cell division patterns are found in embryo and root development, which suggests lineage-based mechanisms of differentiation. However, experimental evidence from Arabidopsis, including cell ablation studies, suggests that positional signals rather than lineage play a more important role in plant cell fate determination (Saulsberry et al. 2002; van den Berg et al. 1995b). The identity of an individual plant cell is provided by its position in the apical/basal axis, the radial axis, and the adaxial/abaxial axis, as well as its specific functions within the group of cells that surround it. However, while the identities of these positional cues are slowly being uncovered, the interactions of the signaling pathway networks governing these processes are still poorly understood.
**Mechanisms for cell fate determination**

The process of cell fate determination has many stages throughout development. In animals, the initial cells that are produced from mitosis of the zygote are totipotent cells and can differentiate into either embryonic or extraembryonic cells. As embryogenesis proceeds, the totipotent cells give rise to pluripotent stem cells which can differentiate into the main germ layers in the body. In the adult, the stem cells that persist are usually limited in the types of cells that can be produced based on their lineage. This is an important difference between plants and animals, in that plants retain stem cells that can give rise to many cell types throughout their lifetime. Plant cells also typically do not move, which means the positioning of cells throughout the plant is determined by the location of actively dividing cells. In plants, the groups of stem cells that produce a majority of the dividing cells are called meristems. Asymmetric division of cells in the meristems replenishes the stem cells and also generates new cells that differentiate and form the various tissues in the plant. This process of differentiation integrates information from positional cues determined by signaling between cells.

In *Arabidopsis*, asymmetric cell divisions are responsible for many important developmental transitions and cell fate identities (De Smet and Beeckman 2011; Ten Hove and Heidstra 2008). Two general types of asymmetric cell divisions can generate new cell identities. A mother cell can divide to regenerate itself and a daughter cell
with a different identity, or a mother cell can divide to produce two new cells that differ from the mother cell. The first cell division of the zygote is an asymmetric cell division producing a smaller apical cell that will give rise to the embryo proper and a larger basal cell which becomes the suspensor, connecting the embryo to maternal tissue (Fig 1) (Mansfield and Briarty 1991). Asymmetric cell divisions are found throughout embryogenesis, including tangential cell divisions that delineate an outer layer of cells from inner cells at the transition from the 8-cell (octant) embryo to 16-cell (dermatogen) embryo (Fig 1) (Mansfield and Briarty 1991). The basal cell of the suspensor becomes

**Fig 1.** RLK functions during embryogenesis. Embryos from the two-cell stage through the heart stage are shown. Dotted arrows represent possible cell interactions and solid lines represent interactions with experimental evidence. Radial layers are first present at the dermatogen stage. Signals from the embryo proper recruit the uppermost suspensor cell to become the hypophysis. RPK1/TOAD2 maintain protodermal cell fate identity in the central domain during the early globular stage. Signals from the protoderm are proposed to repress vascular primordium fate in cells in the position of the ground tissue initials. ACR4/ALE2 promote protoderm cell fate maintenance, particularly in the apical domain. GSO1/GSO2 are important for the maintenance of the epidermis beginning at the heart stage.
part of the embryo and forms the hypophysis, which undergoes an asymmetric cell division to produce an apical lens shaped cell, the progenitor cell for the root meristem (Kawashima and Goldberg 2010). Meristemoid cells, stem cell-like cells with limited self-renewing capabilities in the epidermis, undergo a series of asymmetric cell divisions to produce pavement cells and stomata, cells specialized for gas exchange (Dong and Bergmann 2010). The stem cells found in shoot and root tips and in the vasculature undergo asymmetric cell divisions that produce cells that will differentiate, and also regenerate these stem cells. These stem cell niches have been a major focus of study, and these studies uncovered the role of both intrinsic factors and extrinsic signals regulating the identity and activity of stem cells.

Plants maintain active meristematic regions throughout their development. Meristem cells divide to produce a daughter cell that will differentiate into a more specialized cell while the other cell retains the stem cell fate. The three major meristem populations found in *Arabidopsis* are located in the shoot apical meristem (SAM), root apical meristem (RAM) and the vascular meristem, which is formed in both the procambium and the cambium (Fig 2). One invariant feature of these meristems is the presence of intrinsic factors required for maintaining the identity of the stem cell niches. Members of a subfamily of homeobox transcription factors called WUSCHEL-LIKE HOMEBOX genes (WOX), are required for maintaining each of the meristem niches. WUSCHEL (WUS) is required for maintaining the organizing center (OC) in the SAM, WOX5 is required for quiescent center (QC) identity in the RAM, and WOX4 is involved in maintaining an active procambium in the vasculature (Ji et al. 2010; Mayer et al. 1998;
Extrinsic signals have also been described which are required for the activity or patterning of the meristems, including plant hormones such as auxin and cytokinin.

The plant hormone auxin, is required for the patterning of organogenesis at the SAM, positioning of the QC at the RAM, and for patterning the cells that will become the procambium (Avsian-Kretchmer et al. 2002; Donner et al. 2009; Sabatini et al. 1999).

**Fig 2** CLE-RLK-WOX signaling networks in the SAM (A), vascular meristem (B), and RAM (C). A) CLV3 from the L1 and L2 layers is perceived by CLV1/CLV2/CRN in the L2 and L3 layers and represses WUS in the OC. BAMs likely are active in the peripheral zone and can compensate for loss of CLV1 function. Inner-to-outer feedback mechanism acts to promote CLV3 expression. B) CLE41/44 are secreted from the phloem and bind PXY in the procambium which promotes WOX4 expression and stem cell proliferation. C) The QC is maintained through signals from both the epidermis and the columella. CLE40 is secreted from the columella and likely perceived by ACR4 in the columella and columella initials and represses WOX5 expression. WOX5 expression and QC maintenance is also maintained through non-cell-autonomous effects of BRII in the epidermis.
Additional signals including small secreted peptides and other hormones, have roles in patterning throughout *Arabidopsis* (Matsubayashi and Sakagami 2006).

**Molecular mechanisms for intercellular signaling**

It is evident that cell fate determination processes in plants require extrinsic signals. Determining what these signals are and how they are perceived to direct cell fates is a major area of study. Intercellular signals can be perceived through a variety of very different mechanisms. Transcription factors have been shown to move between cells through cytoplasmic connections between cells called plasmodesmata. For example, the transcription factor SHORTROOT (SHR) moves from the stele to the endodermis in the root to promote endodermal identity (Helariutta et al. 2000; Nakajima et al. 2001). SHR movement is tissue-specific and restricted to one layer (Sena et al. 2004). In maize, the homeobox protein KNOTTED1 (KN1) moves from inner cells to the epidermal layer to regulate leaf formation (Lucas et al. 1995). These examples demonstrate the movement of transcription factors for cell fate determination, although the movement appears restricted to a single cell layer.

A second mechanism for intercellular communication is through the perception of ligands. A common mechanism for perceiving ligands in animals is through G Protein Coupled Receptors (GPCRs), also known as 7-transmembrane receptors. Upon binding of an extracellular signal, GPCRs signal to internal G proteins (alpha, beta and gamma proteins) to activate secondary signals. In humans, 791 GPCRs have been identified thus far (Bjarnadottir et al. 2006). In *Arabidopsis*, GPCRs are not as prevalent as in
metazoans, with only 27 GPCR-related proteins identified in the sequencing of the
Arabidopsis genome (Initiative 2000). Further analysis classified 394 proteins as very
divergent putative GPCRs and only 54 that conform to GPCRs with 7 TMs (Moriyama et
al. 2006). Using a more stringent bioinformatic analysis, 8 of these GPCRs were
identified as putative GPCRs, and 7 of these were experimentally shown to interact with
the sole alpha G protein in Arabidopsis. G PROTEIN COUPLED RECEPTOR 2 (GCR2)
has been the only GPCR identified that binds a ligand (Liu et al. 2007). GCR2 binds the
plant hormone abscisic acid (ABA), which is involved in a wide variety of plant
developmental processes and in stress responses, although it is not clear if GCR2 works
redundantly with other receptors to regulate these processes (Gao et al. 2007). Although
GPCRs make up one of the largest families in animals, they are much less abundant in
Arabidopsis and other plants.

Unlike animals, plants contain Histidine Protein Kinases (HPKs), a third
mechanism for intercellular signaling. Perception of a signal by the histidine kinase
receptor induces autophosphorylation of the histidine kinase. These kinases are part of a
two or multi-component system, where the phosphoryl group on the histidine residue is
then transferred to an aspartate on a receiver domain which then activates downstream
targets. 8 HPKs have been identified thus far in Arabidopsis (Grefen and Harter 2004).
Some of these receptors have been shown to bind the plant hormones cytokinin and
ethylene. Three HPKs including Arabidopsis HISTIDINE KINASE 2 (AHK2), AHK3
and AHK4 are known to bind cytokinin, and have recently been shown to be located in
the endoplasmic reticulum (Wulfetange et al. 2011; Yamada et al. 2001). Two HPKs,
ETHYLENE RESISTANT1 (ETR1) and ETHYLENE RESPONSE SENSOR1 (ERS1) are known to bind ethylene. These hormones, and their receptors, mediate various biological processes including responses to stress, cell proliferation and developmental patterning. The perception of these hormones also demonstrates that the binding of signals may not be restricted to plasma membrane localized receptors. Indeed, some extrinsic signals are not dependent on being perceived on the exterior of the cell. The plant hormone auxin, which is involved in many aspects of development including apical/basal pattern formation, is perceived by a cytoplasmic F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) which forms an ubiquitin ligase complex to regulate auxin signaling (Dharmasiri et al. 2005). Although F-box proteins are the largest class of proteins in *Arabidopsis*, the largest family of genes that demonstrates receptor functions is comprised of the putative plasma membrane localized receptor protein kinases.

In the *Arabidopsis thaliana* genome, 610 genes have been annotated as members of the Receptor-like Kinase (RLK) family (Shiu and Bleecker 2001). Among these are at least 125 Receptor-like Cytoplasmic Kinases (RLCKs), which are not likely to bind intercellular ligands. RLKs are composed of a single-pass transmembrane region, a serine-threonine kinase domain, and a variable extracellular region (Fig 3). The large number of RLKs in *Arabidopsis* and other plants such as rice suggests that signaling through these receptors plays a fundamental role in plant growth and development. Over the past twenty years, functional analysis of RLKs in plants has revealed numerous examples of roles for RLKs in cell fate determination. In this introduction, I will focus on RLK-
mediated mechanisms that specify and maintain meristems and stem cell identity, and those that specify more terminal identities such as epidermal and vascular cell identities.

**RLKs mediate extracellular signaling mechanisms**

RLKs bind a variety of different types of ligands including steroid hormones (brassinosteroids), peptides (phytosulfokine and CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-related peptides (CLEs)), and small secreted proteins (INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)). In tomato, the receptor for brassinosteroids (BR), BRASSINOSTEROID INSENSITIVE1 (BRI1), can bind both BR and the peptide systemin, indicating that even individual RLKs can perceive multiple signals (He et al. 2000; Kinoshita et al. 2005; Scheer et al. 2003; Scheer and Ryan 2002;
Wang et al. 2005; Wang et al. 2001). Closely related RLKs can also show differential binding affinities for a range of peptides (Yamaguchi et al. 2010). The identification of more than 1000 putative small peptides in the *Arabidopsis* genome indicates that a large number of signaling pathways could be initiated by these peptides (Lease and Walker 2006).

RLKs can regulate immediate local responses in the cell or downstream pathways that lead to changes in gene expression. In *Arabidopsis*, BR is perceived by BRII1, a Leucine-Rich Repeat (LRR) RLK, and the downstream signaling pathway initiated by BRII1 has been well characterized (Clouse et al. 1996; Kim and Wang 2010; Li and Chory 1997). One role of BR in plants is to induce cell expansion. A fast local response of BR was identified that leads to cell wall expansion and cell elongation. A BR-mediated interaction of BRII1 with a P-ATPase leads to hyperpolarization of the plasma membrane and induces cell wall expansion that precedes cell elongation (Caesar et al. 2011).

The transcriptional responses to BR include a series of steps leading to the binding of specific transcription factors to BR-induced genes. The perception of BR by BRII1 leads to the formation of a receptor complex with another LRR RLK, BRII1 ASSOCIATED KINASE1 (BAK1). The BRII1-BAK1 kinase complex is activated by transphosphorylation between these kinases, which in turn phosphorylates the RLCKs BR-SIGNALING KINASE1 (BSK1) and BSK2. BSKs then bind to bri1-SUPPRESSOR1 (BSU1), a phosphatase, which dephosphorylates the SHAGGY-LIKE KINASE/BR INSENSITIVE2 (BIN2). The deactivation of BIN2 permits
unphosphorylated BRASSINAZOLE RESISTANT1 (BZR1), and BZR2/BRI1-EMS-
SUPPRESSOR1 (BES1) to accumulate in the nucleus and binds to target BR-induced
genes to regulate transcription. BRI1 has also been shown to interact with additional
proteins that are not members of this canonical BR signaling pathway, indicating the
diversity of proteins that RLKs can regulate (Ehsan et al. 2005; Nam and Li 2004).
Additional downstream proteins have been identified for other RLKs including
CLAVATA1, FLAGELLIN-SENSING2 and SOMATIC EMBRYOGENESIS
RECEPTOR-LIKE KINASE1 (Johnson and Ingram 2005). However, well-defined
signaling pathways that are regulated by RLKs are still few in number.

Meristem maintenance and activity requires signaling through a paradigm of RLK
signaling

Analysis of the mechanisms that regulate meristem structure and maintenance has
uncovered a second paradigm for RLK signaling. The SAM is a dome of stem cells that
consists of three clonally related cell layers, Layer 1 (L1), L2, and L3 in order from outer
to inner layers (Fig 2a). The L1 and L2 divide anticlinally to maintain the cell layers and
generate the epidermis and mesophyll/cortex layers, respectively. The inner-most cells
make up the L3 layer, also known as the corpus, and generate the pith and vascular cells.
Within the SAM, the cells remain relatively undifferentiated and begin to differentiate as
subsequent cell divisions push the cells further from the SAM.
Analysis of mutants with altered SAMs allowed for the characterization of one of the most well-studied ligand-receptor networks, the CLAVATA (CLV) signaling pathway. CLV3, a small secreted peptide, is expressed predominantly in the outer two layers (L1 and L2) of the SAM and binds to the LRR RLK CLV1 in inner layers (L2 and L3) of the meristem (Fig 2a) (Clark et al. 1993; Clark et al. 1997; Fletcher et al. 1999; Trotochaud et al. 2000). The OC, which is located in the center of the meristem, is responsible for maintaining a pool of undifferentiated cells. The binding of CLV3 to CLV1 inhibits the expression of a homeobox transcription factor WUS, which is restricted to the OC (Brand et al. 2002; Schoof et al. 2000). Genetic and biochemical analyses of two additional RLKs, CLV2, a receptor-like protein that lacks a kinase domain, and CORYNE (CRN), an RLCK, indicates CLV2 and CRN form a complex and act in a parallel pathway to bind CLV3 and regulate WUS (Guo and Clark 2010; Kayes and Clark 1998; Muller et al. 2008). However, using fluorescence resonance energy transfer and luciferase complementation imaging analyses, the interactions detected between CRN and CLV1 suggest CLV1-CLV2-CRN can form a receptor complex to regulate WUS activity (Bleckmann et al. 2010; Zhu et al. 2010). Additionally, two RLKs related to CLV1, BARELY ANY MERISTEM1 (BAM1) and BAM2, regulate WUS activity in a CLV2-dependent manner and can compensate for the loss of CLV1 to regulate meristem size (Deyoung and Clark 2008). The CLV1-CLV2-CRN-BAM1-BAM2 receptor network regulating WUS activity and SAM maintenance indicates complex interactions between multiple RLKs can regulate cell fates.
Additionally, the combined mutant phenotype and expression patterns of WUS and CLV3 imply that WUS activates an inner-to-outer layer signaling mechanism, creating a feedback loop promoting CLV3 expression to maintain this signaling mechanism (Mayer et al. 1998). However the details of this feedback mechanism, particularly the inside to outside intercellular signaling, have yet to be identified.

A similar circuit of genes that are related to CLV3-CLV1-WUS has been identified that regulate the RAM, suggesting this type of signaling mechanism is generally responsible for maintaining stem cells. WOX5 was first identified as a transcription factor functioning in a similar fashion to WUS, but in the QC of the RAM (Fig 2c). WOX5 is expressed in the QC of the RAM, and WOX5 expression persists throughout root development (Haecker et al. 2004; Sarkar et al. 2007). Mutations in WOX5 result in loss of QC identity and premature differentiation of cells surrounding the QC (Sarkar et al. 2007). CLEs had previously been shown to regulate root development, with ectopic expression or exogenous application leading to root consumption, premature differentiation and mis-specification of cell identities in the root. Treatment of roots with a CLE closely related to CLV3, CLE40, had specific consequences in the RAM. In particular, increased levels of CLE40 caused ectopic expression of WOX5 in cells above the QC and increased differentiation of cells in the root tip (Stahl et al. 2009).

Additionally, the RLK Arabidopsis CRINKLY4 (ACR4), previously shown to regulate cell proliferation in the epidermis (described below), was proposed to be a receptor for CLE40. Plants homozygous for mutations in acr4 were insensitive to changes in CLE40 levels. It was proposed that CLE40 binds to ACR4 in the cells below the QC which then
inhibits WOX5, similar to the mechanism in the SAM, regulating the meristem population in the root tip (Fig 2c).

A related mechanism was also found to regulate meristem structure in vascular tissues. Vascular cells are generated from asymmetric cell divisions from the vascular meristem or procambium. Cells that divide toward the periphery will become phloem cells and cells that divide toward the interior will be specified as xylem cells. In the vasculature, the CLE41/42/44 genes which produce CLE peptides, related to CLV3, known as TRACHEARY DIFFERENTIATION INHIBITING FACTORs (TDIF), are secreted from the phloem and bind the LRR RLK PHLOEM INTERCALATED IN XYLEM (PXY) in the procambium (Fig 2b) (Fisher and Turner 2007; Hirakawa et al. 2008). However, in the vasculature, a CLV3-related CLE peptide inhibits differentiation and promotes meristem identity and proliferation. The CLE41-PXY signaling pathway regulates the expression of WOX4, which promotes procambium proliferation, and is another example of the conserved CLE (ligand)-RLK (receptor)-WOX (homeodomain protein) signaling circuit (Hirakawa et al. 2010).

The expression of additional WOX genes in cells other than stem cells indicates this type of signaling pathway may be utilized in other developmental contexts. One possibility is that this signaling mechanism may be utilized for regulating the identities of cell types other than stem cells. Before addressing this issue, it is first necessary to review the RLKs with known roles in the determination of cell identity.
Origin of tissue fate begins during embryogenesis

Cells first begin receiving cues about their positions relative to other cells during embryogenesis (Fig 1). The apical-basal axis becomes evident in the first cell division of the single-celled zygote. The zygote divides asymmetrically to give rise to a smaller apical cell and a larger basal cell (Mansfield and Briarty 1991). Directional flow of auxin first specifies the apical pro-embryo cell, and later reverses flow downwards to specify the basal root pole, thereby establishing the apical-basal axis (Friml et al. 2003). The apical cell continues to divide, and gives rise to an octant embryo with an apical and central domain. The basal cell produces a file of cells called the suspensor, an extra-embryonic structure that provides a physical link to the surrounding maternal tissue. Auxin is transported from the maternal tissue through the suspensor and accumulates in the apical cells of the embryo to trigger specification of the apical domain.

The radial axis, which will provide the basis for much of the adult body form, is initially established after the transition from the octant stage to the dermatogen stage of embryo development, with tangential cell divisions delineating the outer cell layer (protoderm) from the inner cell layer (Fig 1) (Laux et al. 2004). The protoderm layer is maintained through periclinal cell divisions along the embryo surface. The two inner cell layers are generated through further tangential cell divisions during the early globular stage, producing the ground tissue and provascular initials. During the globular to heart stage transition, the apical domain of the embryo transforms from a radial structure to a bilaterally symmetric heart-shaped structure. Abaxial(lower)/adaxial(upper) asymmetry is
established initially in the cotyledons, embryonic leaves, and provides cues for
development in lateral adult organs such as floral organs and leaves (discussed below).
The nearly invariant cell lineage of *Arabidopsis* has made it a very useful system for
studying embryogenesis and many recent analyses have indicated that intercellular
signaling by members of the RLK family plays a central role in directing critical stages of
embryo development (Mansfield 1991; Nodine et al. 2011).

Fate-mapping experiments indicate that *Arabidopsis* embryonic cells do not have
fully restricted fates before the octant stage (Saulsberry et al. 2002). This supports the
notion that positional signaling plays a major role in early patterning. Moreover,
*Arabidopsis* mutants with irregular cell divisions throughout the early embryo, before the
initiation of many differentiation programs, (such as *fass* mutants) still form seedlings
with proper body patterns (Torres-Ruiz and Jurgens 1994). Therefore, the location of a
cell in the developing embryo, and presumably its communication with surrounding cells,
plays an important role in regulating its differentiation. The epidermal layer regulates the
post-embryonic development of sub-epidermal layers through non-autonomous signals
(Hantke et al. 1995; Reinhardt et al. 2003; Savaldi-Goldstein et al. 2007) and the
protoderm appears to have similar roles during embryogenesis. The protoderm also
influences the differentiation programs of surrounding cell types. Chemical ablation of
protodermal cells at the dermatogen stage can result in hypophysis (basal cell) cell
division defects at the globular stage (Baroux et al. 2001; Weijers et al. 2003). The
influence of the protoderm/epidermis can further be deduced through the analysis of
RLKs that function to specify this layer.
Epidermal specific homeobox proteins several as markers for cell fate

A subfamily of homeobox genes called HD-ZIP IV transcription factors are integral proteins regulating epidermal development. The HD-ZIP IV subfamily of 19 genes are characterized by a leucine zipper motif downstream of the homeobox domain, and are all expressed in the outer layer (Nakamura et al. 2006). HD-ZIPs, which are plant specific, are part of a larger gene family of homeodomain containing proteins. Although these homeobox proteins do share the presence of a homeodomain, many subfamilies have diverged significantly with the HD-ZIPs showing only distant relationship to the WUS class of homeobox genes (WOX). Whereas WOX genes are found in single-celled green algae, HD-ZIPs are only found in flowering plants suggesting that WOX genes may have initially had a role in basic cellular processes and HD-ZIP may have a more specialized role in flowering plant development (Mukherjee et al. 2009). Several HD-ZIP IV proteins have been utilized as markers of epidermal cell fate due to their specific expression in the outer layer.

Cell-type specific markers are very useful for interpreting the phenotypes of mutants that affect cell fate. Expression of Arabidopsis thaliana MERISTEM LAYER1 (AtML1), a member of the HD-ZIP IV family, has been used to track protodermal (cells that will become epidermal) cell identity (Nodine et al. 2007; Takada and Jurgens 2007). AtML1 expression can initially be detected in both cells of the 2-cell stage embryo via in situ hybridization or by analysis of reporter gene fusions to the AtML1 promoter region (Fig 1). By the dermatogen stage and thereafter, AtML1 is restricted to cells within the
outer cell layer of the embryo (Takada and Jurgens 2007). Although the mechanism for how *AtML1* becomes restricted to the outer cell layer is not yet known, maintenance of protodermal cell identity is now known, at least in part, to be regulated by RLK signaling; there is no current evidence for a lineage-based mechanism.

The *AtML1* and the closely related PROTOGERMAL DETERMINING FACTOR 2 (PDF2) transcription factor are HD-ZIP IV genes together required for epidermal cell fate determination (Abe et al. 2003). In *atml1 pdf2* mutants, the apical domain of the embryo develops abnormally and an outer to inner cell fate transformation occurs in the resulting seedlings, with the epidermal cells of the cotyledons taking on the physical appearance of the internal mesophyll cells. *AtML1* and PDF2 both bind to the "L1 box", an 8-base pair cis-regulatory motif found in the promoters of epidermal specific genes (Abe et al. 2003). *AtML1* and PDF2 also have L1 box binding sites within their own promoters, suggesting that once the protoderm is specified in the dermatogen stage, its identity is maintained, at least in part, via a positive feedback loop.

Once the epidermis is specified, epidermal cells can then be further specified into more specialized cell types, including hair and non-hair cells in the root, and stomata, guard and trichome cells in the aerial tissues. GLABRA 2 (GL2), an HD-ZIP IV transcription factor related to *AtML1*, is involved in specifying non-hair cells in the root (Masucci et al. 1996). As will be described in detail later, GL2 is involved in a network of genes that specify the differentiation of the epidermis into specialized epidermal cell types. Not only is GL2 required for epidermal cell differentiation, but similar to ATML1 and PDF2, it has also been widely used as a marker for epidermal cell fates. These
transcription factors are likely to represent downstream targets of pathways that regulate epidermal cell fate.

Interestingly, these HD-ZIP IV transcription factors also contain a lipid/sterol-binding StAR-related lipid transfer protein domain (START). Although the START domains in plants are not closely related to those of animals, structural conservation of the domain suggests the START domain in plants has a common role in the binding, transport and metabolism of lipids/sterols (Schrick et al. 2004). The presence of the START domain indicates these transcription factors may influence gene expression in a sterol-dependent manner or possibly enable the HD-ZIP proteins carrying this motif to localize to the membrane where they can be regulated (Ponting and Aravind 1999).

**Maintaining the protoderm cell identity: RLKs pave the way**

There is currently very little understanding of the mechanisms that establish the identity of the protoderm during the dermatogen stage of embryo development, or the mechanisms that maintain AtML1 expression in the epidermal layer and lead to its disappearance in the inner cell layers. However, recent evidence from our lab indicates that signaling through the RECEPTOR PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2) LRR RLKs is required for maintaining protoderm cell identity during the globular stage, and for signaling to the inner radial layers (Fig 1) (Nodine et al. 2007). In all of the rpk1 toad2 double mutants and in approximately half of rpk1 toad2/+ mutants, early embryonic lethality results in a phenotype of brown shrunken seeds. Mutant
embryos show extra tangential cell divisions in the protoderm layer, growth cessation at the globular stage, and display a mushroom shape dubbed the “Toadstool” phenotype. “Toadstool” embryos show a loss of AtML1 expression at the early globular stage, although AtML1 is initially expressed and correctly restricted to the protoderm during the dermatogen stage (Nodine et al. 2007). This data demonstrates that RLK signaling by the redundant RLKs RPK1 and TOAD2 is necessary to maintain protoderm cell identity. It is possible that RLKs are also required for specifying the protoderm when it is first formed at the dermatogen stage. However, the specific RLKs that might be candidates for signaling between the protoderm and the inner layer are not known.

**Maintaining the epidermis requires a network of RLKs**

The epidermal cell layer needs to be properly specified for normal plant development. Recent reports suggest a common mechanism for the maintenance of the epidermis in adult tissues and the maintenance of the embryonic protoderm (Becraft et al. 1996; Gifford et al. 2003; Tanaka et al. 2007; Tanaka et al. 2002; Watanabe et al. 2004b). CRINKLY LEAF-4 (CR4) was the first RLK reported to function in epidermal development; maize cr4 mutants have defects in the adult leaf epidermis and also in the epidermal-like aleurone layer of the seed endosperm (Becraft et al. 1996). Five CR4-related genes are encoded in the *Arabidopsis* genome with the closest homolog designated *Arabidopsis CR4 (ACR4)* (Tanaka et al. 2002). acr4 mutants were shown to have similar epidermal defects in adult leaves and ovule integuments as observed in the maize cr4
mutant, including crinkly leaf surfaces, fusions between organs, abnormal outer layer morphology of ovules, and altered cuticle development (Becraft et al. 1996; Watanabe et al. 2004b). Since ACR4 is also expressed during embryo development, primarily in the protoderm, it is possible that ACR4 acts during additional stages of embryo development to maintain epidermal identity. While the arrested growth of acr4 mutant embryos is likely due to their integument defects, embryos expressing ACR anti-sense RNA showed more severe embryonic defects, possibly due to knocking down the expression of other closely related ACR4-like genes (Tanaka et al. 2002). These data suggest that ACR4 may act redundantly with other ACR-related genes in the maintenance of protoderm cell fate. ACR4 may act early in embryonic development to maintain the protoderm either with RPK1-TOAD2 or in an alternate mechanism that is also necessary to maintain this tissue layer.

Genetic analysis has revealed that the ABNORMAL LEAF SHAPE2 (ALE2) RLK acts with ACR4 during embryo development (Tanaka et al. 2007b). ale2 mutant plants show similar adult defects as those seen in the maize cr4 and Arabidopsis acr4 mutants including crinkly leaves, defective cuticle, and abnormal ovule development (Becraft et al. 1996; Tanaka et al. 2007b; Tanaka et al. 2002; Watanabe et al. 2004b). Genetic and biochemical analysis of ACR4 and ALE2 have shown that they physically interact and function in the same pathway to maintain protoderm identity during the early stages of embryo development (Fig 1). Double acr4 ale2 mutants resemble ale2 mutants, indicating along with biochemical interaction, that these RLKs act in the same pathway. However, genetic analysis of ale2 or acr4 mutants with mutations in ALE1, a secreted
protease, results in earlier defects in embryo development that are not apparent in acr4 or ale2 single mutants. Double mutants of ale1 with acr4 or ale2 are defective in protoderm development, as indicated by morphological defects including deformed protodermal cells at the heart stage, as well as by loss of protodermal markers, including loss of expression of AtML1, PDF1 and PDF2 as early as the heart stage.

As described above, RPK1, TOAD2, ALE2, and ACR4 are required for the maintenance of epidermal identity during early and intermediate stages of embryogenesis, and ALE2 and ACR4 are also required for maintaining epidermal identity during adult stages of Arabidopsis development. There is evidence that two redundantly-acting LRR RLKs that are closely related to each other, GASSHO1 (GSO1) and GSO2, are required for epidermal identity by the late heart stage of embryogenesis and during early seedling development. The gso1 gso2 double mutants display morphological defects during embryogenesis, including adherence of the embryo to the inner seed integument, loss of cuticle formation and abnormal cell divisions in early seedling stages, resulting in seedling lethality soon after germination (Tsuwamoto et al. 2008). Although the identity of the epidermal cell layer has not been analyzed for these mutants, it is apparent that their epidermal development is impaired. gso1 gso2 mutant seedlings are abnormally permeable to the dye toluidine blue (TB), likely due to defects in the formation of the cuticle, a waxy, protective covering produced by plant epidermal cells. The lethality of gso1 gso2 seedlings has been proposed to be due to the dehydration caused by defects in epidermal cell differentiation (Tsuwamoto et al 2008).
These studies indicate that a large number of receptor kinases (RPK1, TOAD2, ALE2, ACR4, GSO1, and GSO2) are required for the maintenance of epidermal cell fate during embryonic and adult development (Fig 1). There may well be many more RLKs that play important roles, as many others are expressed at levels equal or higher than RPK1, TOAD2, ALE2, ACR4, GSO1 and GSO2 during embryogenesis (Nodine et al. 2011). Whether the maintenance of epidermal cell fate involves the activation of a series of independent ligand/receptor pathways, or sequential physical interactions between these receptor kinases remains to be uncovered.

**Signaling between layers and domains in embryonic development**

From the early stages of embryogenesis, cell layers must be able to communicate with each other to coordinate plant growth. If coordinated growth relies on numerous conversations between cell layers, it is predicted that developmental disruption of one cell layer could have non-cell autonomous consequences on the development of its neighboring cell layers. As discussed above, signaling through RLK-mediated pathways from the outer L1 layer is required to maintain the inner stem cell population of the shoot apical meristem (SAM). A number of experimental observations have demonstrated that disruption of the protodermal layer affects the development of inner cell types during many stages of development. Chemically-induced disruption of the protoderm in globular embryos resulted in improper division of the hypophysial cell and its progeny (Baroux et al. 2001; Weijers et al. 2003). *rpk1 toad2* double mutant embryos, in addition to their defects in epidermal identity, also show a lack of patterning of inner cell types (Nodine et
al. 2007). The expanded expression of markers specific to the inner cell layers 
(*SHORTROOT* (*SHR*) for provascular cell identity; *SCARECROW* (*SCR*) for ground tissue identity) in *rpk1 toad2* embryos suggests that the fates of the inner cell layers are affected. These results suggest that protodermal identity, mediated by RPK1-TOAD2 signaling, inhibits inner cell identity. One model suggests that signals from the protoderm are perceived by receptors in the inner layers (Nodine et al. 2007).

*BR perception in the epidermal layer is required to signal growth to underlying cell layers*

Analysis of the regulation of growth in adult plants suggests that signals from the epidermis control the growth of inner layers. The plant steroid hormone BR is known to promote growth through stimulation of cell expansion and cell division, and also plays a role in vascular cell fates (Cano-Delgado et al. 2004). Loss of production or perception of BR results in reduced growth and a dwarf phenotype (Choe et al. 1999; Clouse et al. 1996; Li and Chory 1997). When *BRI1* expression is restricted to the epidermal layer by using the *AtML1* promoter to drive BRI1 expression, the dwarf phenotype of *bri1* mutants was rescued (Savaldi-Goldstein et al. 2007). However, the vascular bundle organization defect of *bri1* mutants was not rescued, suggesting BRI1 expression in the inner layers is still required for specification of inner cell types. In contrast, when BRI1 expression, or the expression of genes involved in the production of steroids, was driven by inner layer promoters, there was significantly less rescue of the dwarf phenotype (Savaldi-Goldstein et al. 2007). These data led to a model in which BRI1-mediated perception and signaling
in the epidermis, leads to epidermal signaling to inner cell types in order to coordinate the growth of the entire plant.

Brassinosteroids also regulate root meristem activity and growth of root apices (Gonzalez-Garcia et al. 2011; Hacham et al. 2011). Treatment of plants with brassinolide (BL), the most active BR, also affects the identity of the QC in the RAM (Gonzalez-Garcia et al. 2011). In these experiments, the addition of BL or a reduction in BL signaling, using a mutation in BRI1, altered expression of several markers for QC identity. Exogenous application of BL led to both the expanded expression of several QC markers, including WOX5, as well as the reduction of expression of other markers. Loss of BR signaling also led to a reduction in expression of WOX5. The effects of BR on meristem size and identity can be attributed to the perception of BR solely in the epidermal layer. By expressing BRI1 in specific radial layers using the *GL2* (epidermis), *SCR* (ground tissue and endodermis) and *SHR* (stele) promoters, BR signaling in the outer layer was shown to have the most significant effect on regulating meristem size and QC identity (Hacham et al. 2011). These data, combined with the known effects of BR perception in the L1 of SAMs, suggest perception of BR in the outer layer regulates plant growth through potential inter-layer signaling mechanisms to the meristem cells (Fig 2c), providing a mechanism to coordinate the overall size of the plant.

*Position-dependent RLK signaling pathways regulate root epidermal cell differentiation*
After the identity of the epidermal cell layer is established and successfully maintained, cells within the epidermis differentiate into specialized cell types. While the aerial epidermis is patterned into trichomes, guard cells and pavement cells, root epidermal cells differentiate into hair (root hair) and non-hair cells. Root hair cell patterning is one of the best studied systems for understanding plant cell differentiation, and the transcriptional networks that control this process have been well characterized (Ishida et al. 2008).

In *Arabidopsis* roots, epidermal cell differentiation is regulated by a position-dependent mechanism in which epidermal cells that overlie two cortex cells become hair cells (H-position), and epidermal cells that overlie a single cortex cell become non-hair cells (N-position) (Fig 4) (Dolan et al. 1993; Galway et al. 1994). The first inroads into the mechanisms that produce H- and N-cells came from the analysis of mutants with fewer or additional root hairs. Mutants with extra root hairs were isolated in *GL2*, and in *WEREWOLF* (WER), encoding an R2R3 Myb transcription factor (Di Cristina et al. 2003).
GL2, which is specifically expressed in non-hair cells (Fig 4), and WER, whose transcripts preferentially accumulate in non-hair cells, both act to prevent hair cell formation in the non-hair cell position (Schiefelbein et al. 2009). CAPRICE (CPC), encoding an R3 Myb protein lacking a transcriptional activation domain, is expressed in non-hair cells, and CPC is thought to move to hair cells where it negatively regulates WER (Kurata et al. 2005; Lee and Schiefelbein 2002; Wada et al. 1997). Both positive and negative feedback loops based on interactions of these and other transcription factors further refine the alternating patterning of H- and N-cells laterally within the epidermal layer. Lateral movement of factors, including CPC, through plasmodesmata has been demonstrated and contributes to the feedback mechanisms in the lateral inhibition of cell fates (Bernhardt et al. 2005; Wada et al. 2002). Although epidermal root hair cell differentiation is clearly based on the orientation of epidermal cells with respect to the inner cortex cell layer, how this positional information was perceived was until recently, a mystery.

A series of recent studies have determined that the positional-dependent regulation of root hair cell differentiation is influenced, at least in part, by SCRAMBLED (SCM), an LRR RLK. scm was identified in a screen for mutants that disrupted the expression pattern of GL2 in epidermal cells; mutations in SCM caused ectopic expression of GL2 in hair cells and a decrease in the frequency of N-cells expressing GL2 (Fig 4) (Kwak et al. 2005). The expression patterns of several other root hair/non root hair cell identity genes including CPC, WER, and ENHANCER of GLABRA 3 (EGL3) were also affected in the scm mutant (Kwak et al. 2005). It is worth noting that although the
WER:GFP and GUS markers show specific accumulation in non-hair cells, in situ hybridization of the WER transcript shows some accumulation in hair cells (Lee and Schiefelbein 1999). The conclusion from these studies is that SCM likely regulates epidermal cell fate through its negative regulation of WER in cells in the hair cell position.

Based on these and other studies, there are two clear alternative models for the action of SCM. The first model is that SCM is required in the epidermal cell layer to perceive a signal from underlying cortical cells to determine non-root hair cell fate. Support for this model comes from in situ hybridization and GUS reporter experiments that show that SCM is expressed in the epidermal layer and that a 4 kilobase (kb) SCM promoter region drives expression of GUS in all radial layers of the root (Kwak et al. 2005). Furthermore, an SCM-GFP fusion protein under the control of the same promoter region is sufficient to rescue scm mutant defects (Kwak and Schiefelbein 2008). Expression of an SCM:GFP fusion protein driven by the promoter of GL3, a gene expressed in H-cells, rescues the disrupted GL2 expression pattern in scm roots, whereas altered GL2 expression is still observed when SCM-GFP is driven by inner cell-specific promoters such as SCR and SHR (Kwak and Schiefelbein 2008). In this model, SCM activation would result from the perception in the epidermis of a signal emanating from inner layers.

An alternative model is that SCM, also known as STRUBBELIG (SUB), functions in inner cell layers and is responsible for generating an inner to outer signal. This is the model supported by independent studies of SUB (Chevalier et al. 2005; Yadav et al. 2008). SUB also has roles in stem, floral and ovule development; for example the
outer integument fails to enclose the ovule, leading to infertility. A 3.5 kb promoter region of \textit{SUB} and 0.2 kb of its 3'-untranslated region was found to drive expression of GUS fused to a cyclin destruction box (CDB-GUS transcripts will be degraded after mitosis) in all cell layers of ovules and roots, although the expression in the root epidermal layer was somewhat variable (Yadav et al. 2008). However, a SUB-enhanced GFP fusion protein driven by the same regulatory regions was only found in the inner cells of the ovule and in the root vasculature. These results suggest that SCM/SUB is regulated at the level of translation. To test the layer-specific regulation of SUB, its expression was driven using layer specific promoters. The results were somewhat inconsistent with the previously described model: SUB was determined to primarily function in inner cell layers to coordinate growth of the outer integument (Yadav et al. 2008). However, surprisingly, expression of \textit{SUB} using the epidermal \textit{AtML1} promoter rescued defects in ovules, flowers and stems, although root epidermal patterning was not tested. With the exception of this last result, these experiments lead to the conclusion that SCM/SUB can act non-cell autonomously, and that activation of SCM in inner cells leads to signals that regulate outer fates.

Both of these models emphasize an important role for signaling from inner cell layers to outer layers. However, establishing whether SCM/SUB is at the initiating or receiving end of this signaling mechanism needs to be determined, Both groups tested different SCM/SUB regulatory sequences. The transcriptional fusion using the CDB was intended to remove problems associated with excess stability of GUS, but these sequences may have had other effects on SCM/SUB transcript stability. In addition, the genetic
backgrounds in which these experiments were carried out differed significantly. The SUB experiments were conducted in the Landsberg *erecta* accession, which contains a weak mutation in the ERECTA RLK gene. This receptor functions in the epidermis and interacts with other signaling pathways. Additional experiments that rely on the same constructs in the same genetic backgrounds are necessary to determine the precise role of SCM/SUB in signaling between layers. It is also possible that both models are correct: that SCM/SUB functions both in the epidermis and in signaling from inside layers outwards. Identifying other components of the SCM/SUB pathway and perhaps parallel pathways will ultimately be useful in understanding how epidermal patterning is established and maintained.

*BRs and BRI1 signaling regulate cell fate decisions in epidermal root cell differentiation*

We previously described the role of BRs in regulating plant growth through their function in the protodermal or epidermal layer. BRs also have a role in cell fate specification of root epidermal cells. Global transcription analysis of whole seedlings has revealed that WER gene expression is induced upon treatment with BRs (Kuppusamy et al. 2009; Nemhauser et al. 2004). BR is perceived by the BRI1, BRI1-LIKE1 (BRL1) and BRL3 LRR-RLKs. By analyzing the expression of the root non-hair cell specific genes CPC and GL2, the effects of BR on root hair cell specification were shown to require BRI1 (Kuppusamy et al. 2009). This report showed that BRs and BRI1 act to suppress non-hair cell (N) formation in the hair cell (H) position. *bri1* mutant seedlings carrying a transgene of the *GL2* promoter fused to the β-glucuronidase reporter gene (*GL2:GUS*)
show ectopic expression of *GUS* in hair cells (Fig 4). Furthermore, *CPC* expression was reduced in a *bri1* background, consistent with the role of CPC in inhibiting *GL2* expression. These results provide strong evidence for BRs and BRI1 in regulating the cell fate of epidermal cells. Further experiments will be necessary to determine if BRL1 and BRL3 act redundantly with BRI1 in this process.

An interesting connection between several of the above RLKs is their role in regulating both epidermal development and meristem activity. Genetic analyses of TOAD2, ACR4, and BRI indicate that disruption of these genes, in some cases together with additional genes, leads to mis-expression of epidermal specific HD-ZIP IV genes; TOAD2 and ACR4 regulate *AtML1* and BRI affects GL2 expression. *acr4* and *bri1* mutants affect the expression of WOX5 in the RAM, through both proposed cell-autonomous signaling (ACR4) and non-cell-autonomous signaling (BRI1) pathways. The regulation by these RLKs suggests a regulatory link between the transition to the WOX and HD-ZIP homeobox genes.

**Specifying and maintaining internal cell fates: Analysis of vascular development**

As apical/basal patterning promotes the position of the SAM and RAM and radial patterning contributes to the structure of a majority of adult organs, abaxial/adaxial patterning provides cues for asymmetric development of lateral organs including leaves and some floral organs. The abaxial side refers to the side away from the meristem (lower side of leaves) and adaxial refers to the side near the meristem (upper side of leaves).
Abaxial/adaxial signaling directs patterning of several developmental programs pertaining to leaf development including the vasculature in leaves with the xylem forming adaxial to the phloem.

Although a great deal of attention has been given to developmental patterning of cells in the epidermis, other internal organs in *Arabidopsis* are less well studied. One such organ that has received some attention recently is the vascular system. The vascular system in *Arabidopsis* is comprised of three main tissue types; the xylem and phloem which are the structural and conductive elements specialized for the transport of water, nutrients and signaling molecules, and the intervening meristem called the procambium (Fig 5). The specification of the vascular tissues is determined, at least in part, by oriented cell divisions from the procambium where cells that divide to the interior of the root or stem, or adaxial side of leaves, become specified as xylem and cells that divide to periphery of the root or stem, or abaxial side of leaves, become specified as phloem.

Although the morphological features of vascular development have been known for many years, an understanding of how xylem and phloem are specified and maintained is just beginning.

Procambial meristems arise from the shoot and root apical meristems formed during embryogenesis. The initial specification of the procambium occurs in ground tissue cells from the SAM/RAM, and establishment correlates with the accumulation of the hormone auxin (Sachs 2000). Mutations in genes that affect polar auxin transport such as PIN-FORMED 1 (PIN1), an auxin efflux carrier, and GNOM (GN), a GDP/GTP
exchange factor, result in altered vascular differentiation, and mutations in AUXIN RESPONSE FACTOR 5/MONOPTEROS (ARF5/MP) disrupt continuous xylem cell formation (Galweiler et al. 1998; Przemeck et al. 1996; Scarpella et al. 2006; Steinmann et al. 1999). Green florescent protein (GFP) expression from the auxin responsive promoter DR5 and expression of Arabidopsis thaliana HOMEBOX GENE 8 (AtHB8), a procambium marker that is also directly regulated by MP, precede vascular formation in Arabidopsis (Donner et al. 2009; Scarpella et al. 2006). Analysis of loss-of-function mutants of AtHB8-related HD-ZIP III genes including AtHB15/CORONA (CNA), PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV) and AtHB8 revealed these genes work together to regulate procambium and xylem differentiation (Carlsbecker et al. 2010; Ilegems et al. 2010). Auxin signaling and the group of related HD-ZIP III transcription factors are clearly required for initiation and maintenance of vascular stem cells in Arabidopsis.

The cambial meristem is derived from the procambium and is responsible for radial growth of plants and for wood production in woody plants. The cambium produces secondary phloem and xylem cells and is also responsible for lateral growth of the interfascicular region, the connective tissue in between the vascular bundles. Although the regulation of the transition from procambium to cambium is not well known, several factors are known to regulate cambium activity. Overexpression of AtHB8 results in an increase in the size of the interfascicular region as well as an increased number of xylem cells, indicating a role for AtHB8 in promoting the activity of both procambium and cambium meristems (Baima et al. 2001). high cambium activity
(hca) mutants were identified with a continuous ring of vascular cells, instead of alternating vascular bundles (Pineau et al. 2005). hca mutants also have increased expression of AtHB8 as well as altered sensitivity to both cytokinin and auxin (Pineau et al. 2005). Cambium activity is also regulated by RLKs. Mutations in two RLKs, REDUCED IN LATERAL GROWTH1 (RUL1) and MORE LATERAL GROWTH1 (MOL1), resulted in a reduced interfascicular region and an expanded interfascicular region respectively (Agusti et al. 2011). The involvement of RLKs in regulating cambium activity suggests intercellular communication is required for mediating the activity of this secondary meristem.

**Controlling vascular proliferation through Brassinosteroid and Cytokinin signaling**

Although Brassinosteroid signaling is known to regulate cell elongation and differentiation, BRs are implicated in a separate function in vascular development. BR-deficient mutants have been shown to have increased amounts of phloem and decreased amounts of xylem, thereby indicating BRs in controlling the ratio of phloem and xylem production (Choe et al. 1999; Szekeres et al. 1996). This altered phloem to xylem ratio was also visible in mutant analysis of the BR receptors BRI1, BRI-LIKE 1 (BRL1) and BRL3. While BRI has a wide expression domain in growing cells, BRL1 and BRL3 expression is restricted to vascular tissues (Cano-Delgado et al. 2004). Mutations in bri1 together with brl1 and brl3 result in altered ratios of vascular cells with further reductions in xylem cells and reduced vascular differentiation (Cano-Delgado et al. 2004). In
addition, overexpression of BRI1 results in an increased amount of xylem. VASCULAR HIGHWAY1 (VH1)/BRL2, a close paralog to BRI1 and BRL1 and BRL3, does not bind BR, but mutants in VH1 have vascular defects as well. VH1 appears to regulate early patterns of differentiation in provascular cells (Clay and Nelson 2002).

The phytohormone cytokinin has also been shown to be required for maintaining procambium cell divisions. Mutations in wooden leg (wol)/ahk4, a cytokinin receptor, result in a reduced number of procambium cells and transformation of procambium into protoxylem (Mahonen et al. 2000; Scheres et al. 1995). WOL/AHK4 is required for the asymmetric cell divisions in the procambium that give rise to the xylem and phloem. Two related cytokinin receptors AHK2 and AHK3, also are required for proliferation and maintenance of the procambium layer (Hejatko et al. 2009). Although several pathways are known to regulate procambium development, few genes have been identified that regulate the specification and development of the phloem and xylem.

**Specification of differentiated vascular cells**

Using expression analysis with a Zinnia culture system, factors were isolated that were able to promote the differentiation of multiple cell types into xylem elements. Two genes that were isolated using this system were VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7. VND6 and VND7 were shown to be necessary for xylem-like vessel formation and sufficient to transform multiple cell types into metaxylem (mature xylem) and protoxylem (initial xylem) cells respectively (Kubo et al.
2005). Other factors have been identified that regulate various aspects of xylem formation including ACAULIS5 (ACL5), a spermine biosynthesis gene, which controls xylem differentiation through the regulation of cell death (Muniz et al. 2008). Although several genes have been identified that regulate xylem differentiation, only one gene has been identified thus far that regulates phloem differentiation. ALTERED PHLOEM DEVELOPMENT (APL), a MYB coiled-coil transcription factor, has been shown to be necessary for the specification of phloem cells and for limiting xylem formation (Bonke et al. 2003). However, ectopic expression of this gene is not sufficient to induce phloem differentiation, and how it is regulated remains unknown.

**Fig 5** The CLE41-PXY-WOX4 signaling pathway regulating procambium/cambium activity. CLE41/44 is secreted from the phloem and binds PXY in the procambium which induces WOX4 expression and maintains cell proliferation. A CLE41/44-PXY signaling pathway is also involved in repressing xylem formation in the procambium, although this pathway is independent of WOX4. PXY also appears to act within the xylem to regulate xylem differentiation.
The characterization of the LRR RLK PHLOEM INTERCALATED WITH XYLEM (PXY) was the first indication that RLKs can regulate vascular development. PXY is expressed in the procambium and maintains the proliferation of the procambium, and also regulates xylem differentiation (Fisher and Turner 2007). PXY receives positional cues from the secreted CLE41 peptide expressed from the phloem and surrounding cells to regulate procambium proliferation through the transcription factor WOX4, and regulates xylem differentiation through a WOX4-independent pathway (Fig 5) (Hirakawa et al. 2010; Hirakawa et al. 2008). As seen in the regulation of epidermal and RAM development above, both HD-ZIPs (ATHB8) and WOX genes (WOX) converge on the regulation of developmental processes. Given that many RLKs regulate SAM and RAM maintenance, one would predict there would be other RLKs that regulate xylem and phloem differentiation, but these have not yet been identified. Identification of RLKs specifically expressed in these tissue types should provide candidates that may regulate these developmental programs. Additionally, the vascular meristem is determined during embryogenesis. How the vascular system is initially established and how much cell-to-cell communication is involved in this process remains unknown.

**Unresolved questions regarding the function of RLKs and the regulation of development**

Above I have described several paradigms for RLK signaling, and the function of RLKs involved in epidermal and vascular development. Several of the above described RLKs including CLV1, BAM1, BAM2, GSO1 and 2, PXY, BRI1 and its homologs
BRL1, BRL3, and VH1 belong to a group of related subfamilies of LRR RLKs; subfamilies VII, X, XI, XIII (Fig 6). Many other RLKs within this group are involved in other aspects of patterning throughout development including stomata and pavement cell formation in epidermal cells (ERECTA), anther development (TOAD2, EXTRA SPOROGENOUS CELLS (EXS)/EMS, BAM1, BAM2), and abscission (HAESA). Other RLKs in this group are involved in additional biological processes including response to pathogens (PEP1 RECEPTOR 1 (PEPR1), PEPR2), endosperm development (HAIKU2), and cell wall elongation (FEI1, FEI2) (Canales et al. 2002; Hord et al. 2006; Luo et al. 2005; Mizuno et al. 2007; Shpak et al. 2004; Xu et al. 2008; Yamaguchi et al. 2010; Yamaguchi et al. 2006). This group of RLKs represents the best characterized families of RLKs, however only 29 of the 61 have a described function. With about half of the RLKs in this family still uncharacterized and well over half of the remaining RLKs in Arabidopsis uncharacterized, much is still unknown regarding the processes that RLKs regulate. With many of the RLKs described thus far regulating similar processes, two important questions remain; 1) what are the functions of the many RLKs that are still uncharacterized, and 2) do these RLKs function in parallel networks that converge on specific targets, such as the HD-ZIPs and the WUSCHEL like homeobox genes? While much is still unclear about how cells communicate with one another to regulate development, one conclusion that can be drawn is that social networks of RLKs coordinate many biological processes.
Fig 6 A phylogeny based on kinase domains for subfamilies VII, X, XI and XIII in *Arabidopsis thaliana*. This tree was adopted from a previous analysis performed by Shiu and Bleecker (2001).
CHAPTER 2: PRESENT STUDY

The leucine-rich repeat receptor-like kinases GASSHO1 and GASSHO2 are required for epidermal differentiation and root growth in Arabidopsis thaliana.

How cells communicate to regulate cell fate specification and coordinated growth is a key question in developmental biology. Receptor-like kinases (RLKs) are one of the largest gene families in Arabidopsis thaliana, which make these genes key candidates for regulating various intercellular signaling pathways. Here we show the role of two RLKs, GASSHO1 (GSO1) and GSO2, in regulating the cell fate identity of the epidermis and the root apical meristem (RAM). Using markers for epidermal cell identity and for RAM activity and specification, we demonstrate that gso1 gso2 mutants may show root growth arrest due to the decreased activity of the RAM and abnormal cell identity of cells in the RAM. We propose that GSO1 and GSO2 are required for specifying epidermal and RAM cell fate identity either through direct roles in both tissue, or that loss of both RLKs disrupts signaling events between the epidermis and the RAM required for development, that has been previously shown to be present.
XYLEM INTERMIXED WITH PHLOEM1, a leucine-rich repeat receptor-like kinase required for stem growth and vascular development in *Arabidopsis thaliana*

The receptor-like kinase (RLK) gene family is one of the largest gene families in *Arabidopsis thaliana*, however the functions of more than 50% of these RLKs are not yet known. This published manuscript provides the initial analysis for a previously uncharacterized RLK, we named XYLEM INTERMIXED WITH PHLOEM1 (XIP1). *xip1* mutants were shown to have significant stem growth defects compared to wild-type. Expression analysis of XIP1 indicated that XIP1 is expressed specifically within the vasculature throughout development. Using hand-cut sections and histo-chemical analyses, we determined that *xip1* mutants had significant abnormal secondary wall characteristics in the phloem region, characteristics that are typically found in the xylem or fiber tissues. These defects could be attributed to abnormal differentiation of phloem cells into xylem or fiber cells. In addition, we also demonstrate that *xip1* stems show aberrantly oriented cell divisions that lead to misalignment of the vascular cells, which could contribute to reduced height of the stems. We propose that XIP1 prevents ectopic lignifications of the phloem and is required to maintain the organization of the cell files in the stem vasculature.


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APPENDIX A:

THE LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASES GASSHO1 AND GASSHO2 ARE REQUIRED FOR EPIDERMAL DIFFERENTIATION AND ROOT GROWTH IN *ARABDIOPSIS THALIANA*

Anthony C. Bryan and Frans E Tax

This appendix contains a manuscript to be submitted for publication. The work presented in this manuscript was designed and carried out entirely on my own. Interpretation of results and discussion of the manuscript was assisted by Dr. Frans E Tax and the members of the Tax Lab.
The leucine-rich repeat receptor-like kinases GASSHO1 and GASSHO2 are required for epidermal differentiation and root growth in *Arabidopsis thaliana*.

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Abstract

The epidermal layer is important for coordinated growth in plants. Several receptor-like kinases (RLKs) are essential for maintenance of epidermal identity and for epidermal differentiation in adult organs. Additional RLKs are required to regulate cell fates in the root apical meristem (RAM), either through the perception of intercellular signals in the RAM, or non-cell autonomously through signaling between the epidermis and the RAM. Here we show that GASSHO1 (GSO1) and GSO2, two RLKs that together regulate epidermal formation in the embryo and seedling, are also essential for root development. Using markers for epidermal cell identity, we demonstrate that although epidermal identity of gso1 gso2 mutants appears normal during embryogenesis, seedling roots mis-express GLABRA2 (GL2), a marker for epidermal cell differentiation. In addition to the seedling lethal phenotype of gso1 gso2 double mutants, the morphological features of the RAM and the expression of the cell division marker CYCLIN B1;1 (CYCB1;1) indicate reduced RAM activity. These and further RAM specification defects in gso1 gso2 mutants could contribute to the growth arrest phenotype. In addition, the application of exogenous sucrose is able to rescue the growth arrest defect. Our results demonstrate that GSO1 and GSO2 are required together for epidermal differentiation and for the maintenance of meristem identity in the root, and that growth on sucrose can bypass the lethality of gso1 gso2 mutants.
Introduction

The ability to perceive signals in order to execute specific cell fates and to maintain meristematic cell populations is vital for continuous growth in plants. The shoot apical meristem (SAM) and root apical meristem (RAM), which are sources of the majority of cells in the adult plant, require extrinsic signals as well as internal factors in order to maintain their identity and activity. However, the proteins responsible for perceiving these signals are only now just beginning to be identified. Because of its nearly invariant organization (Dolan et al. 1993), the RAM is an excellent system in which to investigate the roles of signaling proteins that regulate the activity and identity of the meristem region.

The RAM is comprised of various cell types organized into longitudinal cell files originating from the meristematic initial cells surrounding the quiescent center (QC). In Arabidopsis, the QC is a group of 2-4 cells that maintain the activity of the meristem by inhibiting the differentiation of the immediately surrounding initial cells, which are stem cells (van den Berg et al. 1997). The identity of the QC is maintained by various signaling pathways that converge on the homeobox transcription factor WUSCHEL-LIKE HOMEBOX GENE5 (WOX5) (Matsuzaki et al. 2010; Sarkar et al. 2007; Stahl et al. 2009). Expression of WOX5, which is restricted to the QC, is regulated by the extracellular signaling peptide CLAVATA3/ENDOSPERM SURROUNDING REGION-LIKE40 (CLE40), a peptide related to CLAVATA3 (CLV3) that functions in maintaining the SAM (Haecker et al. 2004; Sarkar et al. 2007; Stahl et al. 2009). The secreted CLE40
peptide is likely perceived by *Arabidopsis* CRINKLY4 (ACR4), a receptor-like kinase (RLK) of the CRINKLY4 family (Stahl et al. 2009). Mutations in ACR4, which is expressed in the columella cells below the QC, result in the expanded expression of WOX5 and the formation of extra columella initials (De Smet et al. 2008; Stahl et al. 2009). These data suggest ACR4 perceives CLE40 in the columella initials and represses WOX5 expression, thereby restricting the size of the QC.

ACR4 was initially characterized based on its relation to the CRINKLY4 (CR4) gene in maize. *cr4* mutants have defects in the development of the aleurone layer of the seed endosperm and in the adult leaf epidermis (Becraft et al. 1996). Of the five *Arabidopsis* genes that are related to CR4, ACR4 was found to be most closely related to CR4. Mutations in ACR4 have similar defects as *cr4*, including crinkly leaf surfaces, fusions between organs, abnormal outer layer morphology of ovules and altered cuticle development (Gifford et al. 2003; Watanabe et al. 2004). During embryogenesis, ACR4 is expressed primarily in the epidermal layer (Tanaka et al. 2002). Although *acr4* mutants did not demonstrate any embryo defects, knock-down experiments using ACR4 antisense constructs resulted in embryos with morphological defects, suggesting that ACR4 and related RLKs are involved in embryo development (Tanaka et al. 2002). ACR4 and another RLK, ABNORMAL LEAF SHAPE2 (ALE2), phosphorylate each other *in vitro* and along with genetic evidence, suggests there two RLKs function together in a signaling pathway to maintain epidermal cell identity during the late globular-to-early heart stages of embryogenesis (Tanaka et al. 2007). Mutations in ACR4 or ALE2 together with mutations in the secreted protease ALE1 resulted in abnormal embryos with
loss of expression of several epidermal specific markers, including *Arabidopsis* MERISTEM LAYER1 (*AtML1*) and PROTODERM DETERMING FACTOR2 (PDF2) in the early heart stage (Tanaka et al. 2007). *AtML1* and PDF2 are two related HD-ZIP transcription factors required during embryogenesis to specify the epidermal layer (Abe et al. 2003). Loss of both of these transcription factors results in the epidermis resembling inner cell types in young seedlings. Therefore, ACR4 is required both to perceive signals for specifying and maintaining cell fates in the RAM and for maintaining the epidermal cell fate in embryos and adult development.

Several other RLKs have been found to play roles in the identity of the epidermis. Two paralogous Leucine-Rich Repeat (LRR) RLKs, RECEPTOR PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2) are required to maintain epidermal identity in early stages of embryo development (Nodine et al. 2007). All *rpk1 toad2* embryos and approximately half of the *rpk1 toad2/+* embryos arrest embryo development and lose expression of *ATML1* at the early globular stage. *rpk1 toad2* embryos also show an outer-to-inner cell fate transformation, with the epidermal layer expressing SHORTROOT, a marker for provascular cell identity. In addition to maintaining epidermal identity during embryogenesis, TOAD2, also known as RPK2, regulates SAM activity, through restricting the size of the stem cell domain (Kinoshita et al. 2010). Both ACR4 and TOAD2 exemplify the emerging theme that RLKs that function in the maintenance of epidermal fate during embryogenesis also function in maintaining the RAM or SAM.
RPK1-TOAD2 and ACR4-ALE2 are RLKs that maintain epidermal identity during early stages of embryo development. In later stages of seedling development, SCRAMBLED (SCM), an LRR RLK, is required for perceiving positional signals for directing the differentiation of epidermal cells into non-hair cells and hair cells in the root (Kwak et al. 2005). SCM is proposed to regulate position-dependent signaling between the epidermis and the inner cortex through its effects on downstream transcription factors responsible for the differentiation of hair and non-hair cells (Kwak and Schiefelbein 2007; 2008). Loss of SCM results in roots that mis-express specific epidermal markers including GLABRA2 (GL2), a non-hair specific gene, in hair cells.

Epidermal cell differentiation in roots is also regulated by additional signals including the plant hormone Brassinosteroids (BRs) (Kuppusamy et al. 2009). Perception of BR by the LRR RLK BRASSINOSTEROID INSENSITIVE1 (BRI1) regulates GL2 expression in the root. Loss of bri1 results in epidermal cells with ectopic expression of GL2 in cells that would normally become hair cells, a phenotype similar to that of scm.

Prior to the identification of the role of BRs in epidermal cell differentiation, BRs were known for their role in regulating cell expansion and cell division; defects in the perception or synthesis of BR results in a dwarfed mutant phenotype (Clouse et al. 1996; Li and Chory 1997; Nam and Li 2004; Szekeres et al. 1996). Synthesis of brassinolide (BL), the most active form of BR, strictly within the outer layer of the SAM is sufficient to drive growth (Savaldi-Goldstein et al. 2007). Restricting BRI1 expression to the epidermis is also sufficient to rescue the dwarf phenotype in a bri1 mutant.
background. These analyses were conducted by using the AtML1 promoter to drive the expression of BRI1, and genes involved in the synthesis of BL strictly in the epidermal layer in mutant backgrounds that lacked BRI1 and BR biosynthesis genes respectively (Savaldi-Goldstein et al. 2007). These studies demonstrate the importance of perception and signaling within the epidermis for controlling plant growth. Because plant cells are tightly associated, coordinated cell expansion and proliferation of cell layers is necessary, and would also be expected to occur via intercellular communication. However, how BRs and BRI1 convey signals signaling to inner layers is not known.

BRs and their perception in the epidermis also regulate meristematic activity in the RAM. The size of the RAM is reduced in both loss- and gain-of-function mutants in BR signaling genes, suggesting the balance of BRs is important for the regulation of meristem size (Gonzalez-Garcia et al. 2011). Analysis of bri1 mutants further demonstrated a decrease in mitotic activity in the RAM (Gonzalez-Garcia et al. 2011). Perception of BR in the epidermal layer, by driving BRI1 expression from the GL2 promoter, is sufficient to regulate RAM size and activity (Hacham et al. 2011). Additionally, BR signaling within the epidermis regulates QC and inner cell identity, as demonstrated by changes in cell specific markers for the QC and the stele (Hacham et al. 2011). Together these results substantiate not only the roles of BR and BRI1 in the maintenance and differentiation of the epidermal layer, but the importance of communication between cell layers to coordinate growth.
GASSHO1 (GSO1) and GSO2, two related LRR RLKs, are redundantly required for epidermal development and seedling viability (Tsuwamoto et al. 2008b). *gso1 gso2* double mutants have morphological defects in the epidermis, including abnormal permeability, abnormal cell divisions, and adherence of the embryo to the inner seed integument; these phenotypes are reminiscent of *cr4* mutants. Here we describe post-embryonic epidermal specification defects in *gso1 gso2* double mutants, and further demonstrate defects in specification of the root meristem. In addition, *gso1 gso2* growth arrest defects can be rescued by the exogenous application of sucrose.

**Materials and Methods**

Growth conditions and genetic analyses

The *gso1-1* (SALK_064029), *gso1-2* (SALK_043532), *gso2-1* (SALK_130637), and *gso2-2* (SALK_147249) alleles used in this analysis were identified in the SALK Insertion collection (Alonso et al 2003). The GL2:GUS (CS8851), CPC:GUS (CS6497), QC184:GUS, and QC46:GUS marker lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All lines were grown at 22 °C in a Conviron growth chamber with a 16 hr light/8 hr dark cycle. Seedlings were plated on 0.5x MS media and seeds stratified at 4°C for 48 hrs and grown with a 16hr light/8 hr dark cycle at 22 °C. Seedling roots were analyzed 6 days after germination (DAG). All plate assays used the same growth conditions as above, but also contained indicated concentrations of sucrose, glucose, 3-O-Methylglucose (3OMG), NaCl, or mannitol.
Growth measurements were taken at three and six DAG. To genotype the mutant alleles, three primer polymerase chain reactions were performed using Ex-Taq polymerase (TaKaRa). GSO1 F 5’ GTGAATGTAAAGTGACTAAGCC, GSO1 R 5’ TGACATATCATCAAGAGCATAC, GSO2 F 5’ TCTTTACCATGACTGTT, GSO2 R 5’ CTCTCATAAACATGCTTCG.

Cloning and marker line constructs

The *ArML1* promoter fusion construct was created by PCR amplification (using Prime Star polymerase) of the promoter of *ArML1* as previously described using primers *ATML1-T1*, *ATML1-T2* (Takada and Jurgens 2007) and cloning it into pCR8-TOPO using the TOPO cloning kit (Invitrogen). Using the LR Gateway system (Invitrogen), the *ArML1* promoter was subcloned into the pFYTAG vector. The YFP SCR promoter region was amplified using primers SCR F 5’ AAGGGATAGAGGAAGGGTACTA and SCR R 5’ GGAGATTGAAGGGTTATGG. GSO1 and GSO2 promoter fusions were creating using Prime Star polymerase to amplify putative promoter sequences. The amplified products were each cloned into pCR2.1 and digested with restriction enzymes respectively and ligated into digested pBI101:GUS destination vector. The CYCB1;1 promoter and destruction box (Colon-Carmona et al. 1999) were amplified using Prime Star polymerase and cloned into pCR2.1 (Invitrogen). CYCB1 F 5’ TTTAAAGTTCCTCGAGATGACTAAATT, CYCB1 R 5’ TATATCAGAAGACTTGACGTTTCT. Using restriction enzymes, the product was
ligated into digested pBI101:GUS. All constructs were transformed into Col-0 and/or gso2-1 plants using Agrobacterium-mediated transformation and crossed with gso1-2 to create double mutants where required.

Microscopy and staining

Images of seedlings were taken using a Canon Power Shot SX110 digital camera mounted on a Leica dissecting microscope. Six DAG seedlings containing the GL2, CPC and CYCB1;1 marker lines and GSO1 and GSO2 promoter fusions were fixed in 80% acetone and subsequently stained using a solution containing X-Gluc (1mM X-Gluc (Gold Bio Technology)) dissolved in DMSO, 10mM EDTA, 2mM potassium ferrocyanide, 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 0.1% Triton X-100, 100mM NaH2PO4, pH 7.0) for 2-24 hours and mounted in ddH2O. Images were taken using a Zeiss Axioplan microscope equipped with a digital camera and QCapture Pro 5.0 imaging software. Seedlings with the QC184 and QC46 marker lines were stained with X-Gluc for 2-6 hours and mounted in 6:1 Chloralhydrate:Lugol solution for 15 min and imaged using a Zeiss Axioplan microscope. As a control, wild-type and mutant lines with no marker lines were mounted in 6:1 Chloralhydrate:Lugol for 15 min and imaged using a Zeiss Axioplan microscope. Root lengths were measured 6 DAG using Image J software. Embryos carrying the AtML1 marker line were dissected from siliques and six DAG roots carrying the SCR marker line were stained with 2μM
FM4-64 and 10μM propidium iodine respectively for 5 min each, and mounted in ddH2O and imaged using a Zeiss metaconfocal with LSM510 imaging software.

Statistical analysis

Meristem lengths were determined based on cytoplasmic density. Col: n=28, mean=798.24cm ±12.97, gso1-1 gso2-1: n=31, mean=332.46cm standard deviation (SD)±52.79, gso1-2 gso2-1: n=40, mean=323.37cm SD±49.94. Student’s T-test between Col and gso1-1 gso2-1 p=1.28 x10^-18, and between Col and gso1-2 gso2-1 p=2.22x10^-18. CYCB1;1 expression regions were calculated based on measurements of GUS expressing regions. Col: n=40, mean=689.3cm SD±90.3. gso1-2 gso2-1: n=33, mean=318.1cm SD±113.3. Student’s T-test shows p=2.1x10^-22. The number of columella layers containing starch granules were determined for Col (n=27), gso1-1 gso2-1 (n=30) and gso1-2 gso2-1 (n=42) and using the Chi square test ( p=1x10^-17). gso1-2 gso2-1 mutant roots (35/35) showed expanded expression of QC184 outside the QC (Col (0/36), Chi square test p=3.57x10^-17). gso1-2 gso2-1 mutants roots (60/85) showed scattered expression of GL2 compared to Col (6/44), Chi square p= 8.52x10^-10.
Results

gso1 gso2 double mutants arrest root growth

GSO1 and GSO2 encode two paralogous LRR RLKs that are redundantly required for seedling growth and epidermal surface formation (Tsuwamoto et al. 2008). Although no phenotype was observed for single mutants, mutations in both RLKs together resulted in seedling lethality. Additional defects found in these mutants were primarily related to epidermal function, including extra cell divisions in the epidermal layer of hypocotyls and cotyledons, as well as a highly permeable epidermal layer, which was demonstrated by penetration of toluidine blue into aerial tissues (Tsuwamoto et al 2008). To analyze additional defects caused by mutations in these redundantly acting RLKs, we isolated additional exonic T-DNA insertion alleles for both GSO1 and GSO2 from the SALK T-DNA collection (Alonso et al 2003). gso1-2 (SALK_147249) is located in the LRR region of GSO1 and gso2-2 (SALK_043539) lies in the extracellular region of GSO2 (Fig 1). These additional alleles, along with those previously described, were analyzed to confirm the seedling lethal phenotype.

Consistent with previous results, combinations of gso1-2 gso2-1 and gso1-1 gso2-2 also showed a seedling arrest phenotype (Fig 1b, c). In addition, all of the double mutant combinations showed a reduced root growth phenotype, not previously reported (Fig 1b, c, d). At three days after germination (DAG), double mutant combinations showed significant differences in root growth compared to wild type plants. Although some mutant roots did show some elongation, the vast majority of seedlings completely
arrested growth by 6 DAG (82%, 219 seedling). Similar growth arrest was observed in
the aerial portion of the plant 6 DAG, consistent with previously published results (Fig 1c)
(Tsuwamoto et al. 2008).

*gsol gso2* mutants mis-specify root epidermal cell fates.

Although defects in the aerial epidermal layer have been described for *gsol-l*
*gso2-l* mutants (Tsuwamoto et al 2008), the epidermal identity in these mutant seedlings
has not been examined. To determine if the epidermis is specified correctly in *gsol gso2*
double mutants, we analyzed the expression of *AtML1* using the YELLOW
FLUORESCENT PROTEIN (YFP) coding sequence with an added H2B nuclear
localization signal, downstream of the *AtML1* promoter (Takada and Jurgens 2007).
Since initial defects in *gsol gso2* mutants are detected by the heart stage in
embryogenesis, we first analyzed the expression of *AtML1* at that stage. In *gsol-2 gso2-
1* heart-staged embryos, *AtML1* is expressed in the epidermis similar to wild-type
embryos, indicating at least partial specification of the epidermis (Fig 2a, d).

To address if the epidermal layer is specified correctly in roots, the expression
patterns of markers specific for epidermal cell differentiation were analyzed. GL2,
described above, and CAPRICE (CPC), encoding a single repeat MYB protein, are
expressed in non-hair cells (N-position) (Masucci et al. 1996; Wada et al. 2002; Wada et
al. 1997). Hair (H-position) and non-hair cell types form longitudinal cell files in the root
with their differentiation determined by positional cues from inner cortical cells. Cells in
the N-position are specified due to their position overlapping a single subjacent cortex cell, whereas cells in the H-position overlap two subjacent cortex cells (Dolan et al. 1993; Galway et al. 1994). GL2 expression in epidermal cells indicates non-hair cell fates, whereas in a lateral inhibition mechanism, CPC protein moves to adjacent epidermal cells and represses GL2 expression which results in hair-cell identity. To visualize the expression patterns of GL2 and CPC, plants carrying the GL2 or CPC promoter fusions to the reporter gene β-glucuronidase (GUS) were crossed into gso1-2 gso2-1 double mutants. The GL2 and CPC marker lines in wild-type plants are detected as continuous blue cell files (Fig 2b,c). gso1-2 gso2-1 double mutant seedlings show a scattered expression of GL2 in the roots, indicative of mis-specified root epidermal cells (Fig 2d). Analysis of CPC in gso1-2 gso2-1 mutant seedlings showed a similar scattered patterning of expression (Fig 2f). The abnormal patterning of both reporter lines suggests that epidermal differentiation is disrupted, leading to aberrant patterning of epidermal cell fates. These results indicate that, in addition to regulating epidermal cell divisions and permeability of the aerial epidermis, GSO1 and GSO2 are required for correct patterning of epidermal cell fates in roots.

Root apical meristem architecture is disrupted in gso1 gso2 seedlings

The aberrant differentiation patterns in the epidermis of gso1 gso2 roots do not explain why these double mutants undergo growth arrest in their roots, as scm mutants with similar phenotypes do not arrest (Kwak et al. 2005). We therefore wanted to identify
the cause of the root growth arrest and determine if other cell types in the root also show specification or patterning defects. We analyzed the overall morphology of the RAM using Nomarski optics and analyzed markers for RAM activity and cell identity of the QC and inner cells in gso1 gso2 mutant roots. Comparing wild-type to gso1-2 gso2-1 roots, we initially observed a difference in the overall size of the RAM. Meristem cells in the RAM are characterized by rapidly dividing cells with dense cytoplasm (Fig 3a). gso1-1 gso2-1 and gso1-2 gso2-1 mutants had a significantly reduced meristematic regions (332 μm ±53) and (323 μm ±50) respectively, compared to wild-type roots (798 μm ±128) (p=0) (Fig 3a, b).

The meristematic region in the RAM can also be assayed using a marker for cell division. We fused the promoter and the destruction-box motif of CYCLIN B1;1 (CYCB1) to the GUS reporter and transformed the reporter into wild-type and gso1-2 gso2-1 plants. We measured the region of the roots that expressed the CYCB1;1:GUS marker, to determine the actively dividing zone (Colon-Carmona et al. 1999). The average region expressing the CYCB1;1 marker in wild-type roots was 689 μm ±90 compared to only 318 μm ±113 in gso1-2 gso2-1 roots (p=0) (Fig 3c, d). The length of region of the root expressing the CYCB1 reporter in the double mutant is similar the length of the meristematic region, confirming that the shorter visible meristematic region in gso1 gso2 mutants indicates a reduced activity of the meristematic region.

The morphology of the Arabidopsis root meristem is nearly invariant, with cells organized in longitudinal files originating from initial cells in the RAM. The QC is
positioned at the apex of the RAM and signals to surrounding initial cells to maintain an undifferentiated state. Starch granules accumulate in differentiated columella cells in the root tip below the QC and are absent in columella initials, just beneath the QC. To determine if $gso1$ $gso2$ mutants have defects in columella cell differentiation, wild-type and $gso1$ $gso2$ seedlings were treated with Lugol’s solution, which stains starch granules black, and roots were subsequently cleared to examine their morphology. Wild-type root tips show staining of starch granules in the differentiated columella cells, and no starch granules in the columella initials lying just below the QC (Fig 3e). However, $gso1$ $gso2$ roots stained with Lugol’s solution show a reduced number of columella cells that stain for starch granules (Fig 3e). While wild-type roots have a clear organization of columella cells arranged in cell files, $gso1$ $gso2$ mutants do not have the same alignment of columella cells. $gso1$ $gso2$ roots show the same number of initials between the differentiated columella and QC, but have a reduced number of columella layers or cell layers with starch accumulation (Fig 3g).

To determine if the specification of the QC in $gso1$ $gso2$ seedlings is defective, markers for QC identity were assayed. The identity of the QC can be visualized using QC184, a GUS expressing marker that is specifically expressed in the QC (Sabatini et al. 2003). QC184 expression in wild-type plants shows GUS staining in the two visible QC cells (Fig 4a). In contrast to wild-type plants, $gso1$ $gso2$ roots expressed QC184 in the columella cells as well as in the QC (Fig 4e). 36/36 mutant seedlings show an expansion of the QC184 marker compared to 0/35 wild-type plants (chi square $p=0$). The QC184 expression in the double mutants is coinciding with columella cells that are differentiated,
as determined by the presence of starch granules (Fig 3e). However, an additional marker for QC identity, QC46, was assayed and showed no difference between wild-type roots and *gso1 gso2* roots (Fig 4b, f).

We also used the marker WOX5, which is specifically expressed in the QC, to determine the cell fate of the QC and surrounding initials (Haecker et al. 2004; Sarkar et al. 2007; Stahl et al. 2009). The WOX5 promoter was fused to YFP with an added nuclear localization signal to visualize the expression of WOX5 in wild-type and *gso1-2 gso2-1* seedlings. *gso1 gso2* mutant roots showed expression in the columella initials along with expression in the QC, indicating an expanded expression region of WOX5 compared to the two visible cells expressing WOX5 in wild-type plants (Fig 4c, g). This is consistent with the expanded expression of QC184, as QC184 expression has been shown to be dependent on WOX5 (Sarkar et al. 2007).

In order to determine if inner cell layers also show abnormal patterning defects, we analyzed the expression of SCARECROW (SCR), a transcription factor specifically expressed in the endodermis and the QC in the RAM. We used a construct with the promoter region of SCR driving the expression of a fusion protein with a nuclear localization signal from H2B in frame with the reporter gene YFP, and transformed this construct into wild-type and *gso1 gso2* mutant plants. In wild-type plants, SCR expression was restricted to endodermal cells, including endodermal initials and the QC as determined through medial optic sections (Fig 4d). *gso1 gso2* mutants, however, showed an altered expression pattern of SCR (Fig 4h). SCR is typically expressed in
endodermal cells of gso1 gso2 roots similar to wild-type, but SCR expression is found in younger endodermal cells and the QC, but absent from mature endodermal cells.

The RAM defects seen in gso1 gso2 seedlings could be attributed to indirect effects caused by loss of epidermal patterning or by loss of function of these RLKs within the RAM. Previous analysis of GSO1 and GSO2 indicated that GSO1 but not GSO2 is expressed in roots, based on RT-PCR and promoter-GUS analysis (Tsuwamoto et al. 2008b). Using a similar promoter sequence to drive the expression of GUS, we further refined the expression of GSO1 and GSO2 in roots. We determined that GSO1 is expressed throughout the mature region of the root, with expression in inner cells, and expression in the RAM (Fig 4i). Extending the length of staining for up to 24 hours in several samples resulted in visible expression of GSO1 in the QC of the RAM (Fig 4j, arrowhead). In support of our data, GSO1 was previously determined to be expressed in the QC based on cell sorting and microarray analysis of QC expressed genes (Nawy et al. 2005). We did not detect GSO2 expression in the QC, and based on the QC microarray analysis, GSO2 expression is significantly lower compared to GSO1 in the QC. In addition, GSO2 expression was mostly detected in aerial tissues, however, at very low levels GSO2 expression was detected in the root, in inner cells and in the epidermis of mature cells (Fig 4k, arrow).

**Exogenous application of sucrose rescues root growth arrest**

We hypothesize that defects in either the epidermis or the RAM in gso1 gso2 mutants cause the arrest of cell division and growth in surrounding cells. To see if we
could bypass the growth arrest phenotype, \textit{gso1 gso2} mutant seedlings were grown in the presence of exogenous sucrose and glucose. Wild-type and \textit{gso1 gso2} double mutant root elongation was measured in the presence of 1\% sucrose and 1\% glucose, and both showed similar rates of root growth (Fig 5a, b), indicating the application of exogenous sucrose or glucose was able to rescue the growth arrest defect. Additionally, the aerial portion of the plant was also able to grow producing a fertile adult plant. However, the application of a non-metabolizable glucose analog, 3-O-Methylglucose (3OMG), had no effect on root growth of \textit{gso1 gso2} mutants (Fig 5b). This indicates the metabolism of glucose and not the activation of sugar signaling pathways is required to rescue the growth defect. The ability to rescue the root growth defects was also not due to changes in osmotic conditions, as the application of NaCl or mannitol did not increase root growth (Table 1). The exogenous application of sucrose may have two effects, 1) sucrose may rescue the patterning defects caused by loss of both \textit{gso1} and \textit{gso2}, or 2) sucrose may activate cell proliferation to bypass the growth arrest defects of \textit{gso1 gso2} seedlings. 

In order to address whether exogenous sugar rescues the patterning defects in \textit{gso1 gso2} seedlings, we first assayed for the ability of sucrose to rescue the TB defect that was originally reported (Tsuwamoto et al. 2008b). Wild-type and \textit{gso1 gso2} seedlings germinated on medium containing sucrose were treated with toluidine blue. Mutant seedlings stained purple, indicating the permeability defect in \textit{gso1 gso2} seedlings was not rescued (Fig 5c). We further analyzed the effects of exogenous sucrose on the expression of the QC184 marker, which had expanded expression in \textit{gso1 gso2} seedlings. Growth of wild-type plants with the QC184 marker showed no change in expression,
consistent with sucrose having no effect on patterning in wild-type seedlings (Fig 5d).

Analysis of QC184 in \textit{gso1-2 gso2-1} seedlings in the presence of exogenous sucrose did not show normal expression in the QC, indicating sucrose does not rescue the patterning defects in \textit{gso1 gso2} seedlings.

In \textit{gso1 gso2} seedlings grown in the presence of sucrose, QC184 expression was not restricted to the position of the presumptive QC as in wild-type plants, but was ectopically expressed in cells neighboring this location (Fig 5d, e). However in the presence of sucrose, \textit{gso1 gso2} mutants did not have the same expanded expression of QC184 as seen without sucrose (compare to Fig 4e), indicating an effect of sucrose on the organization of the RAM in \textit{gso1 gso2} mutants. Additionally, the ectopic expression of QC184 in sucrose-treated \textit{gso1 gso2} mutants was not always located in the same region. Sucrose did not restore the epidermal permeability defects of \textit{gso1 gso2} seedlings, although it did have an effect on the identity of the QC.

Although exogenous sucrose was able to restore growth of \textit{gso1 gso2} seedlings, additional defects were uncovered while analyzing \textit{gso1 gso2} double mutants grown in the presence of sucrose. We previously used Lugol’s to stain for starch accumulation in the columella cells in the root tip. Staining \textit{gso1 gso2} seedlings grown on sucrose with Lugol’s solution also demonstrated the abnormal presence of starch granules in the cotyledons and hypocotyls of mutant seedlings compared to wild-type seedlings (Fig 5f). The presence of starch granules in the mutant seedlings could be caused by the inability to utilize the granules formed during embryogenesis or due to the formation of new starch
granules in the seedling after germination. The large accumulation of starch granules was not observed in \textit{gso1 gso2} seedlings grown without sucrose indicating that the starch granules likely accumulated after germination as an effect of growth in the presence of exogenous sucrose. In addition, the aerial tissue of \textit{gso1 gso2} seedlings sometimes showed aberrant growth, including fusions of the stem to leaves and abnormal leaf shapes; however the mutants were able to continue to grow and produce a fertile adult plant. These results indicate that sucrose allows the \textit{gso1 gso2} seedlings to grow, but does not restore patterning defects caused by loss of these two genes.

\textbf{Discussion}

\textbf{GSO1 and GSO2 regulate epidermal differentiation in roots}

In addition to the defects in epidermal cell surface formation previously described, we have shown that GSO1 and GSO2 are redundantly required for specification of epidermal and sub-epidermal cell fates in \textit{Arabidopsis} roots. Although \textit{gso1 gso2} mutant embryos show abnormal adherence to the inner seed coat, the epidermis is at least partially specified normally, as determined by the expression of \textit{AtML1}. However, the post-embryonic root epidermal layer in these double mutants does have specification defects. Cell files in \textit{gso1 gso2} mutant roots that should be specified as hair cells express markers for non-hair cell fates. The scattered expression of non-hair cell markers in the root epidermis of \textit{gso1 gso2} mutants could be due to a role of GSO1 and GSO2 in regulating the perception of position-dependent information, similar or in parallel to SCM.
SCM, although expressed throughout the epidermis, preferentially accumulates in hair cells where it represses the MYB transcription factor WEREWOLF (WER), a positive regulator of GL2 expression (Kwak and Schiefelbein 2007; 2008). The loss of SCM leads to ectopic WER activation of GL2 in hair cells. In gso1 gso2 mutants, only the identity of hair cells appears to be disrupted, as cell files expressing GL2 are still present. Therefore, GSO1 and GSO2 likely regulate the identity of root hair cell identities in either a similar or parallel pathway to SCM. Alternatively, GSO1 and GSO2 could also function within inner layers to promote signals from inner cells to the epidermis to regulate hair and non-hair cell specification, as GSO1 and GSO2 are expressed in inner cell layers.

The mis-specification of epidermal cell identities in gso1 gso2 and scm mutants is also similar to bri1 mutants. However, BRI1 and SCM are proposed to work in separate pathways regulating hair cell fate (Kuppusamy et al. 2009). Application of BR increases the expression of WER, and bri1 scm mutants phenotypes appear mostly additive (Kuppusamy et al. 2009). As SCM likely acts in epidermal cells spanning two cortical cells, BRI1 is thought to function in epidermal cells overlying one cortical cell where it induces WER expression. How GSO1 and GSO2 fit into this model is still unclear. In addition, gso1 gso2 mutants have a decrease in cell elongation in epidermal cells of cotyledons and hypocotyls, as previously described (Tsuwamoto et al. 2008b). Instead of regulating the direct positional cues for hair cell and non-hair cell formation, GSO1 and GSO2 could regulate cell division or cell elongation in the epidermis. Defects in either of these processes may cause a disruption in the alignment of epidermal cells with cortex
cells. The irregular placement of epidermal cells over cortical cells would result in altered positional cues disrupting the hair cell or non-hair cell files.

**Roles of GSO1 and GSO2 in regulating RAM activity and identity.**

It was previously hypothesized that the seedling lethality of gso1 gso2 mutants was caused by dehydration due to defects in cell surface formation. Our analysis of root growth and RAM activity has identified cell proliferation and specification defects that also likely contribute to root growth arrest. In addition to the similarities in epidermal specification defects, gso1 gso2 and bri1 mutants have reduced root growth. Similar to bri1, gso1 gso2 roots also have reduced RAM activity, based on the size of the meristematic region. Restricting expression of BRI1 to the epidermis is sufficient to rescue the RAM defects in bri1 mutants, showing the importance of epidermal function regulating inner cell fates.

The spatial requirements for GSO1 and GSO2 functions are as yet unknown, but it is possible that GSO1 and GSO2 may function in the epidermis in a similar or a parallel pathway to BRI1 to regulate RAM activity. Based on our promoter fusion analysis, GSO1 is expressed in multiple root cell layers in mature cells and in the QC, however GSO2 expression was detected in the epidermis of mature cells but absent in the QC. Alternatively, GSO1 and GSO2 may function in the RAM to directly regulate QC activity and specification, as our promoter fusion analysis and previous microarray analysis detected some expression of GSO1 in the QC (Nawy et al. 2005). GSO1 and GSO2 may
also participate in separate signaling events between cell layers, including signaling from the epidermis to the QC or from the QC to the epidermis. The RAM defects in *gso1 gso2* seedlings may be attributed to non-cell autonomous effects from defects in epidermal development, similar to *bri1*, or be due to the direct loss of function of GSO1 and GSO2 in the RAM. Further analysis of which cells require GSO1 and GSO2 for these functions will answer this question.

The mutant phenotypes of *gso1 gso2* seedlings also resemble phenotypes of mutants in another RLK, ACR4. ACR4 regulates formative cell divisions in the root columella, perceives the CLE40 peptide and represses WOX5 activity in the root tip (De Smet et al. 2008; Stahl et al. 2009). ACR4 likely perceives the secreted CLE40 peptide and signals to repress WOX5 expression in the columella to restrict stem cell fate and permit differentiation. Application of CLE40p to wild-type roots causes a shift of WOX5 expression from the QC to cells just above the QC, and also causes columella initials to accumulate starch granules, an indication of differentiation (Stahl et al. 2009). This evidence suggests signals from the columella are acting from beneath the QC to maintain the position of the QC.

GSO1 is expressed predominantly in the region above the RAM, and exhibits low levels of expression in the QC, and loss of both GSO1 and GSO2 together results in the expansion of QC markers to the columella cells. In addition, loss of starch granules in the columella cells in *gso1 gso2* roots suggests the columella cells are not maintaining columella identity or cell divisions of columella initial cells have arrested. GSO1 and
GSO2 may be required to help maintain the position of the QC similar to ACR4, through responding to signals from above the QC. In this model, opposing signaling from both below and above the QC is required to help maintain the position and identity of the QC.

We also see a loss of SCR expression in the region of cell elongation above the RAM. This suggests GSO1 and GSO2 function to maintain the cell identities of more mature cells. Previous studies have shown that mature cells signal to initial cells to guide their differentiation (van den Berg et al. 1995). Loss of identity of mature epidermal and endodermal cells may disrupt signaling to young differentiating cells and the RAM, leading to specification defects. These results suggest two models for the regulation of RAM activity by GSO1 and GSO2; 1) GSO1 and GSO2 act above the QC in an analogous pathway as ACR4 to position the QC or 2) GSO1 and GSO2 are required for maintaining mature root cell identities and loss of these cell fates results in aberrant non-cell autonomous signaling, disrupting RAM activity. In both models, signaling from above the QC would be disrupted, thus affecting the activity and identity of the QC.

Although the direct impact of loss of *acr4* on QC specification markers is not known, *gso1 gso2* and *acr4* have an effect on the differentiation of stem cells in the root tip, likely through restricting stem cell identity. However, since some QC markers, like QC46, are not affected, other signaling pathways may act in parallel with GSO1 and GSO2 to maintain the position of the QC. The identity of the genes whose expression patterns are represented by the QC184 and QC46 markers, however, remains unknown as these markers were identified as enhancer-trap lines with specific expression in the QC.
A group of RLKs that regulate both epidermal specification and meristem maintenance

ACR4, along with ALE2, is also required to maintain epidermal identity during embryogenesis and adult development. ACR4 is expressed in the columella and columella initials, but its expression is restricted to the epidermal layer during embryogenesis and the L1 layer in the SAM (Gifford et al. 2003; Tanaka et al. 2002). ACR4 and ALE2, which phosphorylate each other in vitro, are required together with the secreted protease ALE1 to maintain epidermal identity during embryo development (Tanaka et al. 2007). Defects during embryogenesis in gso1 gso2 mutants were previously reported based on embryo morphology. It is possible GSO1 and GSO2 are required for regulating other epidermal processes, such as cell proliferation or cuticle formation during embryogenesis. Mutations in ACR4 or ALE2 alone do not show a loss of expression of epidermal markers during embryogenesis, similar to gso1 gso2 mutants. It remains possible that GSO1 and GSO2 may act in a similar or parallel pathway to ACR4 and ALE2. Further genetic analyses of these RLKs should help answer how GSO1 and GSO2 function during embryogenesis. The likely functions of GSO1 and GSO2 in epidermal development during embryogenesis indicate a common group of RLKs is required for epidermal development during embryogenesis and in adult development, and in regulating RAM activity.

Two additional RLKs that are required for epidermal development in embryogenesis are RPK1 and TOAD2. These two RLKs are required for early
maintenance of epidermal identity during the globular stage of embryogenesis, with loss of both RLKs resulting in loss of the epidermal specific marker AtML1 (Nodine et al. 2007). TOAD2, also known as RPK2, has recently been shown to act in the SAM to perceive signals from CLV3 in order to restrict stem cell identity (Kinoshita et al. 2010). In our studies, we show the requirement of GSO1 and GSO2 to restrict stem cell identity in the RAM is analogous to a proposed role of TOAD2/RPK2 in the SAM and ACR4 in the RAM. It is possible GSO1 and GSO2 may regulate similar pathways as TOAD2/RPK2 in epidermal and RAM/SAM development. However, the signals that GSO1 and GSO2 perceive are still unknown. Both the ACR4 and the TOAD2/RPK2 signaling pathways likely involve CLE peptides. gso1 gso2 mutants also resemble the phenotypes of CLE peptide treated roots, including root growth arrest and a reduced meristematic region (Fiers et al. 2005). It is possible that GSO1 and GSO2 perceive a CLE peptide ligand similar to the ACR4 and TOAD2 signaling pathways.

TOAD2/RPK2 also has a role in anther development (Mizuno et al. 2007). Although no known role in anther development has been described for GSO1 and GSO2, both are expressed in anthers (Tsuwamoto et al. 2008b). Therefore, the pathways in which RPK1, TOAD2, GSO1, and GSO2 function may be utilized in other aspects of development. Thus far, it appears that RPK1-TOAD2, ACR4-ALE2, SCM, BRI1, GSO1-GSO2 represent a network of RLKs that act in various stages of epidermal development either to maintain the identity of the epidermis or for spatial-temporal differentiation of the epidermal layer. Determining the downstream signaling pathways,
as well as the genetic interactions among these RLKs will help resolve how this network of RLKs together regulates epidermal development.

**Bypassing defects through activation of cell cycle**

The *gso1 gso2* seedling lethal phenotype was previously shown to be rescued when seedlings were grown under high humidity conditions. In our analysis, seedlings grown on sealed plates, which should create high humidity, do not continue to grow. One difference between these two experiments is the temperature in which the mutants were grown. It is possible that growth under higher temperature, as was used in the previous study, in addition to high humidity conditions, may permit the *gso1 gso2* mutant seedlings to survive. However, our ability to rescue the seedling lethal phenotype with the application of exogenous sucrose does suggest an alternative process that can rescue the *gso1 gso2* double mutants.

A similar seedling lethality, which can also be rescued with growth on exogenous sucrose, was identified for mutations in STIMPY (STIP)/WOX9, a related gene to WUS and WOX4. The phenotypes of *stip* loss of function mutants resemble *gso1 gso2* mutants, including smaller cotyledons and smaller embryos, reduced expression of cell cycle markers in the RAM, arrested seedling growth and the rescue of the growth arrest with the application of sucrose (Tsuwamoto et al. 2008b; Wu et al. 2005). STIP also has overlapping expression patterns with GSO1 in the maturation region in the root and in the epidermis. STIP is a positive regulator of WUS and functions in both the shoot and the
root, as well as during embryogenesis. It has also recently been shown that sucrose is able to rescue *stip* mutants, and that after such treatments, reactivating the cell cycle through promotion of the G2 to M transition (Skylar et al. 2011). Sucrose may have a similar affect on *gso1 gso2* seedlings, allowing reactivation of the cell cycle, thereby permitting growth. Based on the mutant phenotypes, GSO1 and GSO2 could function upstream of the STIP pathway. Further analyses of the downstream signaling components of GSO1 and GSO2 will determine if GSO1-GSO2 and STIP comprise a RLK-WOX signaling pathway similar to those identified in the SAM and RAM.
References


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Tsuwamoto R, Fukushima H, Takahata Y (2008) GASSHO1 and GASSHO2 encoding a putative leucine-rich repeat transmembrane-type receptor kinase are essential for the normal development of the epidermal surface in Arabidopsis embryos. The Plant Journal 54: 30-42


Figures

Fig 1 Mutant alleles in GSO1 and GSO2 and reduction in root growth in gso1 gso2 mutants. a) Gene models of GSO1 (top) and GSO2 (bottom) indicating positions of insertion mutations (arrow heads). b) From left to right, seedling of Col, gso1-1 gso2-1, gso1-2 gso2-1, gso1-2 gso2-2 6 DAG. c) Close up image showing seedling size and growth of Col (left) and gso1-2 gso2-1(right) 6 DAG. d) Measurements of Col, gso1-2, gso2-1 and gso1-2 gso2-1 roots.

Fig 2 gso1 gso2 mutants have defects in epidermal differentiation in roots. a-c) Col and d-f) gso1-2 gso2-1 seedlings. a and d) AtML1:H2B:YFP marker expression in heart stage embryos stained with propidium iodide. b and e) GL2:GUS promoter fusion expression in roots 6 DAG. c and f) CPC:GUS promoter fusion expression in roots 6 DAG.

Fig 3 gso1 gso2 roots have smaller root meristem regions and fewer columella cells filled with starch granules. a and b) Col (a) and gso1-2 gso2-1 (b) roots stained with Chloralhydrate:Lugol’s solution 6 DAG, with cytoplasmicly dense zone marked by the yellow opaque region at root tip, indicated by brackets. c and d) CYCB1;1:GUS expression in Col (c) and gso1-2 gso2-1 (d) seedlings 6 DAG. e and f) starch granule accumulation in columella cells of Col (e) and gso1-2 gso2-1(f) roots 6 DAG. The blue arrowhead represents the location of QC and the yellow arrowhead indicates position of
the columella initials. g) graphical representation of the number of starch granule-accumulating columella cells in Col and gso1 gso2 mutants 6 DAG.

Fig 4 RAM specific defects in gso1-2 gso2-1 roots. a-d) Col and e-h) gso1-2 gso2-1 seedlings 6 DAG. a and e) GUS stained roots carrying the QC184 marker line mounted in Chloralhydrate:Lugol’s solution. b and f) GUS stained roots carrying the QC46 marker line mounted in Chloralhydrate:Lugol’s solution 6 DAG. c and g) Medial optical section of seedlings expressing the WOX5 marker in roots stained with propidium iodide 6 DAG. d and h) Medial optical sections of seedlings expressing the SCR marker in roots stained with propidium iodide 6 DAG. i) GUS expression in roots of plants expressing the GSO1 promoter fusion showing high expression in the mature region of the root (i) and in QC (arrowhead). j) GUS expression in the epidermal layer (arrow) can be detected in GSO2 promoter fusion lines, and within inner cell layers.

Fig 5 Sucrose can rescue the growth arrest defect but not the QC specification defects in gso1 gso2 mutants. A) Addition of 1% sucrose can rescue the root growth arrest defect. From left to right Col, gso1-2 gso2-1, gso2-1, gso1-2 8 DAG. b) 1% sucrose and 1% glucose, but not 1% 3-O-methylglucose (3MOG), can restore root growth in gso1-2 gso2-1 mutants. c) From left to right Col, gso1-2, gso2-1, gso1-2 gso2-1 seedlings grown on 1% sucrose and treated with toluidine blue. gso1-2 gso2-1 seedlings stain blue due to penetration of toluidine blue. d and e) QC184 expression of Col (d) and gso1-2 gso2-1 (e)
seedlings grown in 1% sucrose and mounted in Chloralhydrate:Lugol’s solution. Col sample shows the typical location of the QC as shown by expression of QC184, however gso1 gso2 mutants show abnormal location of QC184-expressing cells. Cotyledons and hypocotyls of wild-type (left) and gso1-2 gso2-1 (left) seedlings stained with Lugol’s solution indicating accumulation of starch granules (black spots) (f).
Fig 3
Fig 4
Fig 5
Table 1: *gso1;2* root growth rescue is not due to changes in osmotic conditions.

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APPENDIX B:

XYLEM INTERMIXED WITH PHLOEM1, A LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE REQUIRED FOR STEM GROWTH AND VASCULAR DEVELOPMENT IN ARABIDOPSIS THALIANA

Anthony C. Bryan, Adam Obaidi, Michael Wierzba, Frans E. Tax

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This appendix contains the reproduction of the manuscript in which I am the primary author. Some initial characterization of this gene was performed by Adam Obaidi under my guidance. The majority of the rest of the experiments were designed and executed on my own. Interpretation of results and discussion of the manuscript were assisted by Frans E Tax and members of the Tax Lab. I am the primary author for this publication.
XYLEM INTERMIXED WITH PHLOEM1, a leucine-rich repeat receptor-like kinase required for stem growth and vascular development in Arabidopsis thaliana

Anthony C. Bryan · Adam Obaidi · Michael Wierzba · Frans E. Tax

Abstract The regulation of cell specification in plants is particularly important in vascular development. The vascular system is comprised two differentiated tissue types, the xylem and phloem, which form conductive elements for the transport of water, nutrients and signaling molecules. A meristematic layer, the procambium, is located between these two differentiated cell types and divides to initiate vascular growth. We report the identification of a receptor-like kinase (RLK) that is expressed in the vasculature. Histochemical analyses of mutants in this kinase display an aberrant accumulation of highly lignified cells, typical of xylem or fiber cells, within the phloem. In addition, phloem cells are sometimes located adjacent to xylem cells in these mutants. We, therefore, named this RLK XYLEM INTERMIXED WITH PHLOEM 1 (XIP1). Analyses of longitudinal profiles of xip1 mutant stems show malformed cell files, indicating defects in oriented cell divisions or cell morphology. We propose that XIP1 prevents ectopic lignification in phloem cells and is necessary to maintain the organization of cell files or cell morphology in conductive elements.

Keywords LRR RLKs · Phloem · Procambium · Vasculature · Xylem

Introduction

To coordinate growth within plant organs, the developmental identities of cells must be specified and maintained. Synchronous growth is especially important for the development of the plant vascular system, where adjoining cells in single longitudinal files share the same cellular identity and form conductive elements that transport various substances throughout the plant body (Ye 2002). Plant structural support and the transport of water, nutrients, and signaling molecules are dependent on the specialized cells of the xylem and phloem. Although these vascular tissues are vital to plant survival, the mechanisms for the specification and maintenance of their identities remain largely unknown.

In adult plants, the majority of cells arise from stem cell populations found in the shoot apical meristem (SAM) and in the root apical meristem (RAM). Although the SAM and RAM stem cell niches are organized differently, both utilize a conserved intercellular signaling mechanism comprised a secreted peptide, a receptor-like kinase (RLK), and a homeobox transcription factor to maintain stem cells (Stahl and Simon 2010). The xylem and phloem arise from a vascular meristem located between them known as the...
procambium. Procambium function is characterized by oriented cell divisions which generate xylem to the center of the organ and phloem to the periphery (Scheres et al. 1995; Galweiler et al. 1998). The initial meristematic cells that contribute to vascular tissues are formed during early embryogenesis, at the globular stage (Scheres et al. 1995). Although vascular cells are present in late embryogenesis, they do not fully differentiate until after germination.

The cambium is a secondary meristem that is derived from the procambium in the adult plant and is responsible for secondary growth in the diameter of stems and roots through the production of phloem and xylem. The cambium also regulates the lateral growth of the interfascicular region, the connective tissue between vascular bundles in the stem. In Arabidopsis, the development of the cambium is confined to the base of the stem, the hypocotyl and the mature root (Altamura et al. 2001), although how cambium formation is regulated is not known. Cambium activity, however, has been shown to be increased in plants overexpressing *Arabidopsis thaliana* HOMEobox Gene 8 (AtHB8), an HD ZIP transcription factor involved in procambium initiation and cell proliferation (Baima et al. 2001; Kang and Dengler 2002; Donner et al. 2009). These plants showed increased proliferation and differentiation of xylem cells and ectopic lignification of phloem and pith cells. In addition, high cambium activity (hca) mutants were identified that have altered vascular bundle organization and increased expression of AtHB8 (Pineau et al. 2005). Mutations in two RLKs, REDUCED IN LATERAL GROWTH1 (RUL1) and MORE LATERAL GROWTH1 (MOL1), resulted in a reduced interfascicular region and an expanded interfascicular region, respectively, suggesting a role of intercellular signaling in regulating cambium activity (Agusti et al. 2011). The precise onset of the initiation of cambium formation during development is not completely understood; nor is it understood how the cambial meristem contributes to regulating the specification of the secondary xylem and phloem cells, nor how the procambium contributes to maintaining vascular cell identity. In general, when compared to the SAM and RAM, much less is known about how the procambial and cambial meristems are specified and maintained.

Although several genes have been identified that regulate the initiation and maintenance of the vascular cells, fewer genes have thus far been associated with the specification of distinct cell types. Two NAC domain transcription factors, VASCULAR-RELATED NAC DOMAIN 6 and 7 (VND6 and VND7), promote the differentiation of various cell types including non-vascular tissues such as leaf epidermis and mesophyll and root cortex into metaxylem (mature xylem) and protoxylem (initial xylem) when overexpressed, respectively (Kubo et al. 2005). The only gene known to be required for determining phloem cell identity is ALTERED PHLOEM DEVELOPMENT (APL), a MYB coiled-coil transcription factor. APL is necessary for the specification of phloem companion cells and sieve elements and limits the differentiation of xylem cells (Bonke et al. 2003). However, the mechanisms that regulate APL are not yet known.

A signaling mechanism has recently been described to regulate procambium activity by controlling cell proliferation and xylem differentiation. The Leucine Rich Repeat (LRR) RLK PHLOEM INTERCALATED WITH XYLEM (PXY), which is expressed mainly in the procambium, controls the oriented cell divisions of the procambium (Fisher and Turner 2007). In pxy mutants, phloem cells are found adjacent to and intermixed with xylem cells in inflorescence stems. PXY is a receptor for the tracheary element differentiation factor (TDIF) peptide, a small secreted peptide originating from the phloem and encoded by the CLAVATA3/ESR 41/44 (CLE41/44) genes. Exogenous application of CLE41/44 peptide to seedlings inhibits formation of xylem vessels in leaves and increases the number of procambium cells in hypocotyls; pxy mutants are insensitive to the effects of CLE treatment (Hirakawa et al. 2008). Additionally, CLE41/44 has been shown to activate WUSCHEL RELATED HOMEobox 4 (WOX4) in a PXY-dependent manner (Hirakawa et al. 2010). However, WOX4 regulates procambium proliferation, and not xylem cell differentiation. Thus, a current model suggests CLE41/44 is secreted from phloem cells and binds PXY in the procambium, activating the WOX4 signaling pathway to regulate cell division in the procambium. This mechanism is similar to the CLV3-CLV1-WUS signaling mechanism controlling stem cells in the SAM and CLE40-ACR4-WOX5 signaling in the RAM (Clark et al. 1993; Clark et al. 1997; Schoof et al. 2000; Brand et al. 2002; Sarkar et al. 2007; Stahl et al. 2009). However, CLE41/44 peptide is a positive regulator of stem cell proliferation in procambium tissue, rather than acting by repressing as CLV3 does in the SAM.

In this study, we identified an LRR RLK that is expressed in the vasculature and, we hypothesize, functions in vascular development. We demonstrate that mutants in this RLK lead to accumulation of cells with ectopic lignification in regions of phloem in the vascular bundles of inflorescence stems. However, mutant alleles in this RLK also lead to phloem cells adjacent to xylem cells, similar to pxy mutants, suggesting it additionally influences organization of tissue identities in vascular bundles. We, therefore, named this RLK XYLEM INTERMIXED WITH PHLOEM 1 (XIP1). Mutant phenotypes suggest XIP1 plays a role in differentiation of phloem cells in vascular development. These phenotypes are mainly apparent at the base of the stem, suggesting the defects could be influenced by the effects of secondary growth regulated by cambium
activity. XIP1 is also necessary for the organization or the morphological development of conductive elements in the vasculature.

Materials and methods

Growth conditions and genetic analyses

The xip1-1 allele was generated by the Seattle TILLING Project (Till et al. 2003). The xip1-2 allele was obtained from the RIKEN transposon collection (RATM11-2459-1 H). The xip1-1 allele was back-crossed seven times into the Col-0 ecotype. All lines were grown at 22°C in a Conviron growth chamber with a 16 h light/8 h dark cycle. To genotype the xip1-1 allele, two primer polymerase chain reaction was performed using NEB Taq and the PCR product was subsequently digested with HpaII (Fermentas), as the xip1-1 mutation disrupts an HpaII restriction site. The xip1-2 allele was genotyped using three primer polymerase chain reaction using Ex-Taq (TaKaRa). Primers used for xip1-1: xip1-1 F 5'-CTCTCTGTTCCATATCC CGTCTCAT, xip1-1 R 5'-ACGTGCAATTGATCGTGC TTTG, and PCR for xip1-2: xip1-2 F 5'-TCAGACGCTGT TAATGCGAGTAAACA, XIP1-2 R 5'-GATGATC TCGAAATTACTTTTAATT, XIP1 TF 5'-GAGTCTTTTGCAGTGATGATC TCT). The amplified product was cloned into pCR8-TOPO and subcloned into the pBIB-GFP-BASTA vector (Gou et al. 2010). The promoter fusion construct was transformed into Col-0 plants and the translational fusion constructs were transformed into Col-0 and xip1-1 via Agrobacterium-mediated transformation (Clough and Bent 1998).

To analyze lines carrying the XIP1 promoter fusion construct, tissue samples were cut and fixed in 90% acetone and stained with GUS staining solution containing X-Gluc [1 mM X-Gluc (Gold Bio Technology) dissolved in DMSO, 10 mM EDTA, 2 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100, 100 mM NaH2PO4, pH 7.0] for 2–14 h and mounted in ddH2O. To visualize the radial pattern of expression, hand-cut cross sections were obtained and stained with GUS staining solution for 2–14 h and mounted in ddH2O on glass slides (Fisher and Turner 2007). Images of plant organs were taken using a Canon Power Shot SX110 digital camera mounted on a Leica dissecting microscope. Hand-cut section images were taken using a Zeiss Axioplan microscope and QCapture Pro 5.0 imaging software.

Roots from lines carrying the translational fusion construct were grown on 0.5x MS plates for at least 5 days and mounted in 10% glycerol on microscope slides. Imaging of roots was performed using a Nikon 510 meta-confocal microscope and LSM imaging software.

Microscopy and histochemical analysis

Hand-sections of inflorescence stems were cut using a double edged razor and suspended in ddH2O as described (Lux et al. 2005). Toluidine blue and aniline blue staining procedures were conducted as described by Fisher and Turner (2007). Staining hand-cut sections with Maule reagent and phloroglucinol-HCl were performed as described previously (Guo et al. 2001). All stained sections were visualized using a Zeiss Axioplan microscope and QCapture Pro 5.0 imaging software. Of twenty samples of each of the genotypes stained with toluidine blue, blue staining cells in the phloem were seen in all twenty xip1 samples for each allele, and no blue cells were found in wild-type controls. Twelve samples for each genotype of phloroglucinol with no staining detected in the phloem for the wild type controls and all samples of xip1 mutants showing staining in phloem. Twelve samples of each genotype were stained with Maule reagent, with no abnormal staining detected in the phloem of wild-type controls, and all xip1 mutants showing red staining in phloem. In aniline blue stained sections, 4/12 individuals showed phloem cells adjacent to xylem cells in xip1-2 mutants, and 8/12 xip1-1 individuals were identified with...
phloem cells adjacent to xylem cells. All aniline blue stained xipl mutants showed blue staining in the phloem.

Thin plastic sections were obtained using the following protocol. Inflorescence stems were cut and fixed in 2% glutaraldehyde/1× PBS solution for 3–4 h and then subjected to an ethanol dehydration series (30, 50, 70, 90, 100% ×2). The tissues were infiltrated with LR white resin (electron microscopy sciences) and placed in plastic molds. 13 μm thin sections were cut using a Sorvall MT2-B Ultra microtome. Sections were stained with TB as described above and visualized using a Zeiss Axioplan microscope and QCapture Pro 5.0 imaging software. Six samples of each genotype were analyzed.

For propidium iodide staining, whole-mount inflorescence stems of 4-week-old plants were treated as described in Przemeck et al. (1996). Stems were fixed in 6:1 EtOH:acetic acid for 2–4 h. After fixation, samples were rehydrated using a series of 10 min washes (100% EtOH, 70% EtOH, 50% EtOH, 30% EtOH, 10% EtOH, ddH2O) and stained with 5 μg/ml propidium iodide for 2–4 h. Six stems of each genotype were cut longitudinally and mounted in chloralhydrate/glycerol/water (8:1:2). Images were taken using epifluorescence optics and a Zeiss axioplan microscope and QCapture Pro imaging software. Misaligned cells were seen in all mutant stems and not present in wild-type controls. Images were taken from the apical portion of stems. Six samples of each allele were analyzed. All Col-0 and Nos (six individuals each of Col-O and Nos) samples showed continuous xylem elements. All samples of xipl mutants showed mis-alignment or discontinuous elements.

For height growth measurements, plants were grown on soil for 3 weeks and individual plants measured from the base to the apex of the main bolt. Samples included 32 Col-0, 33 xipl-1, 25 Nos, and 46 xipl-2. Standard error was determined and t-tests indicate significance between Col-0 and xipl-1, p < 0.001, and between Nos and xipl-2, p = 0.01.

Results

Mutants in At5g49660, an LRR RLK, affect plant growth and cause anthocyanin accumulation in leaves

In the Arabidopsis thaliana genome, over 400 genes have been annotated as RLKs including over 200 LRR RLKs (Shiu and Bleecker 2001). The function of the majority of these RLKs remains unknown. In a reverse genetic screen to determine the roles of unknown RLKs involved in Arabidopsis development, we isolated insertion mutant alleles for a large subclass of LRR RLKs and screened for developmental phenotypes. There were no insertion mutant alleles available for At5g49660, so this gene was investigated further. At5g49660 encodes an LRR RLK with 16 extracellular LRRs flanked by two pairs of cysteine residues, a transmembrane domain and a cytoplasmic kinase domain containing the conserved kinase sub-domains associated with an active kinase (Fig. 1a, S1). Using the amino acid sequence of the kinase domain as determined by pfam (http://pfam.sanger.ac.uk), the phylogenetic relationship of At5g49660 was compared with other closely related LRR RLKs (Shiu and Bleecker 2001) using MAFFT sequence alignment, and a phylogenetic tree was created using PhyML (http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome). The resulting phylogenetic tree confirms At5g49660 is part of the LRR XI family, showing distant similarity to CLAVATA1 (CLV1) (45% amino acid identity) and PXY (49% amino acid identity) (Fig. 2). At5g49660 is most similar to a gene involved in endosperm development, HAiku2 (IKU2), (Luo et al. 2005), but in a pair-wise comparison, the 48% amino acid identity suggests these two kinase domains have diverged significantly in their functions. Based on the high degree of sequence divergence between At5g49660 and LRR X, XI, and XIII subfamilies of RLKs, there was no hint to the role of At5g49660. To determine the function of At5g49660, we isolated seven ethylmethane sulphonate (EMS) mutated lines (Fig. 1a) using the “Targeting Induced Local Lesions In Genomes” (TILLING) resource (Till et al. 2003). Once identified, mutants were backcrossed into the Col-0 accession and screened for phenotypic defects. Of the 7 missense mutations in At5g49660 identified through TILLING, all of the mutations are located in the extracellular LRR region except for one mutation, which is a substitution of a serine to a phenylalanine at position 677 (S677P) (Fig. S1) in the kinase domain. The S677P mutation is adjacent to sub-domain 1 in the kinase domain and eliminates a semi-conserved serine residue (Fig. S1). Arabidopsis plants homozygous for the S677P mutation in At5g49660 were the only plants identified from the TILLING screen that exhibited an obvious mutant phenotype. In these mutant plants, the inflorescence stems were shorter than the Col-0 accession plants (Fig. 1b, f), although general morphology and fertility were normal. In addition, the cotyledons and rosette leaves of plants with the S677P mutation had a purple color, indicative of anthocyanin accumulation (Fig. 1c).

An insertion mutation in At5g49660 was later identified in the RIKEN collection of Arabidopsis DS transposon lines (stock # RATM11-2459-1 H). This insertion mutation is located within the coding region of the kinase domain (at amino acid 769) and therefore should represent a null allele (Fig. 1a). RT-PCR of At5g49660 RNA from seedlings homozygous for the insertion allele does not produce RNA corresponding to the C-terminal region downstream of the
insertion, but does express RNA from the N-terminal region (Fig. 1e, f). Therefore, plants homozygous for the insertion do not express a full length transcript. Comparison of the growth characteristics of plants homozygous for the insertion in At5g49660 to wild-type Nossen (Nos) accession plants showed similar phenotypes as plants homozygous for the S677P missense mutation, including reduced growth and accumulation of anthocyanin (Fig. 1b, d, f).

In order to show that the S677P mutation in At5g49660 is responsible for the phenotypes observed, we complemented plants homozygous for the S677P mutation with a wild-type copy of the At5g49660 gene. The genomic region of At5g49660 including 2.5 kb upstream of the coding sequence was fused with the sequence for green fluorescent protein (GFP), creating a C-terminal translational fusion. Plants with the S667P mutation in At5g49660 transformed with this At5g49660:GFP fusion showed similar height compared to wild-type plants and also lacked noticeable anthocyanin accumulation in leaves (Fig. 1b).

The ability to rescue the phenotypes associated with the missense mutation with a wild-type copy of the At5g49660 gene provides further evidence that the mutant phenotype is due to the mutation in At5g49660.

At5g49660 is expressed in the vasculature throughout development

Gene expression data can provide clues to determine the cellular basis of growth defects. To determine which cell types At5g49660 is expressed in, we generated a promoter fusion by cloning the putative At5g49660 promoter upstream of the β-glucuronidase (GUS) reporter gene. The 2.5 kb sequence upstream of the start codon, identical to that used in the genomic rescue clone, was fused to the GUS coding sequence. After transformation of Col-0 plants, eight independent lines were analyzed for GUS activity. GUS activity was detected in the vasculature, beginning with the mature embryo stage through the adult plant in all eight lines (Fig. 3). At5g49660 was expressed in the vasculature of stems, leaves, sepals, pedals, pedicels, hypocotyls and roots (Fig. 3b–l). Hand-cut cross sections of stems and pedicels show expression within the phloem and procambium regions of the vasculature (Fig. 3c–e, g, i). Vascular expression of At5g49660 in roots was analyzed using the translational fusion to GFP (Fig. 3l) and microarray expression analyses available in the AREX database (Birnbaum et al. 2003; Brady et al. 2007). Based on the AREX data, At5g49660 is restricted to the phloem and

Fig. 1  Gene model of XIP1 and growth defects of xip1 mutants. a Gene model of XIP1 with protein domains and mutant alleles shown. b xip1 mutants show reduced growth compared to wild type plants, from left to right Col-0, xip1-1, xip1-1 XIP1::GFP, Nos, xip1-2. c, d Anthocyanin accumulation in xip1-1 and xip1-2 plants (right) compared to Col-0 and Nos (left), respectively. e RT-PCR of XIP1 transcript from RNA samples from seedlings 6 days after germination; from right to left: Col-0, xip1-1, Nos and xip1-2. f Height of Col-0 and xip1-1 plants and Nos and xip1-2 plants measured 4 weeks after germination. Error bars represent the standard error, and t test analysis shows a significance of \( p < 0.001 \) between Col-1 and xip1-1, and \( p < 0.01 \) between Nos and xip1-2.
and pith cells purple (Fig. 4a–f). In stems from both the xylem blue and the primary cell walls of the phloem which binds lignin and stains the secondary cell walls of old mutants and their corresponding wild-type accessions were hand-sectioned and stained with toluidine blue (TB), attributed to accession differences. Red-staining lignified cells were visible in the phloem region in xip1-1 and xip1-2 mutants, but absent in wild-type controls (Fig. 4g, h, i, j). Although the TB and phloroglucinol-HCl staining of phloem cells may be due to a partial transformation of phloem to xylem, the staining in the phloem is also consistent with the production of phloem fiber sclereids. Other than the intermixing of cells with xylem characteristics into the phloem in the xip1-1 and xip1-2 mutants, the vascular bundles appeared similar to wild type plants in morphology.

To identify the additional defects in vasculature identity in xip1 mutants, hand-cut sections were further analyzed using aniline blue, which causes callose in the xylem to fluoresce blue and sieve plates in the phloem to fluoresce yellow under UV light (Fig. 4k–n). Aniline blue stained xip1-1 and xip1-2 sections showed blue fluorescence in the phloem region, confirming the presence of some cells with altered secondary walls in the phloem (Fig. 4l, n). Yellow fluorescing cells in the phloem of xip1 mutants indicate that at least some of the cells in this region retain phloem identity, while other cells show aberrant lignification. In addition, in xip1-1 mutants, phloem cells were located adjacent to xylem, in the region normally occupied by the procambium (8/12 plants) (insert in Fig. 4j). This phenotype, which is typical of pxy mutants, was less obvious in xip1-2 plants (4/12 plants) and never seen in wild-type controls. The differences between the xip1-1 allele and xip1-2 allele could be attributed to accession differences.

The type of lignin deposited in the cell walls of xylem cells differs from the lignin found in the interfascicular region, the lignified tissue formed between xylem bundles in cambial growth. Interfascicular fibers stain red after treatment with Maule reagent due to the presence of syringyl type (s-type) lignin, and xylem cells appear brown due to a reduction or absence of s-type lignin. Hand-cut sections of wild-type and mutant inflorescence stems were stained with Maule reagent due to the presence of s-type lignin (Fig. 4o–r). Cells in the phloem region of xip1-1 (12/12) and xip1-2 (12/12) sections showed red staining similar to the stained regions of interfascicular fibers, indicating the presence of s-type lignin (Fig. 4p, r). Red stained phloem cells were never observed in wild-type sections (n = 12 each for Col-0 and Nos).

**Fig. 2** Phylogenetic analysis of kinase domains related to XIP1 in LRR RLK families X, XI, and XIII. The kinase domain, as determined by pfam database, was used to align the amino acid sequences of a subset of related RLKs. The phylogeny was determined using phylogenies by maximum likelihood analysis with bootstrap values provided. This phylogenetic tree includes RLKs involved in many different processes including responses to stress (PEPR1, PEPR1), abscission (HAE), SAM maintenance (CLV1, BAM1, BAM2, BAM3), endosperm development (IKU2), brassinosteroid perception (BRI1, BRL1, BRL3, BRL3), and vascular development (BRI1, BRL1, BRL3, VH1, PXY, PXL1, PXL2)
Longitudinal sections of *xip1* mutants show malformed vascular cells

Vascular cells need to be aligned with one another at their apical and basal ends to create conductive elements for transport of water, nutrients and small molecules. To determine whether *xip1* mutants have an altered arrangement of vascular cells that contributes to their growth defects, thin longitudinal sections of mutant and wild-type stems cut from the base were examined. The apical and basal boundaries of cells will be clearly visible if cells lie in the same optical plane and therefore constitute a continuous cell file, as can be seen in the pith cells in the sections from both wild-type and *xip1* plants (Fig. 5a–d). In sections from wild-type plants, vascular cells lie within the same optical plane and both apical and basal boundaries of the cells are visible (Fig. 5a, c). In contrast, *xip1* vascular cells have less visible apical and basal cell boundaries and instead have a “bottle-shaped” appearance, whereas the pith cells appear wild-type in the *xip1* sections (Fig. 5b, d). These data may indicate that vascular vessels show an alteration in the orientation and alignment of cells or the morphology of the vascular cells in *xip1* stems is disrupted.

We further examined the structure of the vasculature in whole-mount stems. After staining stems with propidium iodide, we were able to visualize xylem elements in apical stems (Przemeck et al. 1996). In wild-type stems, xylem elements were visualized as continuous vessels (Fig. 5e, g). *xip1* mutants showed discontinuous or misaligned xylem cells (Fig. 5f, h). Misalignment of xylem elements in the *xip1* mutants was visible in the apical portion of the stems, suggesting XIP1 is necessary for vascular development throughout the stem. Therefore, in addition to phloem specification defects, xylem vessel formation is also disrupted in *xip1* mutants.

**Discussion**

XIP1 is a regulator of vascular development

Coordinated cellular patterning and growth are necessary for proper formation of the vascular system in plants. In this study, we have identified a previously uncharacterized LRR RLK, XIP1, which is expressed mainly in the vasculature in *Arabidopsis*. We identified a mutant allele in XIP1 using TILLING and a transposon insertion that resulted in plants with several growth defects, including slower growth of the inflorescence stem and accumulation of anthocyanin in leaves. Further analysis showed defects in the development of the vasculature, specifically in phloem and xylem development. Using histochemical analyses, we showed that *xip1* phloem cells develop modified secondary walls resembling xylem or fiber cells. In addition, *xip1* phloem cells were sometimes adjacent to xylem cells, in the position of procambium cells, and the
vessel elements do not appear to align in linear cell files or show abnormal morphology.

The xip1-1 mutant allele is a missense mutation that results in a substitution of a serine to a phenylalanine in the kinase domain, which could eliminate an important phosphorylation site affecting the activity or function of XIP1. This serine residue shows some conservation in other related LRR RLKs including HAIKU2 (IKU2), involved in endosperm development, PXY-LIKE 2 (PXL2), related to and proposed to be redundant with PXY, and PEP1 RECEPTOR 1 and 2 (PEPR1/2), involved in defense response (Fig. 2) (Luo et al. 2005; Yamaguchi et al. 2006; Fisher and Turner 2007; Yamaguchi et al. 2010). The similar phenotypes seen in the insertion allele of XIP1,
xip1-2, compared to xip1-1 suggests both represent loss-of-function alleles. The slightly stronger xip phenotype observed with xip1-1 mutant may be due to differences in the background accessions or due to altered activity of XIP1 in this mutant. However, xip1-1 phenotypes are recessive, suggesting the differences are mostly likely due to accession differences.

We propose that the disrupted specification and organization of the vascular cells in xip1 leads to less efficient vascular transport that impairs growth of inflorescence stems. The requirement for coordinated cell division and specification of vascular cells to maintain cell files would imply that disruption of these developmental patterns would lead to a disorganized vascular system. Indeed, several other examples of defects in vascular development result in plants with shorter inflorescence stems (Hanzawa et al. 2000; Pineau et al. 2005; Fisher and Turner 2007). In particular, pxy mutants, which show altered orientation of the divisions in the procambium, have reduced inflorescence stem height as well as the accumulation of anthocyanin in the cotyledons, similar to what we observe for xip1 mutants (Fisher and Turner 2007). Defects in vessels, due to misaligned vascular cells or abnormal morphology, may form a vasculature that does not function as effectively in transport which may lead to shorter plants with more physiological stress, as indicated by the anthocyanin accumulation.

Fig. 5 Longitudinal sections of xip1 inflorescence stems. Longitudinal sections of Col-0 (a), xip1-1 (b), Nos (c) and xip1-2 (d) 6-week-old inflorescence stems. Apical and basal end of xylem (xy) and pith (pt) cells can be seen in the plane of the section in wild type. Connected vessels (v) can be seen in both Col-0 and Nos sections. Apical and basal end of cells appear in focus only in pith cells in xip1-1 (b) and xip1-2 (d) sections and vascular cells the boundaries appear out of the plane of section (arrows). Cells with aberrant morphology can be seen in the xylem of xip1-1 (b) and xip1-2 (d). Whole-mount apical regions of stems stained with propidium iodide show visible xylem tracheary elements as fluorescent cells. Col-0 (e) and Nos (g) show continuous vessels compared to discontinuous elements (white arrow heads) seen in xip1-1 (f) and xip1-2 (h). i Model depicting likely locations and functions of XIP1 within the vascular tissues. CLE41/44 peptide originates from phloem cells and signals to PXY in the procambium to regulate WOX4, and to orient cell divisions and to maintain the fate of the procambium. XIP1 receives an as yet unknown signal, and prevents xylem or fiber formation and regulates vascular differentiation, which may have downstream consequences for phloem identity and CLE41/44 expression. a–d Scale bar 50 µm, e–h scale bar 20 µm.
XIP1 and other receptor kinases in vascular development

Recent reports have described the LRR RLK PXY/TDR to be part of a signaling pathway required for maintaining procambium/cambium function, including promoting proliferation within the procambium and suppressing xylem cell differentiation (Hirakawa et al. 2010). In this pathway, the CLE41/44/TDIF peptide is secreted from phloem cells and interacts with PXY/TDR in the procambium/cambium (Fig. 5i). The CLE41/44/TDIF-PXY/TDR network induces the expression of WOX4, a WUS-like transcription factor, specifically in the procambium/cambium and promotes proliferation (Fig. 5i) (Ji et al. 2009; Hirakawa et al. 2010). The histochemical analyses of the xip1 mutants indicate the abnormal development of lignified phloem cells could be caused by either a phloem to xylem cell fate transformation, or due to abnormal differentiation due to cambium activity. Because xip1 mutants also develop a phloem intermixed with xylem phenotype similar to pxy mutants, the XIP1 name is appropriate, although it is still an open question as to whether there is a phloem to xylem transformation in xip1 mutants.

The appearance of phloem cells adjacent to xylem cells and the malformed alignment/morphology of cells in xip1 stems is similar to what is seen in pxy mutants, indicating that in xip1 mutants either (1) PXY signaling may be disrupted, perhaps by loss of CLE peptide signaling, thus affecting cambium activity and oriented cell divisions or (2) XIP1 may be involved in the regulation of cambium proliferation directly. Procambium activity/formation has been previously postulated to be regulated through the action of several KANADI genes (Eshed et al. 2001; Ilegems et al. 2010). These genes are predominantly expressed in the phloem and they are hypothesized to restrict procambium precursor cell formation through suppression of the auxin efflux carrier PIN1. In our model, XIP1 may be acting in a feedback mechanism through the CLE41/44/TDIF peptide-PXY-WOX4 pathway or through a separate pathway that regulates cambium activity and phloem cell differentiation (Fig. 5f).

A similar feedback pathway has been postulated for regulating meristem maintenance in the SAM. Although the effect on the WUS/WOX transcription factors is different, meristem maintenance of the SAM requires signaling from CLV3-CLV1/3/CLAVARIOL2 (CLE-LRR RLK interaction) to repress the expression of WUS. There is also a feedback mechanism that maintains this pathway. Our model, based on previous analysis of PXY/TDR suggests a CLE-LRR RLK module acts in a mechanistically similar way to maintain the meristem cells throughout the Arabidopsis vasculature. One extension of our model is that XIP1 could perceive signals from the procambium/cambium, to regulate phloem differentiation. A better understanding of how XIP1, PXY and PXL2 signaling pathways function independently or together to regulate the procambium/cambium and differentiation of phloem/xylem will contribute greatly to the mechanisms regulating vascular stem cell populations in plants.

Possible XIP1 function in secondary growth

The presence of the xip1 phenotype only at the base of the inflorescence stem suggests the defects may be attributed to abnormal secondary growth, a process regulated by the cambial meristem. The procambial meristem develops into the cambial meristem, which in turn regulates the formation of secondary xylem and phloem tissues. This secondary meristem is responsible for radial growth in adult plants. In Arabidopsis, cambium development appears restricted to the base of the inflorescence stem, hypocotyls and upper portion of the root. Our observation that defects are restricted to the base of the stem in xip1 plants suggests XIP1 may be important for secondary growth and development in stems. However, XIP1 is expressed in the vasculature throughout development, which suggests that XIP1 may play a redundant role in vascular development.

The abnormal phloem cell phenotype that we see in xip1 plants is reminiscent of the phenotype associated with AtHB8, a transcription factor involved in procambium/cambium development. Over-expression of AtHB8 is proposed to increase vascular cell differentiation and secondary growth. The staining of stem cross-sections from AtHB8 over-expressing plants with toluidine blue showed the presence of blue stained cells in the phloem, similar to xip1 mutants (Baima et al. 2001). The authors propose these cells as phloem fiber sclereids because of their thick secondary cell walls. Phloem fiber sclereids can originate from primary or secondary meristems such as the cambium (Lev-Yadun 1994). Ectopic fiber sclereid formation in the pith cells of wild type Arabidopsis due to repeated cutting of the stems (Lev-Yadun 1994) is likely a result of enhanced cambium activity. The staining pattern of s-type lignin in xip1 mutants is also consistent with a defect in phloem fiber sclereid formation in xip1 mutants. It is possible that XIP1 may function to perceive signals from the procambium/cambium to regulate the differentiation of phloem cells, or that XIP1 perceives a signal that restricts procambium/cambium activity. The presence of cells in the phloem with modified secondary cell walls raises the question if the procambium/cambium meristems are also “over-active” in xip1 plants. Indeed, mutants with over-active procambium and cambium, including over-expressing AtHB8 and high cambium activity (hca) mutants, show a significant decrease in plant height (Baima et al. 2001; Pineau et al. 2005).
The lignified secondary cell walls seen in the phloem of xip1 mutants are similar to the phenotype of psy-like l (pxl1) double mutants with either psy or pxl2, as described in Fischer and Turner (2007). It is not yet clear if XIP1 acts independently to repress ectopic lignification in the phloem, or acts together with PXY, PXL1 or PXL2 in the vasculature to regulate various aspects of procambium/cambium activity and vascular cell fate determination. Both psy and xip1 mutants also show defects in oriented cell division shown by disorganized cell files and phloem located near xylem cells, the psy phenotype. Loss of either psy or xip1 results in reduced plant growth and this phenotype may be due to defects in cell division throughout the vasculature of the plant or to the disrupted transport system. These results indicate PXY and XIP1 may play a global role in the vasculature of all other organs as well. Continued analysis of these receptors and their interactions will provide further insight in the gene networks required for vascular development.

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References


XYLEM INTERMIXED WITH PHLOEM1, a leucine-rich repeat receptor-like kinase required for stem growth and vascular development in *Arabidopsis thaliana*

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**Fig S1**

.60 identity threshold, Identical=Green, similar=Magenta, Conserved=Red

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